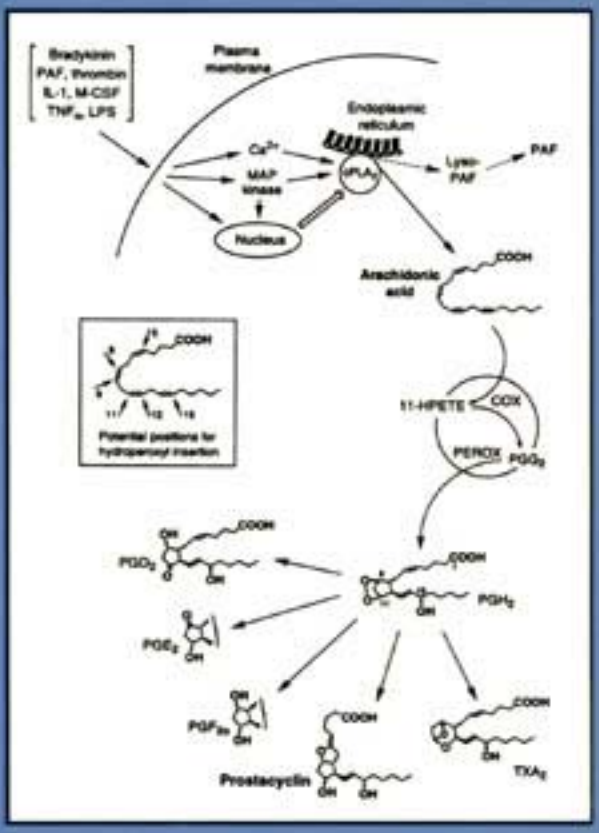
Prostacyclin and Its Receptors



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and
Robert L. Jones

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Preface

The 1970's were dramatic times for prostanoid research, with the discovery of prostacyclin and the elucidation of the mode of action of aspirin by Sir John Vane and his colleagues. As the most potent endogenous antiplatelet agent, prostacyclin received much attention, and the race was on to develop nonhypotensive analogues for thrombosis therapy. Promising leads were invariably confounded by the possibility of species differences in prostacyclin (IP-) receptors, and then a molecular biology revolution occurred in the early 1990's. Shuh Narumiya and his colleagues presented us with a full hand of cloned prostanoid receptors, and the exact identity of prostacyclin receptors in blood vessels and platelets finally became clear. Nevertheless, interest in the biology of prostacyclin remained high, but was focused more on its excitatory actions. Could prostacyclin exert some of the pro-inflammatory actions, for example enhancement of pain sensation, traditionally associated with prostaglandin E_2? The demonstration of IP-receptors on central and peripheral neurones, the existence of two neuronal IP-receptor subtypes with quite different agonist specificities, and the reduction of pain sensation in the IP-receptor gene-knockout mouse has triggered a new wave of prostanoid research. In addition, the development of much-needed IP-receptor antagonists may turn out to be more than rumours. This monograph has been prepared to put all of these data into context and to set the scene for progress into the next millenium.

Helen Wise

Robert L. Jones

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Acknowledgments

We wish to thank industrial colleagues who over the years have supported our research with generous gifts of prostanoids, and in particular Professor Helmut Vorbrüggen of Schering AG and Dr. Nick Meanwell of Bristol-Myers Squibb. RLJ is also appreciative of the camaraderie of Drs. Norrie Wilson and Roma Armstrong, who were involved in the development of the EP analogue series at the University of Edinburgh. The excellent assistance of Mrs. Joresa Ng in the preparation of the manuscript and the patience of Dr. John Rudd in all-things-Macintosh are gratefully acknowledged. Finally, we should like to thank the following publishers for giving permission to use their copyright material: American Society for Biochemistry and Molecular Biology, Birkhäuser, Blackwell Science Ltd, Elsevier Science Ltd, Kluwer Academic / Plenum Publishers, Lippincott Williams & Wilkins, Macmillan Magazines Ltd, Macmillan Press Ltd, and Prous Science.

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Contents

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Chapter 1

An introduction to prostacyclin and its receptors

1. INTRODUCTION

The platelet inhibitory and hypotensive actions of prostacyclin are by now so well established that it is surprising to find that prostacyclin was actually the last member of the primary (2-series) prostanoid family to be identified. By coincidence, the prostacyclin (IP-) receptor was also the last of the prostanoid family of receptors to be cloned.

Interest in prostacyclin as an antithrombotic agent has resulted in an intensive search for more clinically useful IP agonists, with high chemical and metabolic stability, and selectivity of action on platelets. As a result of these studies, a large amount of structure-activity relationship data is available, which we have attempted to summarise in Chapter 2 (prostacyclin analogues) and in Chapter 3 (nonprostanoid prostacyclin mimetics). The ready availability of human platelets for screening potential IP agonists, and the variable choice of agonists used by different groups, has perhaps limited a thorough assessment of the existence of species-specific IP-receptors. Therefore, we have attempted to redress the balance by closely examining the data on the cloned human, mouse and rat IP-receptors (Chapter 4), and compared this with twenty year's worth of platelet aggregation studies (Chapter 5). Despite such a wealth of knowledge on prostacyclin and its platelet receptors, we are constantly hampered in our interpretation of data by the lack of IP-receptor antagonists. So we caution the reader to bear this in mind, and would encourage a re-examination of our conclusions when IP antagonists become available.

The hypotensive property of prostacyclin is generally considered a drawback to the use of IP agonists as antithrombotic drugs. However, there are conditions (e.g. pulmonary hypertension) where its vasodilator action contributes to the therapeutic effect. Prostacyclin can also claim to belong to the family of endothelium-derived relaxing factors (EDRFs), of which nitric oxide (NO) is probably the most well known. As an EDRF, prostacyclin may therefore have an important role in controlling blood flow. These aspects are discussed in Chapter 6.

Aside from the cardiovascular properties of prostacyclin, its other functions are less well established. As an inflammatory mediator, the role of prostacyclin has long been overshadowed by that of prostaglandin E2 (PGE_2), yet both prostanoids are produced at sites of inflammation, and both have unique profiles of activity. Prostacyclin as an inhibitor of the activity of a range of inflammatory cells will be discussed in Chapters 7 and 8, where it will become clear that the source of cells being studies (e.g. elicited or nonelicited) has a crucial influence on their responsiveness to IP agonists. We would also like to draw the reader's attention to the role of prostacyclin in sensory neurones (Chapter 9), and to present evidence showing that IP-receptors have an important role to play in nociception, as least as important as PGE receptors.

Of most recent interest is the role of IP-receptors in other neuronal systems and the possible existence of IP-receptor subtypes. This information may be found in Chapter 10, where we present evidence for the presence of IP-receptors on enteric neurones and in Chapter 11, where we look at IP-receptors in the central nervous system.

Throughout this monograph, we have tried to consider the potential therapeutic application of drugs acting on IP-receptors, but the topics cannot be considered exclusive, they simply represent the best studied areas to date. Because prostacyclin is just one member of an important family of products derived from arachidonic acid (the eicosanoids), and because many of the other members are mentioned throughout this monograph, we would like to set the scene by outlining the metabolic pathways for eicosanoids, the properties of prostanoid receptors, and how certain anti-inflammatory drugs interfere with eicosanoid systems.

2. EICOSANOID BIOSYNTHESIS

The 20-carbon polyunsaturated fatty acid arachidonic acid has two quite different functions in the body. As a component of cell membrane phospholipids, its *cis* double bonds disorder the hydrophobic core of the membrane, thus influencing fluidity, permeability and the behaviour of

embedded proteins.[1] However, following release from internal membranes by phospholipase A_2 (PLA_2), arachidonic acid can enter several oxidation pathways to yield a diverse range of products, some of which have important physiological and pathological roles (Fig. 1 and 2). These products are termed eicosanoids to indicate the presence of a 20-carbon backbone (Greek *eicosi* = 20); related products derived from 06 fatty acids other than arachidonate and from some ω and ω9 fatty acids also belong to the eicosanoid family.

Two types of oxygenase enzyme are responsible for the initial steps in eicosanoid pathways, lipoxygenases (LOXs) and cytochrome P-450 mono-oxygenases. The basic lipoxygenase reaction involves stereospecific attack of an hydroperoxy radical on a methylene-interrupted diene unit (-CH=CH-CH_2-CH=CH-) in the fatty acid substrate. By attacking one end of one of the three diene units present in arachidonic acid (Fig. 1, inset) individual LOXs produce unique products. P-450 mono-oxygenases on the other hand appear to show less specificity, producing a range of epoxides of arachidonic acid (Fig. 2), as well as its 20-hydroxy derivative.[2]

2.1 PGHS or COX pathway

The prostaglandin H synthase (PGHS) or cyclo-oxygenase (COX) pathway, which generates the prostanoids (Fig. 1), was first reported in 1964 by two independent research teams led by Bergström & Samuelsson at the Karolinska Institute in Sweden[3] and by van Dorp & Nugteren at the Unilever Research Laboratories in Holland.[4] They were able to isolate and identify PGE_2 from homogenates of sheep seminal vesicles incubated with arachidonic acid. The choice of seminal vesicles stemmed from observations made some 30 years earlier that smooth muscle contractile and relaxant activities attributable to acidic lipids were present in extracts of male reproductive tissues.[5-8]

Prostaglandin H synthase contains both COX and 15-hydroperoxidase activities. The COX site essentially catalyses two consecutive lipoxygenase reactions; the first occurs at C11 to give 11(R)-hydroperoxy-eicosatetraenoic acid (11-HPETE).[9,10] The second is more complex, involving insertion of an hydroperoxy group at C15 and generation of an endoperoxide ring system. The product PGG_2 is reduced at the 15-hydroperoxidase site to PGH_2. In general, the terms PGHS and COX are used loosely to describe the enzyme system that forms prostaglandin endoperoxides.

The 9,11-peroxide bond in PGH_2 is chemically labile and spontaneous isomerisation readily occurs to give PGD_2 and PGE_2. However, specific synthase enzymes also generate PGD_2 and PGE_2 and two further isomers prostacyclin and thromboxane A_2 (TXA_2). The defined stereochemistry of

PGH_2 (with oxygens below the plane of the cyclopentane ring, 8α /12β side-chains, and S-configuration at C15) is passed on to these primary prostanoids (see Chapter 2 for further stereochemical information). Both prostacyclin and TXA_2 are rapidly hydrolysed under physiological conditions with loss of biological activity ($t_{½}$ = 3 and 0.5 min respectively). Enzymatic reduction of the 9,11-peroxide bond of PGH_2 yields the fifth primary prostanoid $PGF_{2\alpha}$, which may also be formed from PGD_2 or PGE_2.[11]

2.2 Leukotriene pathway

A smooth muscle contractile substance is released from guinea-pig lungs by cobra venom (a source of PLA_2)[12] and by anaphylactic challenge.[13-15] The identity of this "slow-reacting substance of anaphylaxis" (SRS-A) remained elusive for many years until the discovery of the leukotrienes by Samuelsson's group. It was shown that a 5-lipoxygenase (5-LOX) first converts arachidonic acid into 5(S)-HPETE and then abstracts water to generate leukotriene A_4 (LTA_4) (Fig. 2).[16-18] At this point the pathway splits. LTA_4 hydrolase generates the 5,12-dihydroxy compound LTB_4,[19] which has potent chemotactic activity for polymorphonuclear leukocytes.[20] Alternatively, LTC_4 synthase catalyses the opening of the epoxide ring by the SH group of glutathione (y-Glu-Cys-Gly) to yield LTC4;[21] metabolism of the glutathione unit yields LTD_4 and LTE_4. These three peptidoleukotrienes activate specific leukotriene (CysLT) receptors to elicit contraction of intestinal and bronchial smooth muscle, thus accounting for the bioactivity of SRS-A.

The leukotriene pathway is well developed in white cell populations, including neutrophils, eosinophils and pulmonary alveolar macrophages, and there is good evidence for a role for peptidoleukotrienes in the bronchoconstriction of human asthma.[22-25] Recently, several CysLT antagonists, (e.g. zafirlukast[26] and a 5-LOX inhibitor zileuton[27] have been approved as anti-asthma drugs.[28]

Whether COX, leukotriene or other arachidonate products are formed within a cell would be expected to depend on the relative levels of active oxygenases present. However, more subtle mechanisms such as "substrate channelling" may have a significant influence. Consecutive enzymes in a pathway may be located in or directed to internal membranes in such a way that the product of the first enzyme feeds directly into the catalytic site of the second. This will increase the substrate concentration of the primary product, and also protect unstable primary products from chemical reaction in the surrounding aqueous phase. Substrate channelling appears to be particularly important in leukotriene biosynthesis.[29] During cell activation,

PLA_2 and 5-LOX are translocated to the nuclear envelope and adjacent endoplasmic reticulum, where they are anchored by an integral membrane protein, 5-LOX-activating protein or FLAP.[30] In this way, the utilisation of both arachidonic acid and 5-HPETE is maximised. Compounds such as the indole MK-886 inhibit 5-LOX activity by binding to FLAP, whereas zileuton directly inhibits 5-L0X.[31]

2.3 12-LOX and 15-LOX pathways

Arachidonic acid is converted to 12(S)-HPETE by the 12-LOX of human[32] and cow[33] platelets; a leukocyte-type of 12-LOX also exists.[34] Isomerisation (either enzymatic or non-enzymatic) then yields two epoxy-hydroxy derivatives named hepoxilins A_3 and B_3.[35-37] Epoxide hydrolase activity then creates the corresponding trihydroxy acids, trioxilins A_3 and B_3. Hepoxilin A_3 can induce hyperpolarisation of neurones and potentiate glucose-induced insulin secretion[34] and there is evidence for specific binding sites on human neutrophils.[38]

The 15-LOX pathway from arachidonic acid first yields 15(S)-HPETE,[39] while further reactions generate trihydroxy acids known as lipoxins (LXs), which characteristically possess four conjugated double bonds. Lipoxins can also be generated by 12-LOX action on LTA_4. It has been suggested that LTA_4 released by polymorphonuclear leukocytes may proceed either to the classical leukotrienes or to lipoxins by interaction with 12-LOX in neighbouring platelets (an example of transcellular biosynthesis).[40] LXA_4 and LXB_4 exhibit endothelium-dependent vasodilator activity[41] (see Chapter 6) and also release prostacyclin from human umbilical endothelial cells.[42]

The elucidation of these eicosanoid pathways has been described in greater detail by Smith & Borgeat,[43] and information on individual enzymes may be found in the 1995 monograph "Molecular Biology of the Arachidonate Cascade".[44] Many of the eicosanoids exhibit high chemical reactivity and establishing their structures has presented considerable challenges to the biological chemist. Advances in chromatographic techniques and in the spectrometric analysis of microgram amounts of organic compounds over the past 30 years have been crucial to success in this area, and the skilful use of combined gas chromatography - mass spectrometry deserves special mention.[45]

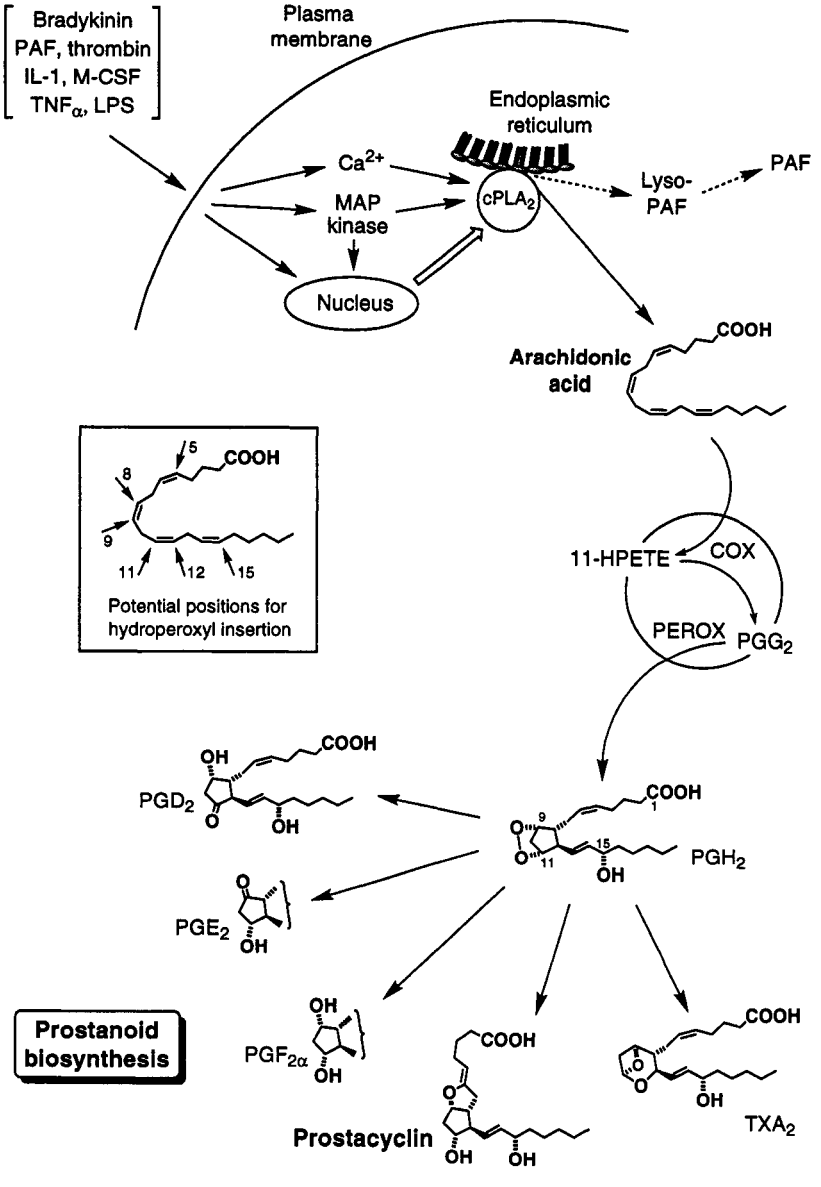


Figure 1. Activation of cytosolic phospholipase A_2 ($cPLA_2$) to release arachidonic acid and subsequent metabolism by the cyclo-oxygenase (COX) pathway to prostanoids. The brackets against the structures of PGE_2 and $PGF_{2\alpha}$ indicate that their α and ω -chains are identical to those in PGH_2. The open arrow indicates nuclear expression of $cPLA_2$. The inset shows the potential positions of lipoxygenase attack on arachidonic acid.

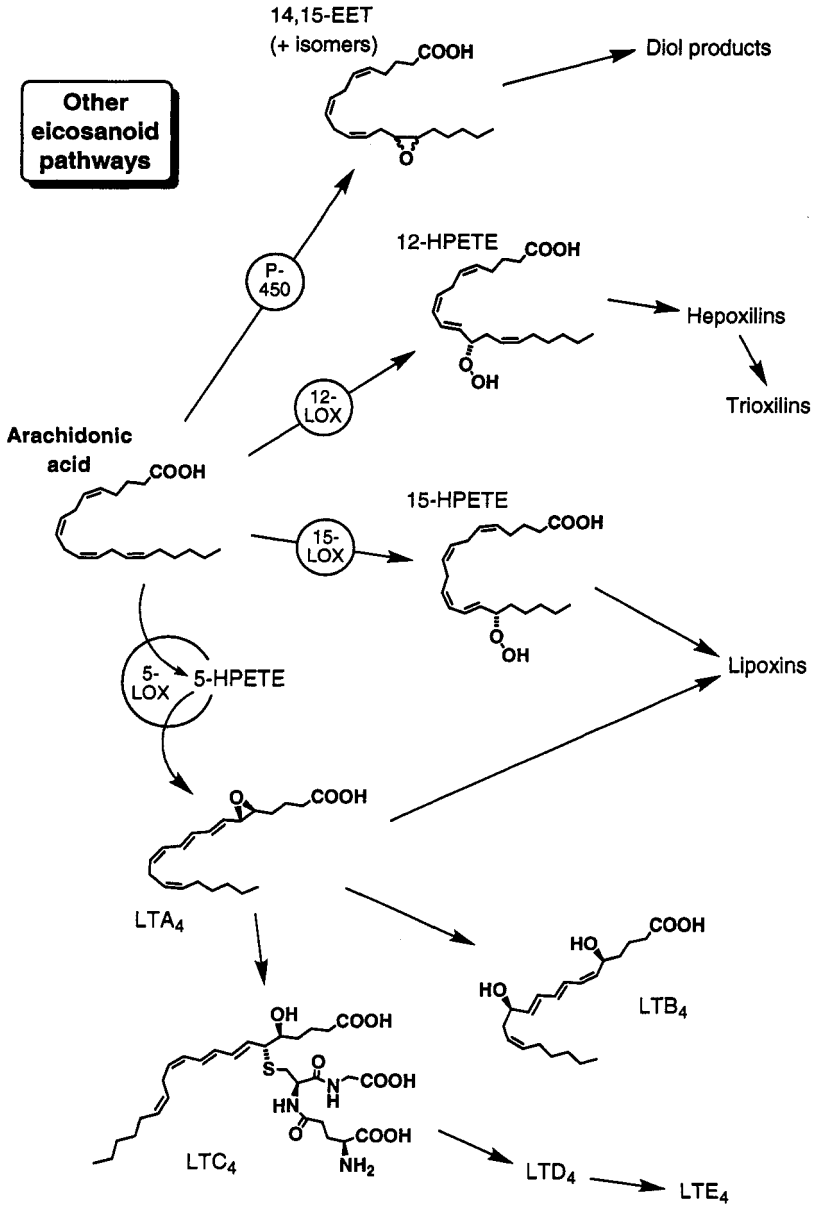


Figure 2. Metabolism of arachidonic acid by 5-, 12- and 15-lipoxygenase pathways and by cytochrome P-450 mono-oxygenase pathway. The latter enzyme system can oxygenate each of the double bonds in arachidonic acid to yield four epoxy-eicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) each with two stereoisomers.

3. DISCOVERY OF PROSTACYCLIN

At this point it is rewarding to consider how prostacyclin was actually discovered, since the simplicity of the methods used contrasts sharply with the complexities of the chemical investigations just described. Over a number of years, a research team led by Sir John Vane at the Royal College of Surgeons of London had perfected the cascade superfusion technique for detecting and quantifying biologically-active substances released from organs perfused with either a Ringer solution or blood.[46,47] The essence of the technique is that a substance in the effluent from an organ generates a distinctive response pattern as its passes over two or three isolated smooth muscle preparations arranged vertically above one another (Fig. 3).

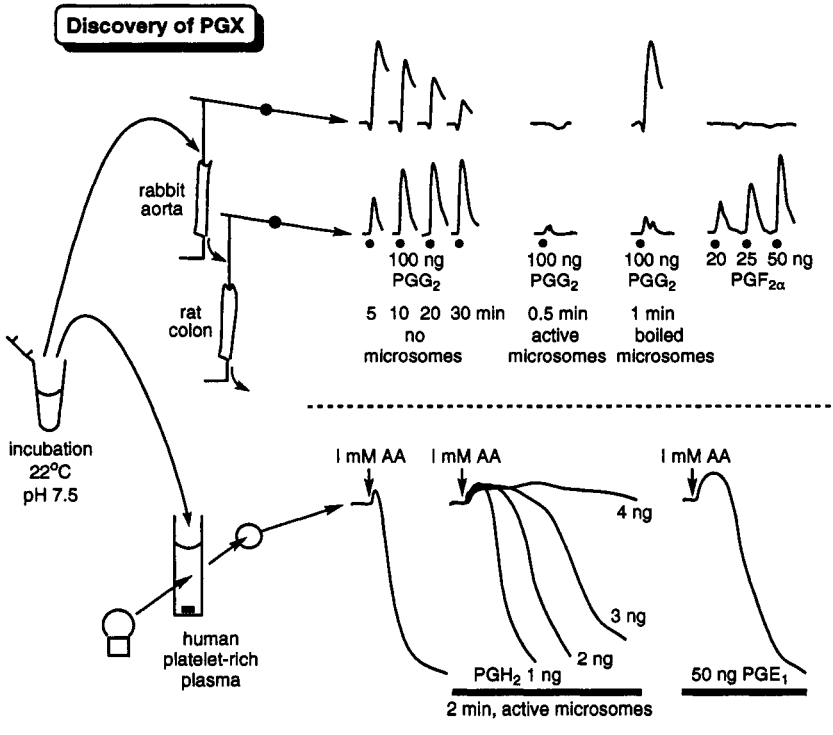


Figure 3. Generation of PGX (subsequently called prostacyclin) from PGG_2 or PGH_2 by microsomes from rabbit aorta. The upper panel shows isotonic contractions of superfused rabbit aorta and rat colon. The lower panel shows light transmission responses of human platelet-rich plasma; the aggregating agent is arachidonic acid (AA). Redrawn from Moncada et al.,[48] with permission.

Judicious choice of preparations allows more than one agent to be detected simultaneously, and also increases the chance of detecting a new endogenous agent. In addition, the "instantaneity" of the method[49] allows the detection of unstable substances, which would be unlikely to survive an extraction procedure. Receptor antagonists can also be included in the superfusion fluid to annul the actions of particular agents.

Using the cascade superfusion method, Piper & Vane[46] demonstrated the release of an unstable "rabbit-aorta contracting substance" (RCS) from isolated guinea-pig lungs during anaphylaxis. Further experiments by Gryglewski & Vane[50,51] indicated that the contractile activity of RCS was similar to that of PGG_2 and PGH_2. However, the biological half-lives of RCS (< 2 min) and PGG_2/PGH_2 (~ 5 min) were different. This and other findings stimulated Samuelsson to search for another unstable contractile agent (see Moncada et al.[47]). Through a brilliant piece of chemical detective work, TXA_2 was identified from the incubation of PGG_2/PGH_2 with washed human platelets.[52]

Vane's group soon showed however that not all artery strips responded to prostaglandin endoperoxides and TXA_2 in the same way.[53] On superfused rabbit coeliac and mesenteric arteries, TXA_2 was always contractile, whereas the endoperoxides induced transient contraction followed by relaxation. It was also found that incubation of either PGG_2 or PGH_2 with rabbit aorta microsomes caused contractile activity on rabbit aorta to disappear rapidly, without a corresponding increase in PGE_2- or $PGF_{2\alpha}$-like activity on the rat colon (Fig. 3, upper panel).[48] In the absence of microsomes, the activity of PGG_2 and PGH_2 decayed much more slowly and PGE_2 and $PGF_{2\alpha}$ were detected. The aortic microsomal activity appeared enzymatic in nature since short-term incubation of PGG_2 with boiled microsomes did not affect its contractile activity. The formation of a novel prostaglandin endoperoxide metabolite seemed likely. Conclusive evidence for this came from a different bioassay system involving addition of small quantities of a PGH_2/aortic microsome incubation mixture to human platelet-rich plasma prior to challenge with arachidonic acid (Fig. 3, lower panel). The aggregation response to arachidonate was delayed and with increasing dosage abolished. The new agent, which was called PGX, was a more potent inhibitor of platelet aggregation than either PGD_2 or PGE_1, and could be distinguished from the former by its instability. It was suggested that the generation of PGX by blood vessels could account for their inherent ability to resist platelet adhesion. The structure of PGX was elucidated in collaboration with chemists at the Upjohn Company, and the name prostacyclin was applied.[54]

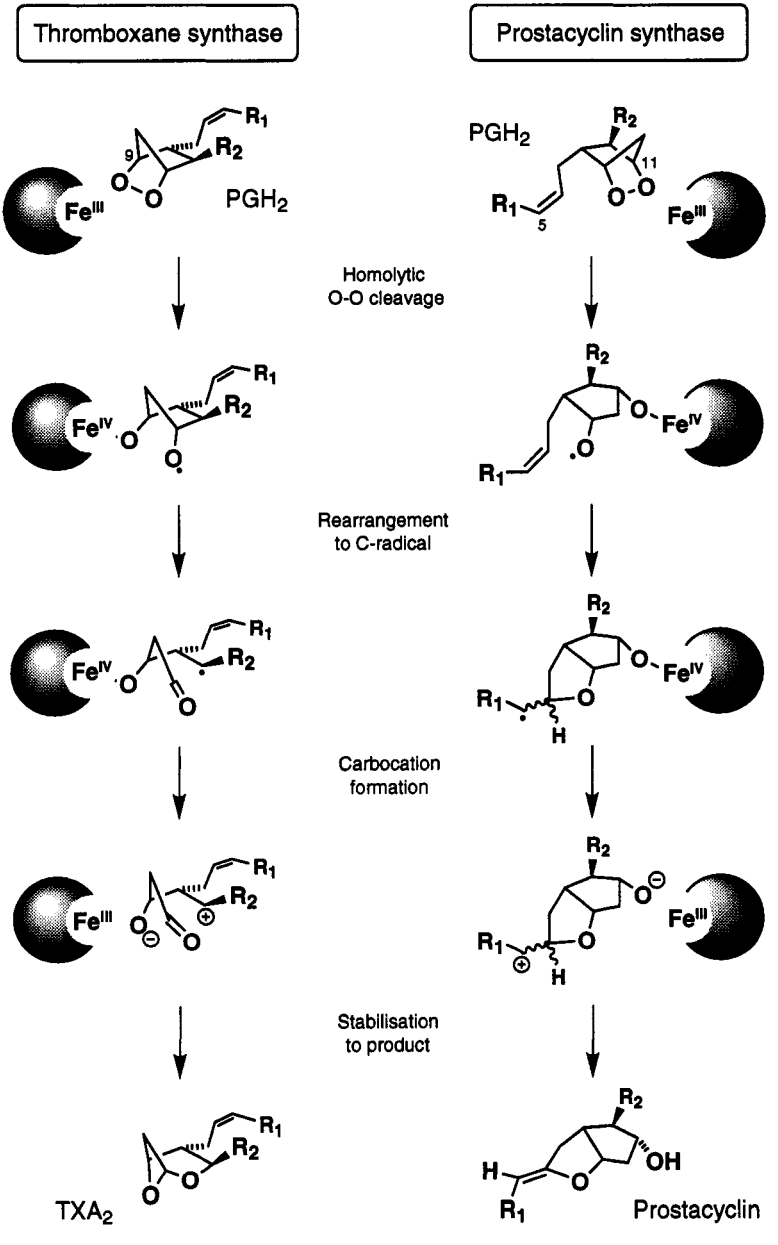


Figure 4. Conversion of PGH_2 to TXA_2 by thromboxane synthase (left reaction scheme) and to prostacyclin by prostacyclin synthase (right reaction scheme). R_1 = -$(CH_2)_3$-COOH, R_2 = complete prostaniod ω- chain. Modified from Tanabe & Ullrich,[55] with permission.

Although TXA_2 and prostacyclin often have diametrically opposite biological actions, they are derived from PGH_2 by similar P-450 mono-oxygenase mechanisms[55,56] In the primary step, the ferric atom of thromboxane synthase co-ordinates with the C9-oxygen of PGH_2 to generate a C11-oxygen radical (Fig. 4, left pathway); prostacyclin synthase on the other hand co-ordinates with the C 11-oxygen of PGH_2 to generate a C9-oxygen radical (right pathway). Subsequent steps involve rearrangement to a carbon radical, formation of a positively-charged carbon centre, and stabilisation to give the product. In the prostacyclin synthase mechanism, the C9-oxygen radical attacks the 5,6-double bond, and in the final step a new double bond is formed with a geometry that gives prostacyclin its unique shape (see Chapter 2). Prostacyclin synthase is inhibited by 15-HPETE (IC_{50} = 1.4 μM), whereas 12-HPETE is inactive (zero inhibition at 300 μM).[57]

4. PROSTANOID RECEPTOR CHARACTERISATION

As early as the mid-l960's, PGE_1, PGE_2 and $PGF_{1\alpha}$ had been shown to have distinctive pharmacological profiles, explicable in terms of interactions with different receptors.[58] There was however some reluctance to talk about prostaglandin receptors. Many pharmacologists doubted that prostaglandins acted on cell surface receptors in the same way that other smooth muscle-active agonists such as acetylcholine and catecholamines did. Physico-chemical mechanisms were thought to be more likely, and jibes such as "which of your hair-oils are you testing today?" are well remembered. These attitudes were to change over the next 15 years.

In the 1970's, binding experiments with tritium-labelled PGE_1, PGE_2 and PGF established the existence of stereospecific, saturable binding sites in many tissues.[59] Over the same period, researchers in several laboratories used the classical pharmacological approach of comparing the potency rankings of natural agonists and selective mimetics to distinguish different prostanoid receptors. In the main, isolated smooth muscle preparations were used, although platelet aggregation experiments also yielded important information. These studies culminated in the proposal in 1982 by Coleman and colleagues at the Glaxo Research Laboratories of a receptor classification scheme in which there are 5 types of prostanoid receptors.[60] These are denoted as DP, EP, FP, IP and TP, indicating the greater potency (by at least 10-fold) on specific preparations of each of the primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (Fig. 5). Receptor

subtypes were designated by subscript numerals, and have been best characterised for the EP-receptor.

Molecular biological studies have confirmed the pharmacological classification and also provided a more definitive picture. In 1989, the purification of the TP-receptor present in human platelets was reported[61] and in 1991 its cDNA was cloned.[62] The TP-receptor was identified as a member of the G-protein-coupled rhodopsin-type receptor superfamily, characterised by seven relatively lipophilic polypeptide domains spanning the plasma membrane. Subsequently, other prostanoid receptors and their subtypes were cloned and shown to belong to the rhodopsin superfamily.[63] The technique of amino acid sequence comparison has been used to infer the phylogeny of the prostanoid receptors and to explain their known preferences for particular second messenger systems (Fig. 5).[64,65]

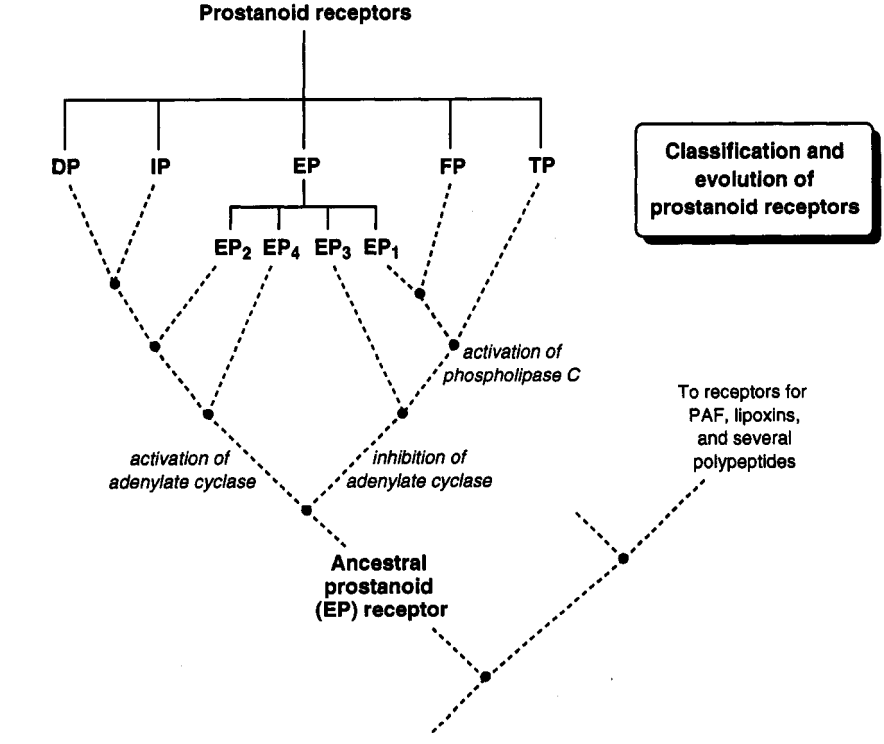


Figure 5. Correlation of the proposed evolution and current classification scheme of prostanoid receptors. The lower portion of the figure shows a phylogenetic tree based on amino acid sequence comparisons; second messenger systems are shown in italics. The upper portion shows the pharmacological classification scheme based on agonist potency ranking and antagonist studies.

It is suggested that the ancestral prostanoid receptor responded to PGE_2 generated in the COX pathway, and that the second messenger system was adenylate cyclase. One branch of the phylogenetic tree contained a receptor that activated adenylate cyclase via G_s, and further branching gave rise to two of the four EP-receptor subtypes, EP_2 and EP_4, and to DP- and IP-receptors; their respective natural ligands all show potent smooth muscle relaxant activity. Interestingly, a single amino acid change in the seventh transmembrane domain of the EP_2-receptor results in a selective gain in function for the IP agonist iloprost.[66] The other main prostanoid branch involved inhibition of adenylate cyclase via G_i; the EP_3-receptor retained this link, while EP_1, FP and TP-receptors graduated to a new second messenger system, G_q/phospholipase C. The natural ligands for the latter three receptors are all potent contractile agents on smooth muscle. EP_3-receptors show the greatest diversity of biological action and this may be partly due to the existence of isoforms that differ in the structure of their cytosolic carboxyl termini.[67] The isoforms, which arise by alternative splicing during RNA transcription, couple to other G-proteins/second messenger systems besides G_i/adenylate cyclase, and show different rates of desensitisation and levels of constitutive activity.[68-70]

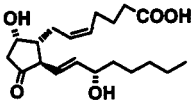
The Receptor and Ion Channel Nomenclature Supplement accompanying the January 1999 issue of Trends in Pharmacological Sciences (TIPS) contains a revised version of the prostanoid receptor classification scheme and also provides an up-to date list of agonists and antagonists suitable for studying prostanoid receptors. Some of the important pharmacological findings relating to each prostanoid receptor type are described below and summarised in Figures 6-8.

4.1 DP-receptors

Early studies showed that both PGD_2 and prostacyclin elevated cyclic AMP levels in human platelets and both agents inhibited aggregation. However, PGD_2 was very much less potent than prostacyclin on rat platelets, indicating the presence of a DP-receptor in the human platelet only.[71-75] A cyclohexyl unit in the ω-terminus is well accommodated by the DP-receptor, as shown by the high potency of the selective DP-receptor agonist ZK 110841 .[76,77] The same moiety is also present in the high affinity DP-receptor antagonist BW A868C,[78] which was developed from BW 245C, another selective DP agonist with an hydantoin ring system.[79] DP-receptor subtypes probably exist,[80-82] but no formal subdivision has yet been proposed.

DP-receptor

Endogenous prostanoid



PGD_2

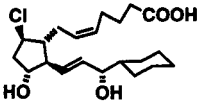
Transduction

G_s / activation of adenylate cyclase

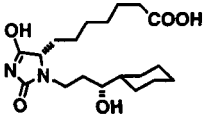
Biological actions

Inhibition of platelet aggregation
Vasodilatation
(Vasoconstriction)

Selective agonists

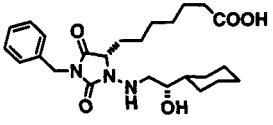


ZK 110841



BW 245C

Antagonist



BW A868C
$pA_2 = 9.3$

Comments

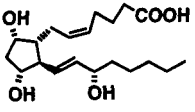
DP-receptors present on human but not rat platelets

Receptor subtypes likely

Dehydration of PGD ring gives PGJs, which have different biological actions

FP-receptor

Endogenous prostanoid



$PGF_{2\alpha}$

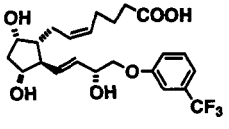
Transduction

$G_{q/11}$ / activation of phospholipase C, Ca^{2+} influx

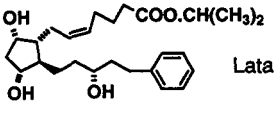
Biological actions

Contraction of uterine, intestinal and ocular smooth muscle
Luteolysis

Selective agonists



Fluprostenol



Latanoprost

Antagonist

None

Comments

Other 16-phenoxy PGF analogues have potent EP_1 and TP agonist activity

Later PGF analogues developed for lowering of intra-ocular pressure in man

Figure 6. Ligands and characteristics of DP- and FP-receptors.

4.2 EP-receptors

PGE_2 has by far the largest variety of biological actions. Initially two receptor subtypes, EP_1 and EP_2, were proposed, mainly to account for contractile and relaxant effects respectively on smooth muscle preparations. The action of PGE_2 at the EP_1-receptor is blocked competitively by SC 19220, a dibenzoxazepine synthesised in the laboratories of G.D. Searle and investigated well before the emergence of the current classification scheme.[83,84] The PGE analogue sulprostone was identified as a selective EP_1 agonist, but its effects on chick ileum (contraction) and guinea-pig vas deferens (inhibition of transmitter release) were not blocked by SC 19220; in addition, the potent EP_2 agonist misoprostol was also highly active on the these two preparations. It became clear that another EP-receptor (EP_3) was necessary to account for the discrepancies.[85] 17-Phenyl-ω-trinor PGE_2 and sulprostone show some selectivity for EP_1- and EP_3-receptors respectively (Fig. 7).[86] However, any characterisation study should include SC 46275, the only highly selective EP_3 agonist reported to date.[87] Finally, the EP_2-receptor was diversified into EP_2 and EP_4 subtypes: butaprost and AH 13205 are selective agonists for EP_2-receptors but are only moderately potent, and there are no selective agonists for EP_4-receptors.[88]

Returning to EP-receptor antagonists, potency within the Searle dibenzoxazepine series has been increased,[89] and a xanthone carboxylic acid AH 6809 has found use as a moderately potent EP_1 antagonist,[85,90] although it blocks DP- and EP2-receptors as well.[64,91] The potent TP antagonist AH 23848 is also a weak antagonist at EP4-receptors.[88,92] Overall, however, we do not possess an arsenal of potent and subtype-specific EP antagonists, which is surprising given the great strides made in antagonist development for other G-protein-coupled receptors over the past 30 years.

4.3 FP-receptors

The finding that $PGF_{2\alpha}$ was involved in controlling oestrus cycle length in the guinea-pig and sheep through regression of the corpus luteum suggested a possible contraceptive use in woman.[93,94] This stimulated much research into metabolically-stable PGF analogues with reduced contractile (EP) activity on smooth muscle. One of the agents developed, 16-*m*-trifluoromethylphenoxy-ω-tetranor $PGF_{2\alpha}$ ICI 81008, fluprostenol),[95] has been adopted as a selective FP agonist for receptor characterisation studies. It is somewhat more selective than the corresponding 16-*m*-chlorophenoxy analogue (ICI 80996, cloprostenol) and much more selective than the 16-*p*-fluorophenoxy analogue (ICI 79939), which is a potent EP1 and TP agonist.[96]

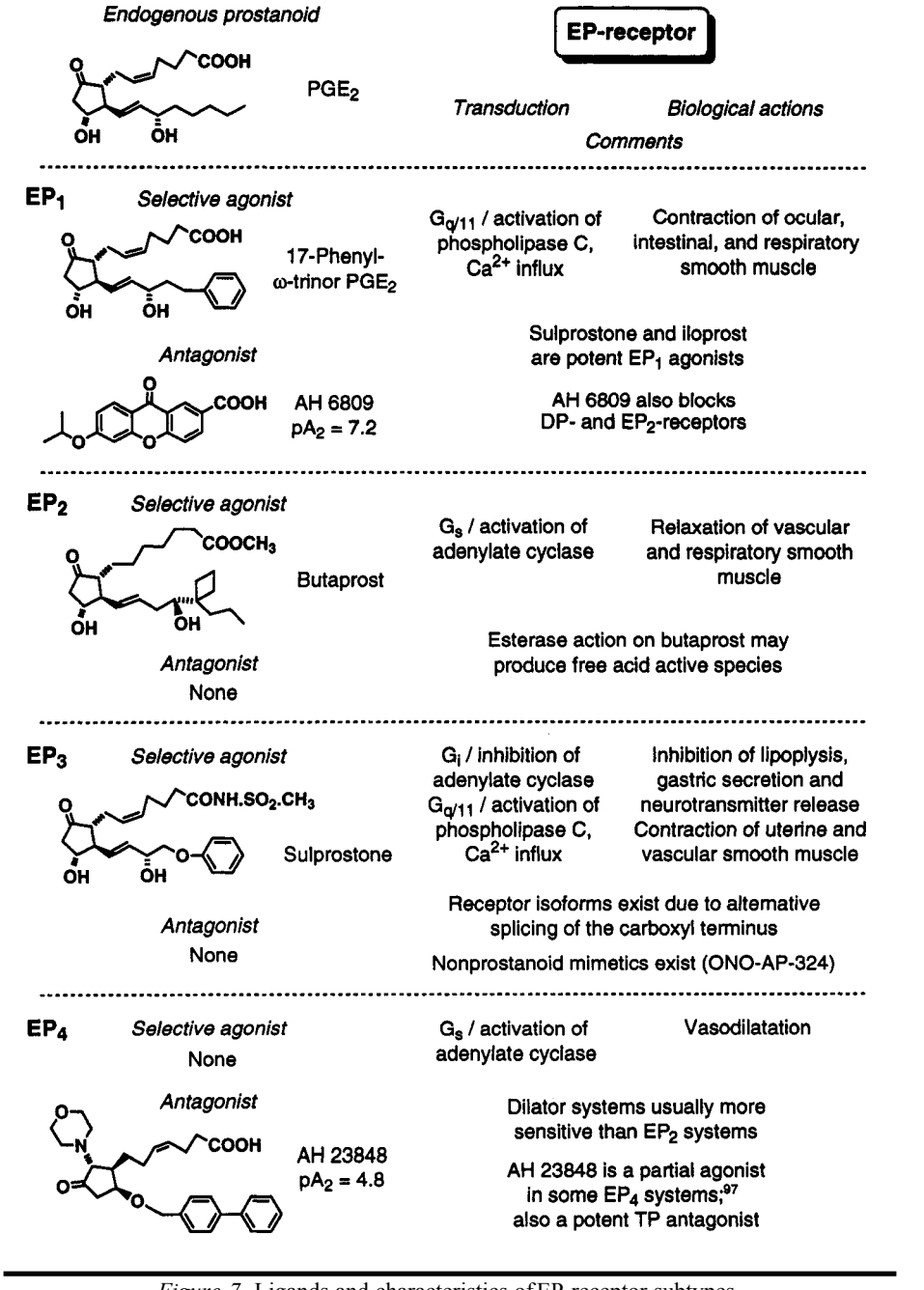


Figure 7. Ligands and characteristics of EP-receptor subtypes.

4.4 IP-receptors

Information on IP-receptors is found throughout the monograph and particularly in Chapters 4 and 5. Suffice to say at this point that the major group of IP receptor agonists is the "carbacyclins", in which the ring oxygen in prostacyclin is replaced with a methylene group. Iloprost,[98] which exhibits the high potency of prostacyclin as an inhibitor of platelet aggregation and a vasodilator,[99] has been studied the most. However, it has the disadvantage of having potent agonist activity at EP_1-receptors and to a lesser extent at EP_3-receptors.[86,100] At present, cicaprost (Fig. 8) is the most selective IP agonist available.

IP-receptors can be activated by a group of carboxylic acids with either a 1,1 -diphenylmethyl or a 1,2-diphenylethyl a-terminus. Since they show little structural similarity to prostacyclin, they have been called nonprostanoid prostacyclin mimetics (see Chapter 3).

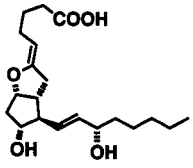
4.5 TP-receptors

Both PGH_2 and TXA_2 are potent agonists at TP-receptors, inducing platelet aggregation, bronchoconstriction and vasoconstriction. U-46619, a chemically-stable analogue of PGH_2[101] is usually employed as a selective TP agonist,[102] although the thromboxane analogue STA_2 (Chapter 2, Fig. 12) has also been used.[103]

All-carbon ring analogues of TXA_2 and PGH_2 are partial agonists at TP-receptors and this has been the starting point for the development of TP antagonists such as EP 169 (see Chapter 3, Fig. 21).[104,105] However, quite a diverse range of molecules afford thromboxane block and the area has been reviewed by Hall[62] and Armstrong & Wilson.[106]

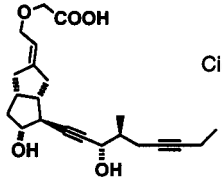
IP-receptor

Endogenous prostanoid

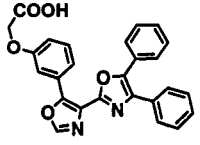


Prostacyclin

Selective agonists



Cicaprost



BMY 45778

Antagonists

Under development

Transduction

G_s / activation of adenylate cyclase

Biological actions

Inhibition of platelet aggregation
Vasodilatation
Activation of neurones

Comments

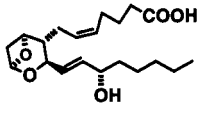
Some prostacyclin analogues (iloprost) are also potent EP_1 agonists

Nonprostanoid prostacyclin mimetics exist (BMY 45778, ONO-1301)

Evidence for receptor subtypes in rat brain and rat enteric nervous system

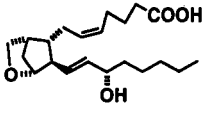
TP-receptor

Endogenous prostanoid



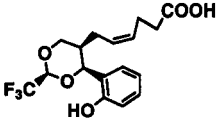
TXA_2

Selective agonist



U-46619

Antagonist



ICI 185282
$pA_2 = 8.5$

Transduction

$G_{q/11}$ / activation of phospholipase C, Ca^{2+} influx

Biological actions

Platelet aggregation
Vasoconstriction
Bronchoconstriction

Comments

PGH_2 also activates TP-receptors

All-carbon ring analogues of PGH_2 and TXA_2 (CTA_2) are often partial agonists

Large range of potent antagonists available (EP 169, SQ 29548, GR 32191, Bay u3405)

Figure 8. Ligands and characteristics of IP- and TP-receptors.

5. NONSTEROIDAL AND STEROIDAL ANTI-INFLAMMATORY DRUGS

One of the greatest boosts to prostanoid research was the discovery in 1971 by Vane and colleagues[107,108] and by Smith & Willis[109] that the nonsteroidal anti-inflammatory drugs (NSAIDs or aspirin-like drugs) inhibit COX.[110] Thus the therapeutic efficacy of the NSAIDs could be largely explained by reduction of the biosynthesis of pro-inflammatory prostanoids. Traditionally, PGE_2 has been considered as the prime mediator through its ability to dilate arterioles and enhance plasma exudation and pain.[111] However, in Chapter 9 (sensory neurones) we explore the possibility that in many situations prostacyclin rather than PGE_2 may serve this function.

The biosynthesis of prostanoids in inflamed tissue can be activated by several inflammatory mediators. For example, bradykinin, platelet-activating factor (PAF) and interleukin-1 act on cell surface receptors to elevate intracellular Ca^{2+}, which in turn activates PLA_2 (Fig. 1). The 85kD Ca^{2+}-sensitive cytosolic form, $cPLA_2$, acting on the endoplasmic reticulum and nuclear envelope, is thought to be most important.[112] $cPLA_2$ can also be activated by the phosphorylating activity of MAP kinase, especially in the presence of Ca^{2+}.[113] The link between the cell surface receptor (e.g. PAF-receptor) and MAP kinase may be either a G_q/PLCβ/protein kinase C or a G_i/MEK kinase/MAP kinase kinase system.[112,114,115] Cytokines (IL-1 and TNFα) and mitogens (macrophage colony-stimulating factor, M-CSF) can also enhance eicosanoid biosynthesis by increasing the nuclear expression of $cPLA_2$ and COX.

The importance of COX expression has become clearer of late with the demonstration that cells can produce two isozymes of COX, termed COX-1 and COX-2. In general, COX-I is a constitutive enzyme responsible for producing prostanoids that serve a physiological function, whereas COX-2 is an inducible form producing prostanoids that contribute to pathological processes.[116-120] Investigation of well-established NSAIDs showed that aspirin, indomethacin and piroxicam have a degree of COX-1 selectivity, whereas flurbiprofen, naproxen and diclofenac have a degree of COX-2 selectivity.[121,122] Over the same period, the chemical development of NSAIDs within the pharmaceutical industry was dramatically reactivated for the purpose of identifying more selective COX-2 inhibitors. One such agent, meloxicam, was recently introduced into clinical practice, and appears to show less gastro-intestinal side-effects than previously used agents.[123]

Aspirin, the oldest of the NSAIDs, has a therapeutic use not shared by its companions. At low-to-moderate oral doses (0.3 - 3 mg kg^{-1} day^{-1}),[124] it reduces myocardial infarction in at-risk-patients.[125,126] The rationale for this convenient, cheap and relatively safe therapy involves the high sensitivity of

COX in human platelets to irreversible inhibition by aspirin. Following absorption from the intestine, aspirin acetylates COX in platelets present in the hepatic portal circulation, thus preventing the generation of PGH_2 and TXA_2 when these platelets reach thrombogenic sites in pulmonary and systemic vascular beds. However, dilution with systemic blood coupled with extensive metabolism means that plasma levels of aspirin in pulmonary and systemic beds remain low; prostacyclin biosynthesis by blood vessels and the consequent vasodilatation and suppression of platelet aggregation are therefore not compromised. The success of low-to-moderate dose aspirin therapy has meant that the clinical application of TP antagonists in cardiovascular disease has been less than anticipated.

Steroidal anti-inflammatory drugs, such as hydrocortisone, prednisolone and dexamethasone, also impinge on eicosanoid pathways. Firstly, they can suppress expression of COX-2 in inflamed tissues.[127] Secondly, they can induce expression of lipocortin, a polypeptide with PLA_2 inhibitory properties.[128-130] These actions contribute to their powerful anti-inflammatory effects by decreasing the production of COX- and 5-LOX-derived mediators, as well as PAF.

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Chapter 2

The development of prostacyclin analogues

1. INTRODUCTION

Since the discovery of prostacyclin in 1976, hundreds of its analogues have been synthesised and biologically evaluated. The reasons for this are three-fold. Firstly, prostacyclin's biological half-life of a few minutes is only advantageous in certain clinical situations. For example, when prostacyclin is used in an extracorporeal circuit to reduce platelet consumption (see Chapter 5), spill-over into the systemic circulation does not result in serious hypotension. For most therapeutic purposes however, a longer action has been considered desirable and drug development programs have led to many chemically and metabolically stable analogues.[1-5] Secondly, there have been numerous attempts to alter the biological specificity of prostacyclin, particularly to maintain its platelet inhibitory potency while reducing its vasodilator potency. Thirdly, there has been the intellectual challenge of the chemistry itself how can one elegantly construct a synthon related to the bicyclic ring of prostacyclin (Fig. 9), which also allows the α- and ω-chain structures to be easily modified?

We shall discuss some of these strategies in this chapter, and also describe the prostacyclin-like activities of other prostanoids, including PGE_1 and its analogues, and certain analogues of PGH_2/TXA_2. In addition, we will attempt to explain what this large amount of information tells us about the receptor-active conformation(s) of prostacyclin.

In many cases, information on the biological activities of the prostacyclin analogues shown in Figures 10 - 18 is meagre, often consisting of a few lines at the end of a chemical paper. In assessing the relative potencies of

these analogues, it may be assumed that prostacyclin has IC_{50} values of about 2,4 and 10 nM as an inhibitor of ADP-induced aggregation in human, rat and rabbit platelet-rich plasmas (PRP) respectively.

2. NOMENCLATURE OF PROSTACYCLIN ANALOGUES

The unique fused ring structure of prostacyclin can create difficulties in naming analogues simply and correctly. It is often convenient to use trivial names, such as iloprost and taprostene, as well as drug company codes such as RS-93427. However, more information can be conveyed by using PGI_2 as the stem name and indicating the positions of structural changes based on α-carboxyl = 1 and terminal ω-methyl = 20. This method works well for simple changes, such as introduction of a methyl group at C15 (15-methyl PGI_2) or conversion of the 13,14-double bond to a triple bond (13,14-didehydro PGI_2), and we have attempted to use it whenever possible. An alternative stem is prostanoic acid, a "template molecule" in which the chains attached to the cyclopentane ring at C8 and C12 have α and β orientations (down and up relative to the picture plane in Fig. 9) respectively, as in all prostanoids produced by the cyclo-oxygenase (COX) pathway. However, both these systems become cumbersome when the bicyclic ring is radically altered (see Fig. 12) and it may be more economical to use the IUPAC name; examples for the commonly encountered prostacyclin analogues may be found in Martindale's Extra Pharmacopoeia.

It is worth mentioning a few more points about structure and nomenclature here.

(a) The configuration of a chiral centre in a prostacyclin analogue can often be specified by the α/β method (e.g. 7β-fluoro PGI_2, Fig. 11). However, where ambiguity could arise the absolute system of Cahn, Ingold and Prelog is preferred. An important example is the configuration at C15 in the ω-chain (Fig. 9). All mammalian *natural* prostanoids have the 15S (as opposed to the 15R) configuration and this configuration usually confers the greater bioactivity and also susceptibility to oxidation by 15-hydroxy-prostaglandin dehydrogenase (15-PGDH) (Fig. 15).

(b) Replacement of the oxygen atom in the furan ring of prostacyclin by a methylene group gives carbacyclin (6a-carba PGI_2 or 6(9)-methano PGI_2), the archetype of an important series of analogues called the carbacyclins.

(c) The geometry of the 5,6-double bond in prostacyclin analogues cannot be unambiguously described by the simple *cis/trans* system. Again we must resort to the atomic number priority rules of the Cahn-Ingold-

Prelog system (Fig. 9). Examination of the groups attached to C5 and C6 in prostacyclin shows that the 4-carbon ranks over the 5-hydrogen and the 6a-oxygen ranks over the 7-carbon respectively. Thus the higher ranking atoms are on the same side (German: *zusammen)* of the double bond giving a Z designation. The corresponding designation for the 13,l4-trans-double bond is E, since the higher ranking atoms are on opposite sides *(entgegen)* of the double bond.

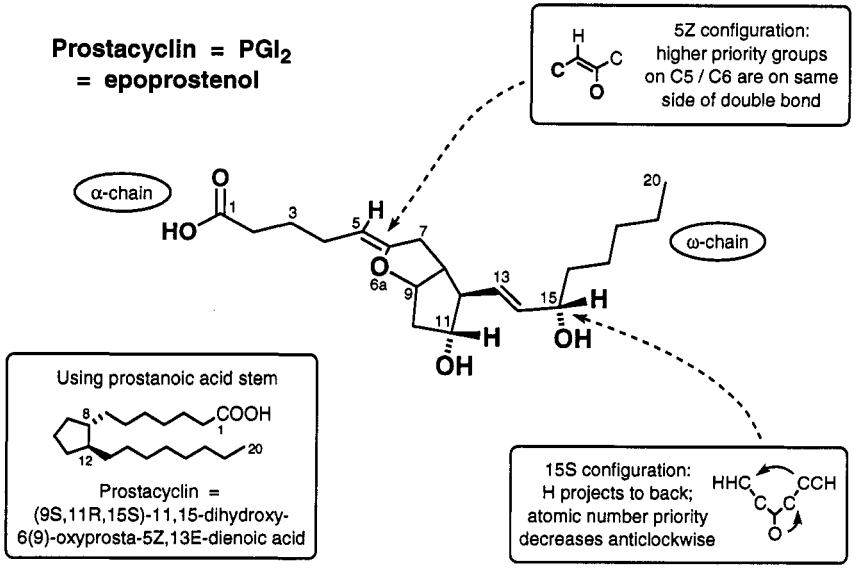

Figure 9. Structure and nomenclature of prostacyclin.

3. CHEMICALLY STABLE PROSTACYCLIN ANALOGUES

3.1 Acid-instability of prostacyclin

The loss of prostacyclin's biological activity in physiological salt solutions ($t_{½}$ = 5 min at pH 7.4) is due to hydrolysis of its vinyl ether unit (Fig. 10).[6] The product, 6-oxo PGF $_{1\alpha}$(preferred name to 6-keto $PGF_{1\alpha}$ can readily interconvert between its cyclic hemiacetal and open-chain oxo forms.[7] As with other vinyl ethers, the rate-limiting step for hydrolysis is

protonation of the *pi*-bond and increasing acidity increases the hydrolysis rate of prostacyclin.[8] Prostacyclin is unusually unstable for a vinyl ether, its $t_{½}$ for acid-catalysed hydrolysis being about six-fold less than that of the model compound 2-methylenetetrahydrofuran (exocyclic double bond). Cho & Allen[8] attributed this difference to release of strain within the bicyclooctane ring system upon protonation at C5. However, during synthesis of 13,14-didehydro PGI_2, it was observed that the methyl ester derivative was much more acid-stable than the free acid, and this led to the suggestion that hydrolysis was facilitated by transfer of a proton from the molecule's own carboxylic acid group (Fig. 10).[9] Correspondingly, the half-lives of the free acid and methyl ester of prostacyclin are 1.7 and 112 min respectively at pH 6.0.[10] Further support for intramolecular proton transfer derives from the greater acid stability of PGI_2 dimethylamide, which has no protonating ability, compared to the corresponding decarboxy-dimethylaminomethyl derivative, which in its quaternary ammonium form can deliver a proton to the double bond (Fig. 10).[11]

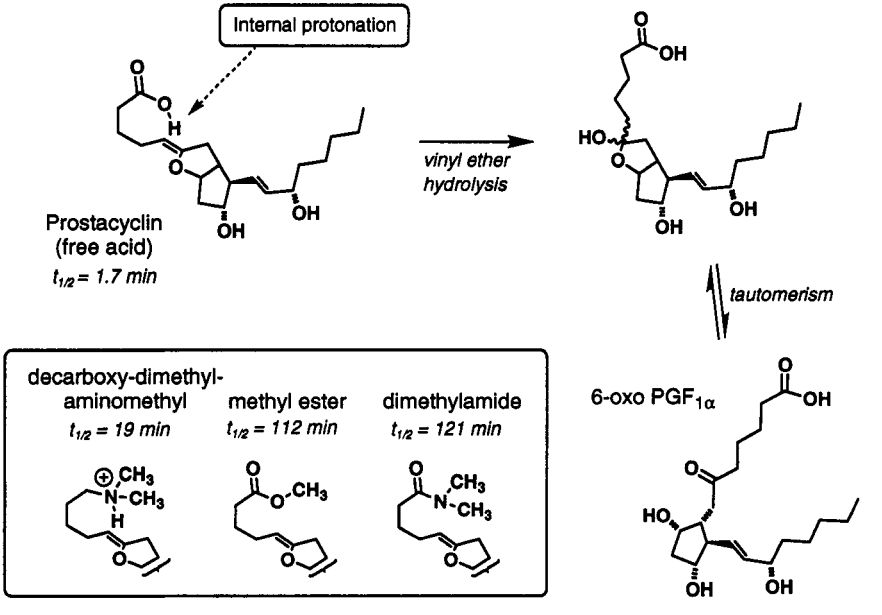

Figure 10. Hydrolysis of prostacyclin due to acid instability of its vinyl ether unit. In the inset, the quaternary ammonium C1-analogue (left) can also internally deliver a proton to the 5,64-double bond and consequently undergoes fairly rapid hydrolysis, whereas the other two C1-analogues are not proton donors and show enhanced stability. The bracket indicates a lower ring and ω-chain identical to prostacyclin.

In the original characterisation studies, prostacyclin was generated enzymatically by incubation of PGH_2 with pig aorta microsomes and then isolated as the methyl ester; the ester is stable on silica gel thin-layer chromatography, and also on Florisil column chromatography provided that the eluting solvent contains a trace of triethylamine base.[6] In the vast majority of studies, prostacyclin generation in tissues has been estimated by measuring 6-oxo $PGF_{1\alpha}$ by radio-immunoassay. More rigorous identification of 6-oxo $PGF_{1\alpha}$ can be achieved by gas chromatography-mass spectrometry after methyl ester formation, oximation of the 6-oxo group, and protection of the three hydroxyls as trimethylsilyl ethers.[12,13]

Ensuring the stability of aqueous stock solutions of prostacyclin is important in biological studies and a common procedure is to dissolve the sodium salt of prostacyclin in 50 mM Tris-HC1 (pH 9.0) buffer, store in aliquots at -20°C, and then dilute a thawed aliquot with ice-cold saline immediately before use. On vascular smooth muscle preparations, prostacyclin's relaxant action is observed to wane over 10 - 20 min, and its biological half-life is estimated to be about 3 - 4 min.[14] Clearly, its potency may be underestimated in isolated tissue preparations, particularly in thick preparations where diffusion into the interior of the tissue is slow. In contrast, the half-life of prostacyclin is extended in blood or plasma[15] probably due to binding within an alkaline micro-environment of the albumen molecule produced by basic amino acids such as lysine, arginine and histidine.[16]

3.2 Stabilisation of the vinyl ether unit

The C1-modified prostacyclin analogues shown in Figure 10 have only minimal biological activity and other ways of increasing the acid stability of the vinyl ether unit (without replacing the 6a-oxygen) have been sought.

The first involves reducing the electron density of the double bond of the vinyl ether so that it is less likely to accept a proton. Substitution of either one or two electron-withdrawing fluorine atoms on or adjacent to the vinyl ether achieves remarkable increases in stability as shown in Figure 11 (upper section).[17-19] The potent prostacyclin mimetic 13,14-didehydro- 10,10-difluoro PGI_2 is of interest since its *gem*-difluoro unit is situated some distance from the 5,6-double bond.[20] Conjugation of the double bond of the vinyl ether with an oxo or cyano group also enhances acid stability by spreading the charge distribution as well as rendering the 5,6-double bond less electronegative (Fig. 1 1, lower section). 7-Oxo PGI_2 retains much of the potency of PGI_2 as both an inhibitor of human platelet aggregation and a relaxant of bovine coronary artery.[21] In contrast, 4-oxo PGI_2 methyl ester is about 400 times less potent than PGI_2 methyl ester as an inhibitor of rabbit

platelet aggregation.[22] The 5-cyano analogue nileprost has only weak IP agonist potency;[23] however the IP-receptor can certainly accommodate the cyano-vinyl ether unit as shown by the 50-fold greater potency of the 18,19-triple bond analogue of nileprost as an inhibitor of ADP-induced aggregation in human PRP.[24]

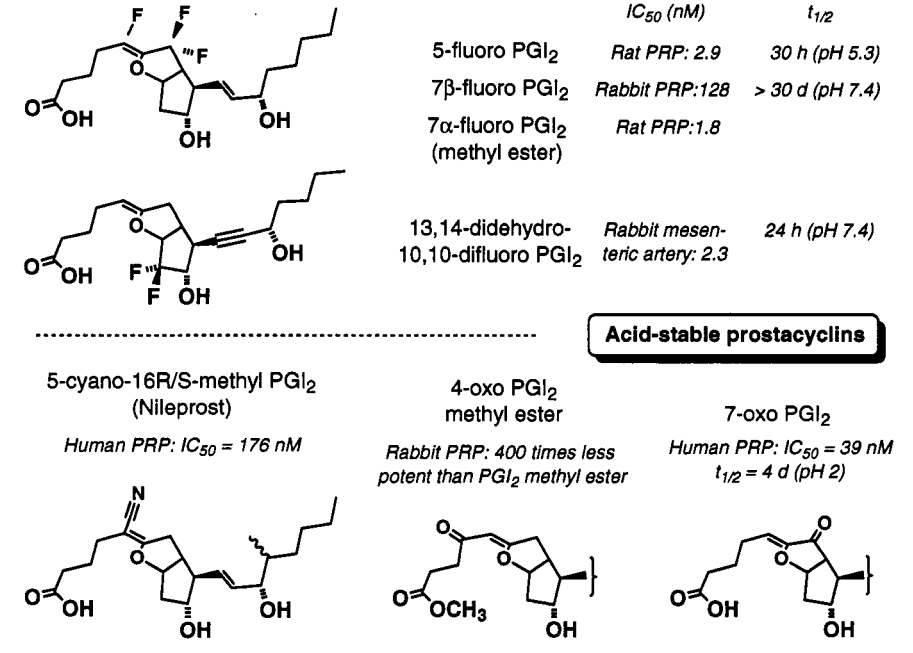

Figure 11. Prostacyclin analogues in which the vinyl ether has been stabilised to acid attack by introduction of an electron-withdrawing group and/or conjugation with another unsaturated group. The vertical bracket indicates an ω-chain identical to that in prostacyclin. IC_{50} values refer to inhibition of ADP-induced aggregation in platelet-rich plasma (PRP) and relaxation of $PGF_{2\alpha}$-induced tone for rabbit mesenteric artery (PGI_2 IC_{50} 3.3 nM).

The second stabilisation method involves transformation of the vinyl ether into a phenyl ether. An example is beraprost (Fig. 12, upper right), a moderately potent IP agonist.[25,26]

The third method involves restricting the mobility of the carboxyl group so that it cannot protonate the 5,6-double bond. This has been achieved in the 1,5-interphenylene series (Fig. 13), where a benzene ring conjugates to both the carboxyl and vinyl ether units. The *meta* (SE 54) and *para* (SE 58) interphenylene analogues of 15-methyl PGI2 are highly stable, whereas in complete contrast the *ortho* analogue (SE 59) is highly unstable since its geometry favours internal protonation of the vinyl ether (Fig. 13).[1]

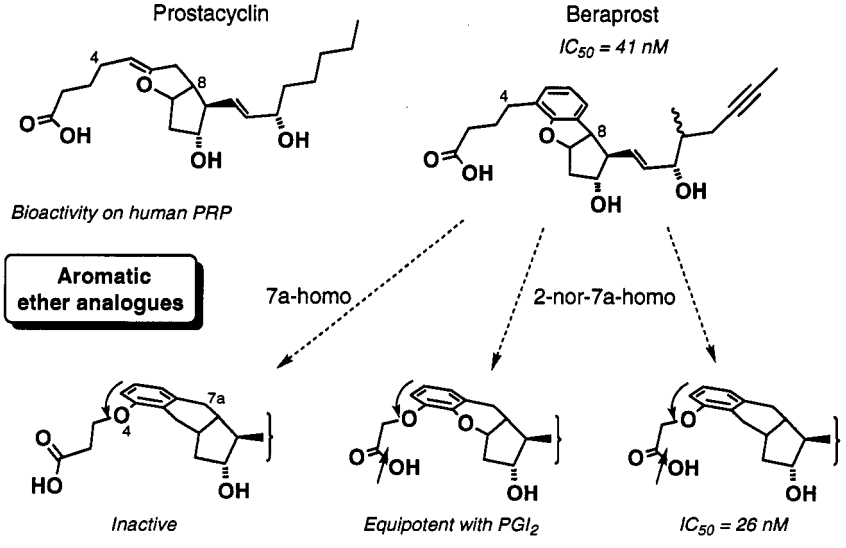

Figure 12. Conversion of the vinyl ether to an aromatic ether to create stable prostacyclin analogues. A curved arrow indicates the movement of the 4-atom due to homologation at C7 and a straight arrow the corresponding adjustment of the 1-carboxylate position due to shortening of the α-chain. The bracket indicates an ω-chain identical to that of prostacyclin.

3.3 The carbacyclins

Replacement of the vinyl ether oxygen with less electronegative atoms has provided a large selection of stable analogues, many of which are highly potent IP agonists. For example, substitution with sulphur gives 6a-thia PGI_2 (Fig. 14, middle left) ($t_{½}$ > 3h at neutral pH and 25ºC), which is 2 - 5 times less potent than prostacyclin as an inhibitor of aggregation in human PRP.[27]

By far the greatest number of prostacyclin analogues are carbacyclins, in which a methylene group has been substituted for the 6a-oxygen, so affording complete chemical stability.[2] 6a-Carba PGI_2 or carbacyclin (Fig. 14, lower left) is about 10 times less potent than prostacyclin.[28-32] Iloprost and cicaprost (Fig. 15) are other potent carbacyclins that have been used as standard IP agonists in many pharmacological studies.

Moving the 5,6-double bond of carbacyclin into the 6,7-position in the ring system maintains the planarity at C6 and obviates the need to separate 5E/5Z geometric isomers during synthesis. However, both the 6a-carba and 6a-thia analogues (Fig. 14, centre) show only weak IP agonist activity.[33,34]

This is not the case when the double bond is switched to the 6(6a)-position in the ring. 6a-Carba-$\Delta^{6(6a)}$PGI$_1$ or isocarbacyclin is a potent IP agonist (Fig. 14, lower right)[35] and the prototype for a large series of isocarbacyclins, some of which may distinguish IP-receptor subtypes (see Section 6.2). For valency reasons, 6a-thia isocarbacyclin cannot exist, but the 6a-aza (Fig. 14, middle right)[36] and 6a-aza-5-thia (upper right)[37] analogues of isocarbacyclin are also potent IP agonists.

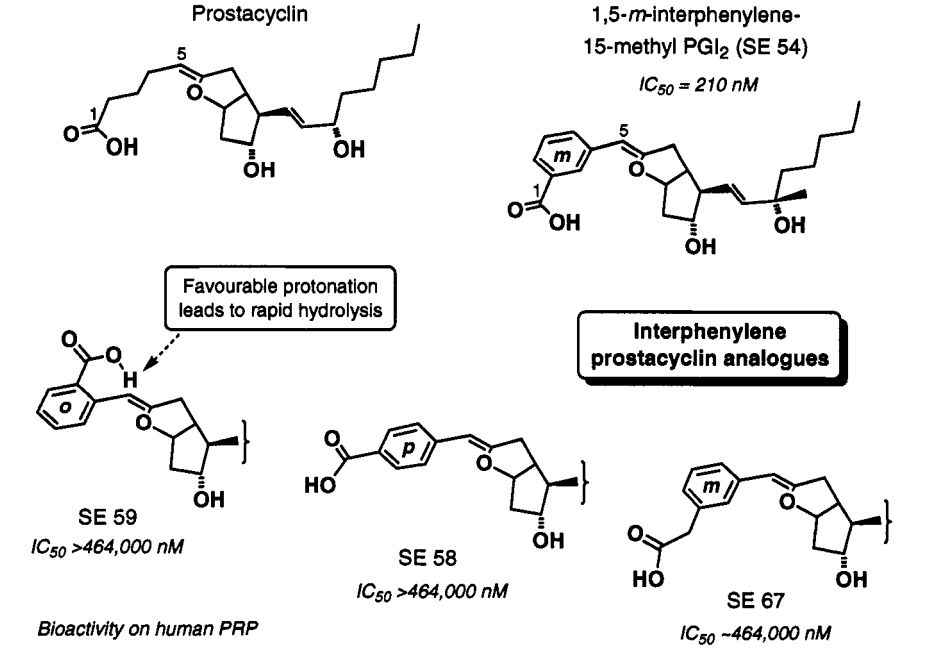

Figure 13. Chemically stable 1,5-*m*-interphenylene prostacyclin analogues, with the exception of the ortho-substituted analogue. The importance of the correct orientation of the I-carboxyl group for high IP$_1$ agonist potency is also shown. IC_{50} values refer to inhibition of arachidonic acid-induced platelet aggregation; >464,000 nM signifies no effect. The bracket indicates an ω-chain identical to that of SE 54.

4. METABOLICALLY STABLE PROSTACYCLIN ANALOGUES

Like other natural prostanoids, prostacyclin is metabolised (to a large extent sequentially) in the following ways: 15-oxidation/13, 14-reduction, β-oxidation of the α-chain and ω-oxidation (Fig. 15).[38-40] When vinyl ether

hydrolysis is included, the end-metabolites are highly water soluble dihydroxy-dicarboxylates. The metabolism of prostacyclin has been reviewed by Granström & Kumlin.[41]

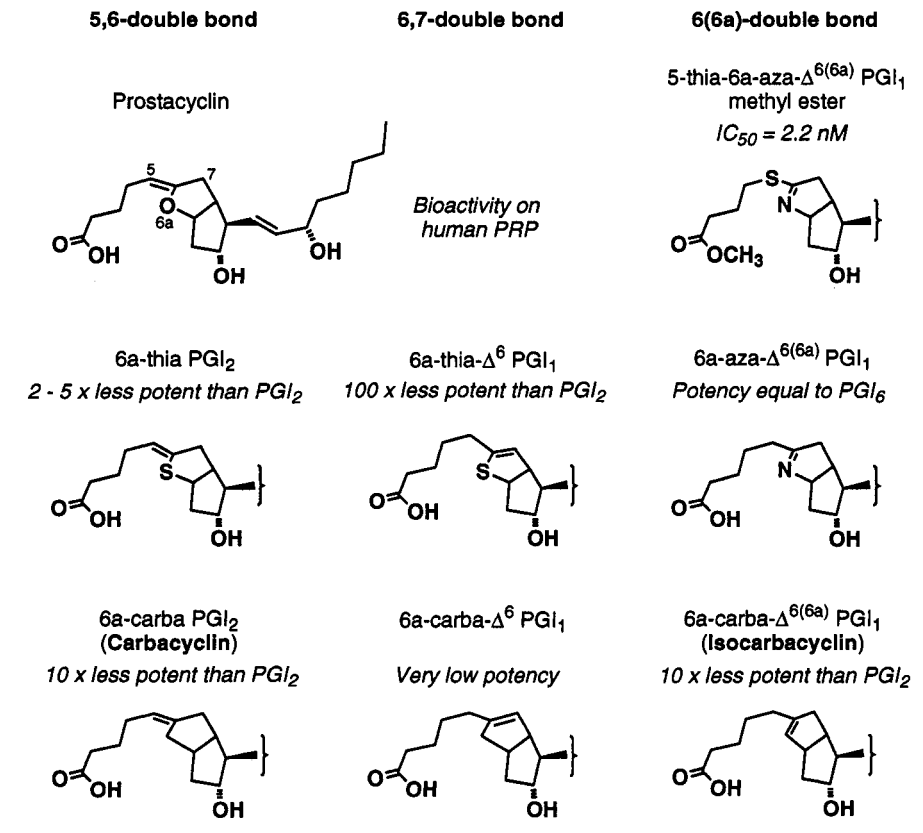

Figure 14. Prostacyclin analogues in which the 6a-oxygen has been replaced by sulphur, nitrogen or carbon to enhance acid-stability. On the left, the double bond is exocyclic to the ring, in the centre it occupies the 6,7-position and on the right the 6(6a)-position in the ring. The bracket indicates an ω-chain identical to that of prostacyclin. Potency values refer to inhibition of ADP-induced aggregation in platelet-rich plasma.

Initially 6-oxo $PGF_{1\alpha}$ was assumed to be the initial substrate for 15-PGDH based on the low chemical stability of prostacyclin. However, in vitro, prostacyclin is at least as good a substrate as PGE_2 for monkey lung 15-PGDH,[39] as good as $PGF_{1\alpha}$ and $PGF_{2\alpha}$ for human placenta 15-PGDH,[1] and a much better substrate than 6-oxo $PGF_{1\alpha}$ in both systems. More direct evidence stems from the elegant studies of Sun & colleagues,[39] in which the metabolism of prostacyclin and 6-oxo $PGF_{1\alpha}$ specifically labelled with

tritium at 15β was studied in conscious rats. 15-PGDH attack on these prostanoids releases tritium, which can be measured in the urine as tritiated water. The authors concluded that "a portion of the administered prostacyclin is oxidised by 15-PGDH before the hydrolysis to 6-oxo $PGF_{1\alpha}$. The remainder which is not oxidised within the short life-span of prostacyclin under physiological conditions was converted to 6-oxo $PGF_{1\alpha}$, which was then further oxidised by I5-PGDH, but at a reduced reaction rate".

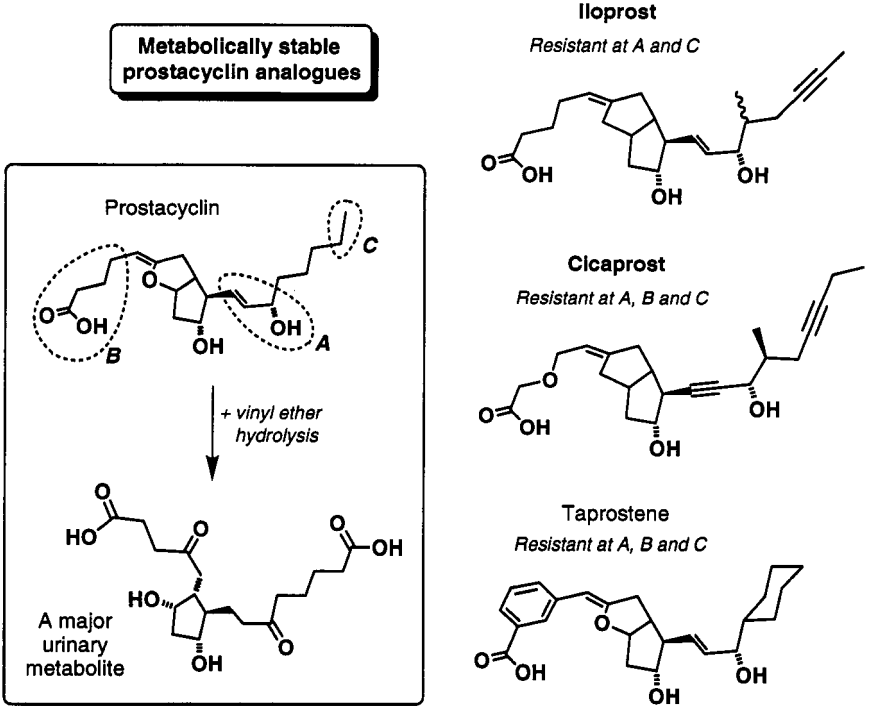

Figure 15. The insert shows the metabolism of prostacyclin by (A) 15-hydroxy-prostaglandin dehydrogenase /Δ 13-prostaglandin reductase, (B) β-oxidation and (C)ω·-oxidation, coupled with vinyl ether hydrolysis, to give a major urinary metabolite. The three analogues on the right were designed to be both chemically and metabolically stable.

The lung is a major site for deactivation of blood-borne PGE_2 and $PGF_{2\alpha}$ by 15-PGDH,[41] but not for prostacyclin since it is not taken into lung cells by their active uptake process(es).[42-44] However, other tissues, including blood vessel wall and kidney, are able to oxidise the 15-hydroxyl of prostacyclin.[39,45]

Figure 15 shows three prostacyclin analogues, iloprost,[46] cicaprost[47,48] and taprostene,[49] that are resistant to 15-PGDH attack and have extended durations of action in vivo. Alkylation at C16 creates analogues that are very poor substrates for human 15-PGDH. Thus the major metabolites of iloprost retain the 15-hydroxyl,[50] and whereas 1,5-m-interphenylene PGI_2 was rapidly oxidised, its 16-ethyl and 15-cyclohexyl-ω-pentanor (taprostene) derivatives had reaction velocities close to zero.[1]

β-Oxidation is the primary route of metabolic inactivation for iloprost, with loss of 4 carbon units leading to a mixture of 6α- and 6β-carboxy metabolites (C6 becomes a new chiral centre).[50] In cicaprost this route is blocked by substitution of an oxygen atom at C3, resulting in a 4-fold slower systemic clearance in man.[48] Similarly, the benzoate moiety of taprostene is resistant to β-oxidation.

In addition to the 20-carboxy metabolite of prostacyclin shown in Figure 15, the corresponding 19- and 20-hydroxy metabolites were also found in urine.[38] These ω-oxidations are reduced in analogues containing either a ω-cyclohexyl group or an 18,19-triple bond, although a 17-hydroxy-α-tetranor metabolite of iloprost was tentatively identified.[50]

5. RECEPTOR-ACTIVE CONFORMATIONS OF PROSTACYCLIN

5.1 The extended conformation of prostacyclin

In prostacyclin, the closed or semi-closed hairpin conformation attainable by the other natural prostanoids is not possible due to the Z-configuration of the 5,6-double bond. Coupled with this, the bicyclic ring of prostacyclin has greater rigidity than the single cyclopentane ring of other prostaglandins. Thus the molecular modelling fraternity felt that predicting the conformations in which prostacyclin and its analogues combine with the IP-receptor would stand a better chance of success than, for example, the conformation of PGE_2 bound to an EP-receptor.

Essentially, the modelling process involves (a) determining which groups in the prostacyclin molecule are likely to bond to the IP-receptor using structure-activity data, (b) computing the minimum energy conformations for a small selection of structurally rigid and potent prostacyclin analogues, and (c) deciding which of the more stable conformers have the greatest overlap of important bonding groups.

Using biological potency data for about 50 prostacyclin analogues, the group led by Tsai & Wu[3,51] concluded that the 1-carboxyl and 11- and 15-hydroxyls of prostacyclin are important groups for binding to the IP-receptor, while the ring structure and 5,6- and 13,14-double bonds are a framework for maintaining a unique spatial orientation of these groups. They then proposed the receptor-bound prostacyclin conformer depicted in Figure 16, in which the α-chain is extended away from the ω-chain rather than at right-angles (the L-shaped conformation).

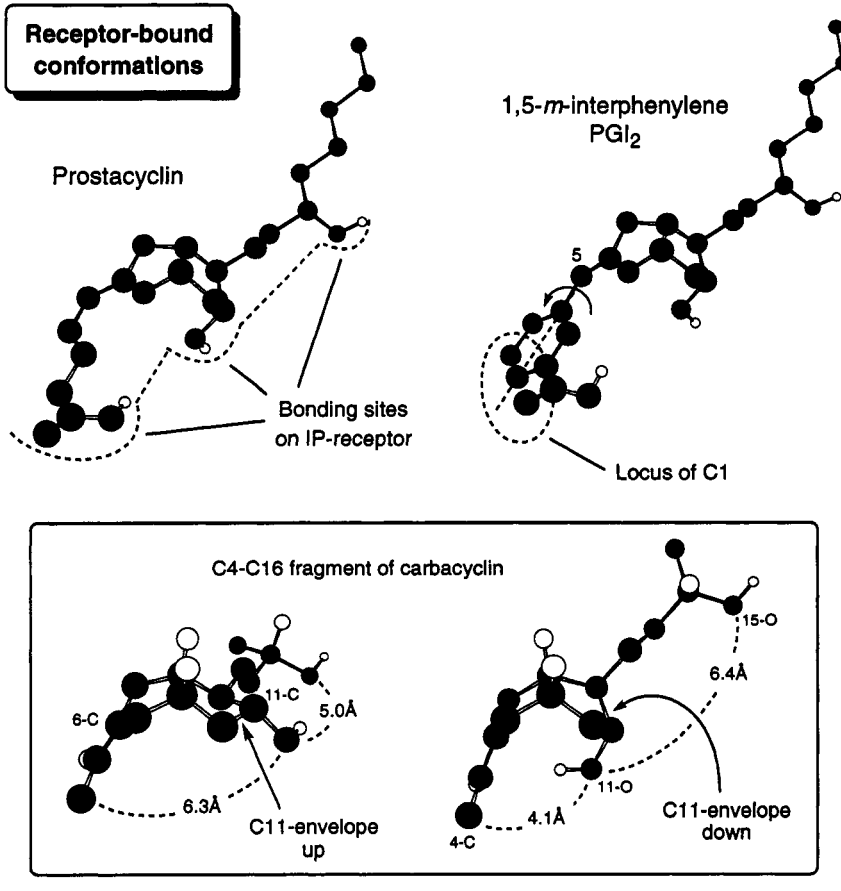

Figure 16. Receptor-bound conformations of prostacyclin and 1,5-*m*-interphenylene PGI_2 by Tsai et al.[51] The 1,5-*m*-interphenylene group restricts the orientation of the carboxyl group relative to the 11- and 15-hydroxyls. The inset shows the effect of flipping the C11-envelope of the C4-C16 fragment of carbacyclin on the relative positions of C4 and the 11- and 15-oxygen atoms.

Two aspects were studied in detail: the conformation of the flexible portion of the a-chain and the spatial relationship between the 11- and 15-hydroxyls. With respect to the former, modelling of l,5-*m*-interphenylene PGI_2 played a critical role, since its carboxyl carbon can follow only one circular orbit arising from rotation of the C4-C5 bond, as shown in Figure 16.[52] It was concluded that the prostacyclin molecule binds with its C3 atom orientated upwards, and looking along the C1-C20 axis in a space-filling model one sees a compact structure with the 1-carboxyl and 11- and 15-hydroxyls roughly in line.

The relative positions of the 11- and 15-hydroxyls is to a large extent determined by the conformation of the cyclopentane ring. As shown for the carbacyclin fragment in Figure 16, an upward flip of the C 1 1 -envelope of the cyclopentane ring brings both the 11-OH and the ω-chain fragment into approximately the same plane as the ring (pseudo-equatorial orientations), whereas with the C 11 -envelope deflected downwards, the 11 -OH and the ω-chain fragment are orientated below and above the ring plane (pseudo-axial) respectively. Tsai & Wu strongly favoured the C11-envelope-down conformation, in which the 11-OH is further from the 1 5-OH, but nearer to C4 (the first fixed carbon proceeding from the 1-carboxyl). During the computation, it also became apparent that for good overlap to occur the 15-hydroxyl must point downwards as shown in Figure 16.

5.2 Support for the active conformation of prostacyclin from structure - activity relationships

To what extent does the available biological potency data support the proposals of Tsai & Wu on the receptor-active conformations of prostacyclin, particularly the extended structure shown in Figure 16? Firstly, in the 1,5-interphenylene series (Fig. 13),[1] both the *para* analogue (SE 58) and the one-carbon homologated *meta* analogue (SE 67) are inactive, and clearly cannot position their carboxyl groups to coincide with that of the moderately potent *meta* analogue (SE 54).

Secondly, contraction (nor) or expansion (homo) of either ring in the bicyclooctane system will tend to swing the 1-carboxylate away from its preferred position for binding to the IP-receptor, resulting in loss of potency. An example of this is found in relatives of beraprost, where expansion of the furan ring on the C7 side produces an inactive carbacyclin analogue (Fig. 12, lower left).[53] However, this unfavourable carboxylate movement can be counteracted by shortening the α-chain by one carbon unit, giving potent prostacyclin (lower centre) and carbacyclin (lower right) analogues.[54]

Further examples of this approach are shown in Figure 17. In 6a-homo PGI_2 (upper right), expansion of the furan ring on the 6a-oxygen side

dramatically swings the molecule into a more L-shaped conformation; this analogue has minimal IP agonist potency.[55] The corresponding 6b-homo PGI_2 (methyl ester), in which the methylene group is introduced between the 6a-oxygen and the 9-carbon, is also inactive.[56] Deletion of the 6b-oxygen to form a 4-membered ring, as in 5Z-6a-nor PGI_2, swings the α-chain in the opposite direction, producing a very weak IP agonist. However, transposing the normal length α-chain to the opposite side of the 5,6-double bond to give 5E-6a-nor PGI_2 (lower left) reorientates the carboxyl group and produces a 10-fold increase in potency.[57] This is an exception to the rule of the naturally-configured C5 geometric isomer having the greater biological potency. For example, natural 5E-carbacyclin is 64 times more potent than its 5Z isomer.[58] Finally, in RS-93427 (lower right) we have the complexity of furan ring contraction / cyclopentane ring expansion (bicyclo[4.2.0]octane system) and one-carbon truncation of the α-chain combining to produce a moderately potent IP agonist.[59]

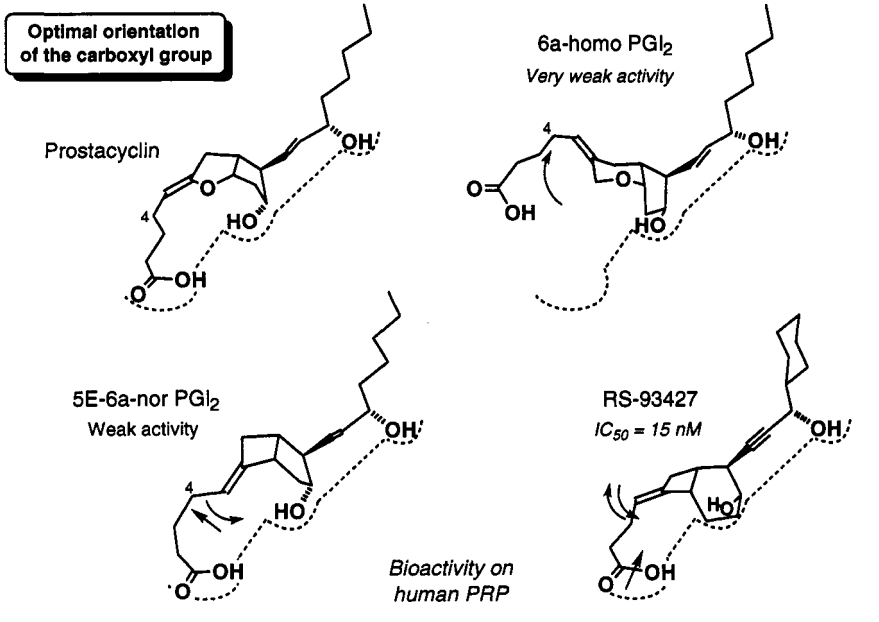

Figure 17. Binding of the 1-carboxylate and 11- and 15-hydroxyl groups of ring-expanded and ring-contracted prostacyclin analogues to postulated binding domains of the IP_1-receptor. A curved arrow indicates the movement of the 4-atom (as in prostacyclin) due to ring expansion/contraction and a straight arrow the corresponding adjustment of the carboxylate position due to either shortening of the α-chain or inversion of the double bond geometry. The 1-carboxylate in the energetically less stable twist-boat conformation of 6a-homo PGI_2 does approach closer to its binding domain.

5.3 Role of the vinyl ether oxygen

The high potencies of iloprost and cicaprost (Fig. 15) indicate that the 6a-oxygen is not essential for IP agonist potency. But does the finding that carbacyclin is about 10 times less potent than prostacyclin imply that the 6a-oxygen makes a specific contribution to binding to the IP-receptor? Not necessarily, since the geometry of the two molecules is subtly different. For the C4-Cl6 fragments of carbacyclin shown in Figure 16, there is general agreement for a plane of symmetry roughly in the plane of the paper and passing through C6 and C1 1 . However, in the vinyl ether of prostacyclin, the planarity of the 5,6-double bond is extended over the 6a-oxygen and this introduces asymmetry into the bicyclic ring. Beierbeck et al.,[60] have proposed a C7-envelope-up/C 12-envelope-down conformation for prostacyclin, while E. Eckle (Schering AG, personal communication) suggests that a CS-envelope-down/C 12-envelope-down conformation is appropriate (MM3 force-field program). Thus, the different bicyclic ring conformations of prostacyclin and carbacyclin could alter the relative positions of the 1-carboxyl and 11- and 15-hydroxyl groups, so accounting for the observed difference in potency without the need for a specific interaction of the 6a-oxygen with the IP-receptor.

6. VARIATIONS IN -CHAIN STRUCTURE

6.1 The iloprost ω- terminus

Many routes to synthetic prostanoids are designed for the ω--chain to be added last, this being the most economical way to examine the effect of varying ω·-chain structure on biological activity. Modification of the somewhat bland *n* -pentyl terminus has dramatic effects on IP agonist potency. Introduction of two methyl groups at C16 in both prostacyclin[61] and 6a-aza-5-thia isocarbacyclin37 reduces potency considerably and there are further reductions when an 18-oxa or 19-oxa group is also present.[62] The two methyls create a bulky *t* -butyl structure at C 16, which may not allow the 15-hydroxyl to interact correctly with its binding domain on the IP-receptor. In contrast, dimethyl substitution at either C17 or C18, which involves less crowding of the 15-hydroxyl, results in retention of potent IP agonist activity.[61]

Introduction of both a single methyl group at C16 (which creates a chiral centre) and a triple bond at C18 has been a popular strategy for producing potent and metabolically stable IP agonists. Thus, beraprost (Fig. 12) is

about 18-fold more potent than it *n* -pentyl relative.[25] Iloprost (Fig. 15), perhaps the most widely studied prostacyclin analogue, also has a 16-methyl-1 8,19-tetradehydro ω--terminus, and has been used as the 16R/16S epimeric mixture in almost all investigations. Skuballa & Vorbrüggen[63] synthesised both isomers and showed that the 16S epimer (C16 configuration identical to cicaprost in Fig. 15) was five times more potent on human platelets than the 16R epimer. Later, Tsai et al.[64] separated the epimers by reversed-phase HPLC and found that the 16S isomer was 20 times more potent than the 16R epimer on human platelets. They also analysed the kinetics of the binding of both isomers to human platelet membranes and showed that the association rate for the 16S isomer was much faster than that for the 16R isomer under identical conditions. Tsai & Wu have suggested that (since the encounter frequencies of the isomers with the IP-receptor are likely to be similar; *authors' addition)* the observed difference in rates of binding is likely to be steric in nature; an incorrect orientation of the approaching 16R epimer would drastically reduce the fraction of successful collisions with the IP-receptor. It should be remembered that [^{3}H]-ioprost used in receptor binding studies is also a 16R/16S epimeric mixture.

6.2 C15-epimers and IP-receptor subtypes

15-O-Methyl prostacyclin has very weak inhibitory activity on human platelets (Fig. 18),[65] demonstrating the importance of the 15-hydroxyl for high IP agonist potency. In addition, the 15-hydroxyl must be part of a 15S chiral centre. Inversion of this natural stereochemistry reduces IP agonist potency (e.g. 33-fold for carbacyclin, Fig. 18),[66] and this is also the usual trend for agonist action at other prostanoid receptors.

Recently, the existence of an IP-receptor that is activated well by both 15R and 15S prostacyclin analogues, and even more surprisingly by analogues lacking any C15 substituent, has been demonstrated in certain areas of the rat brain (e.g. thalamus).[67-69] This receptor has been called an IP_2 subtype to distinguish it from IP_1-receptors present in platelets and blood vessels, and in other areas of the rat brain (e.g. nucleus tractus solitarius, NTS). The crucial ligands in these studies are derivatives of isocarbacyclin in which the *n*-butyl ω-terminus is replaced by a *m*-tolyl group (Fig. 18). The 15S and 15R epimers of 16-*m*-tolyl isocarbacyclin (15s-TIC and 15R-TIC) were comparable competitors for [3H]-isocarbacyclin binding in thalamus tissue sections, whereas 115R-TIC was a much poorer competitor in NTS sections. 15R-TIC is also a weak inhibitor of human platelet aggregation.[68] 15-Deoxy TIC appears to be the most selective ligand for the

IP_2-receptor, having IC_{50} values of 3 and 1000 nM in the thalamus and NTS binding assays respectively.[69]

A similar lack of biological stereoselectivity at C15 has been reported only once previously; the DP-receptor that induces systemic vasoconstriction in the sheep is potently activated by both the 15R and 15S epimers of PGD_2 and also by several C15-modified analogues, such as 15-O-methyl PGD_2 and 13,14-dihydro-15-oxo PGD_2 and its 15-ethylene ketal derivative.[70] PGD_2 (natural 15S) is the only one of these PGD analogues that activates the classical DP-receptor on human platelets. Thus it would be of interest to determine whether the corresponding prostacyclin / isocarbacyclin analogues activate the IP_2-receptor.

We must be careful however not to overgeneralise in our structure-activity relationships. The 15S/15R potency ratio on human platelets varies from 14 for 20-methyl-benzindene prostacyclin[53] to >2000 for 13,14-dihydro-13-thia PGI_2.[71] Moreover, potency losses range from 1.4 to >2000-fold on substitution of a 15-C-methyl group into different 15S prostacyclin analogues.[1,71]

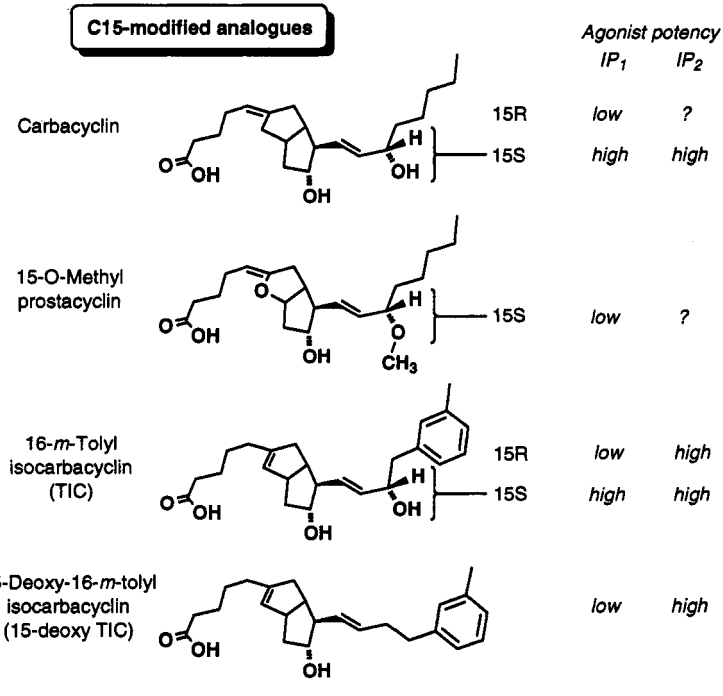

Figure 18. Potencies of the C15-epimers of carbacyclin and 16-*m*-tolyl isocarbacyclin (TIC) for the classical IP_1-receptor (e.g. in human platelets) and the newly discovered IP_2-receptor in rat brain.

7. OTHER PROSTANOIDS AS IP AGONISTS

7.1 PGE analogues

The potent anti-aggregatory activity of PGE_1 was reported in 1967, well before the discovery of prostacyclin and IP-receptors.[72] Indeed, in 1971 van Dorp[73] reported the inhibitory activity on human platelets of a number of α-chain-modified PGEs, including 5-*trans* PGE_2, prepared by incubating the corresponding polyunsaturated fatty acids with the COX/PGE isomerase system of sheep vesicular glands. It is now clear that PGE_1 acts as an IP agonist and that PGE_1 and 5-*trans* PGE_2 are able to mimic prostacyclin more effectively than PGE_2, since the 5,6-cis bond in PGE_2 tends to swing the 1-carboxylate away from its binding domain (Fig. 19). 6-Oxo PGE_1 (Fig. 19), which may arise naturally due to oxidation of 6-oxo $PGF_{1\alpha}$,[74] has IP agonist potency approaching that of prostacyclin[75,76] and is discussed in more detail in Chapter 6.

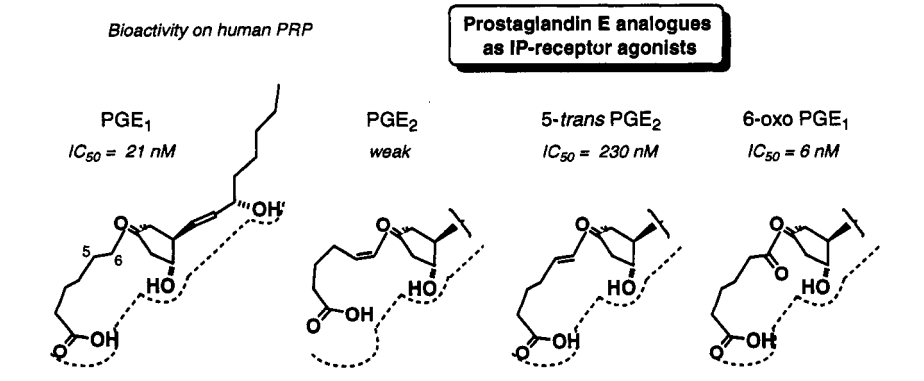

Figure 19. IP agonist potencies of prostaglandin E analogues differing in structure at C5/C6 Docking to the proposed binding domains of the IP-receptor is also shown. The bracket indicates an ω-chain identical to that of PGE_1.

More potent PGE_1 analogues have been developed with antithrombotic use in mind. ONO 1206 (limaprost; Opalmon) is a 17,20-dimethyl analogue with S-configuration at C17 and is 39 times more potent than PGE_1 on human platelets.[77] TFC-6 12 is 17,20-dimethyl-7-thia PGE_1, but in this case the C17 configuration is R. It has 6.4 times greater inhibitory potency than PGE_1 on human PRP, and in contrast to PGE_1 is orally active in the guinea-pig.[78]

7.2 TXA_2 analogues

The platelet aggregatory and vasoconstrictor actions of TXA_2 are mimicked by prostanoids with a 7-oxa-bicyclo[2.2.1] heptane ring system. The highest potency is found when the ring oxygen is located on the α-face of the molecule and the side-chains have the natural $8\alpha, 12\beta$ configuration (see SQ 26655 in Fig. 20, inset). [79,80] However, when the chiral centres at C8 and C12 are both inverted, a switch to weak prostacyclin-like activity is seen.[81] Figure 20 attempts to show how such a molecule fits the Tsai & Wu model for the receptor-active conformation of prostacyclin. The effects on IP agonist potency of modifying the ω–chain in the $8\beta,12\alpha$ series are interesting. Of particular note are the slight loss of IP potency on saturating the 5,6-double bond, which is opposite to that found in the PGE series, and a much more marked loss of IP potency upon saturation of the 13,14-double bond. Presumably the 13,14-double bond is important for orientating the ring ether oxygen and the 15-hydroxyl for effective interaction with the 11- and 15-hydroxyl binding sites on the IP-receptor. In contrast to the prostacyclin series (e.g. iloprost), a 16-methyl-1 8,19-tetradehydro ω-terminus results in low IP agonist potency. The compound with the highest IP agonist activity is the 1 5-cyclohexyl-ω-pentanor analogue, which as the pure enantiomer (SQ 27986, Fig. 20) is about 50 times more potent than its parent *(n*-pentyl) enantiomer.[81] It would be of interest to test SQ 27968 on the IP^2-receptor found in rat brain.

The TXA_2 analogue STA_2 (Fig. 20, inset) is a potent full TP agonist on smooth muscle preparations.[80,82,83] On human washed platelet suspensions it induced irreversible aggregation at concentrations around 100 nM, whereas at higher concentrations only large reversible aggregation waves were seen.[80] When TP-receptors were blocked by a high concentration of the TP antagonist EP 092, STA_2 (100 - 2700 nM) partially inhibited aggregation elicited by platelet-activating factor (PAF). Under similar conditions, another TP agonist U-46619 (up to 3000 nM) did not affect PAF-induced aggregation. In CHO cells, STA_2 (1 - 10 μM) displaced [^{3}H]-iloprost binding to the stably expressed human IP-receptor.[84] Thus it appears that STA_2 may weakly activate the human IP_1-receptor as well as potently activating TP-receptors. The use of such an agonist to characterise a truly competitive TP antagonist may result in a noncompetitive profile if the preparation contains functionally-opposed TP- and IP-receptors.

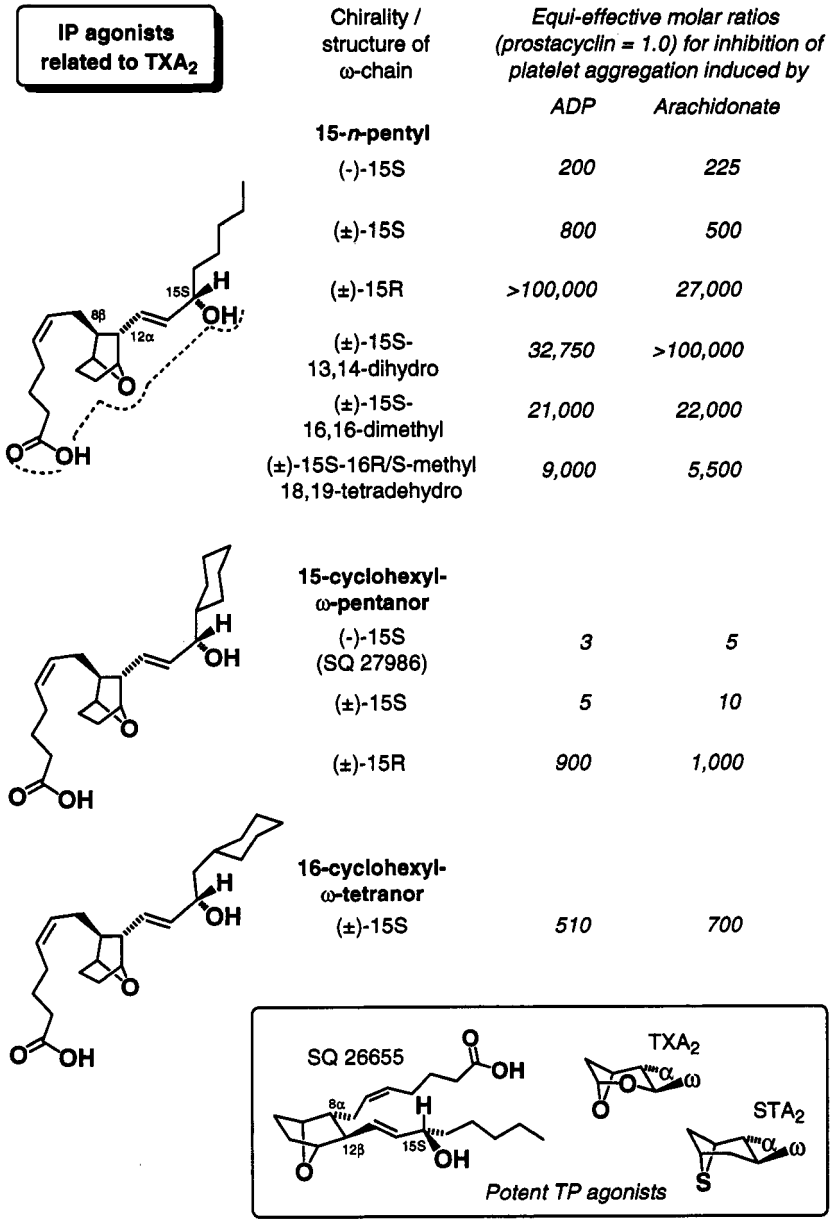

Figure 20. 7-0xa-bicyclo[2.2.1] heptane prostanoids showing IP agonist activity. The IC_{50} values for prostacyclin were 10 nM for ADP and 2 nM for arachidonate; a high value for the equi-effective molar ratio indicates low agonist potency. Data from Haslanger et al.[81]

8. SPECIFICITY OF PROSTACYCLIN ANALOGUES

8.1 DP-receptors

The human platelet has DP-receptors on its cell surface linked to adenylate cyclase. It is thus possible that some of the platelet inhibitory actions of the prostacyclin analogues discussed so far are due (at least in part) to activation of DP-receptors. One way to resolve this problem is to determine whether a specific DP antagonist, such as BW A868C (pA_2 = 9.26 on human washed platelets, Chapter 1, Section 4.1),[85] shifts the log-concentration-response curve for the prostacyclin analogue to the right. In the case of RS-93427 (Fig. 17), exposure to 1 μM BW A868C resulted in a dose ratio of 3.4; the curve for PGD_2 was shifted much further to the right (dose ratio = 68), whereas that for PGE_1 was unchanged.[86] BW A868C also produced small but statistically significant reductions in the amounts of cyclic AMP generated by the lower concentrations (3.2 - 32 nM), but not the higher concentrations (100 - 10,000 nM) of RS-93427. These findings are consistent with activation of both IP- and DP-receptors by RS-93427 over a similar concentration range.

RS-93520 is a diastereoisomer of RS-93427 in which the 15S configuration is retained whilst the four chiral centres associated with the ring system are inverted. This analogue is about half as potent on human PRP as RS-93427 and its log concentration-response curve is shifted far to the right by 1 μM BW A868C (dose ratio = 40).[86] Its log concentration-response curve for elevation of cyclic AMP is distinctly biphasic and the more sensitive portion is shifted well to the right (dose ratio ~ 100) by BW A868C. These findings are consistent with moderate agonist potency of RS-93520 at DP-receptors coupled with low agonist potency at Preceptors.

8.2 EP-receptors

The first real indication that prostacyclin analogues might activate EP-receptors came from the studies of Dong & Jones,[87,88] who showed that iloprost (called ZK 36374 at that time) and isocarbacyclin were moderately potent contractile agents on bullock iris sphincter, guinea-pig trachea (spirally-cut) and rat stomach fundus pre arations. Two PGE receptor antagonists, SC 19220 and SC 25191,[89] later characterised as EP_1 antagonists, blocked responses to the prostacyclin analogues and 16,16-dimethyl PGE_2 to a similar extent. In contrast to iloprost and isocarbacyclin,

cicaprost (ZK 96480) showed little EP_1 agonist activity and was therefore recommended as the preferred standard IP agonist in prostanoid receptor characterisation studies.[88]

Iloprost appeared to behave as a partial agonist at the EP_1-receptor on the three previously mentioned smooth muscle preparations, opposing contractions induced by PGE_2, 16,16-dimethyl PGE_2 and prostacyclin, but not contractions induced by U-466 19 or carbachol.[87,88] However, in later experiments using ring preparations of guinea-pig trachea, which were more sensitive to EP_1 agonists, iloprost behaved as a potent full agonist (Table 1).[90,91] Other potent full agonists included carbacyclin and 17S, 20-dimethyl isocarbacyclin (TEI-9063),[92] whereas taprostene, benzodioxane prostacyclin[93] and cicaprost had minimal EP_1 activity (Table 1). Isocarbacyclin, along with PGE_1 and PGE_2, showed a bell-shaped dose-response curve on the trachea, probably due to activation of both contractile EP_1 and relaxant EP_2-receptors.[88] Relaxation of the guinea-pig trachea by high concentrations (>2.4 μM) of beraprost has also been reported.[94] Experiments with the mouse cloned EP1-receptor expressed in CHO cells have shown that the abilities of 17-phenyl-ω-trinor PGE_2, sulprostone and iloprost to compete for [^{3}H]-PGE_2 binding parallel their EP_1 agonist potencies on isolated smooth muscle preparations (Table 1).[95,96]

The only prostacyclin analogue reported to show potent EP_3 agonist activity is TEI-3356. The major structural change in this isocarbacyclin analogue is the transfer of the 15-hydroxyl to the 16-position; the PGE_1 analogue misoprostol, which is a potent EP_2/EP_3 agonist[97] has an identical ω-chain. TEI-3356 competed for [^{3}H]-PGE_2 binding to mouse cloned EP_3-receptors expressed in CHO cells with an affinity equal to sulprostone, one of the most potent EP_3 agonists;[98] the affinities of TEI-3356 for cloned mouse EP_1-, EP_2- and IP-receptors were much lower. Forskolin-induced cyclic AMP formation in the CHO cells was also inhibited to the same extent by sulprostone and TEI-3356 (IC_{50} ~ 1.01nM), consistent with negative coupling of the EP_3-receptor to adenylate cyclase. On the field-stimulated guinea-pig vas deferens, the archetypal EP3 preparation, TEI-3356 (1 - 100 nM) mimicked the inhibitory action of sulprostone on twitch tension and [^{3}H]-noradrenaline overflow, but was about 50 times less potent.[99] Three other prostacyclin analogues, iloprost, carbacyclin and cicaprost also showed evidence of EP_3 agonist activity on the vas deferens, with cicaprost being the weakest (Table 1). However, activation of IP-receptors in this preparation leads to enhancement of twitch responses, which opposes the EP_3 inhibition, and as a result, accurate relative potencies for EP_3 agonism could not be obtained. Iloprost, carbacyclin and isocarbacyclin show moderate binding affinities for mouse EP_3-receptors, with cicaprost being somewhat weaker (Table 1),[96,100] thus complimenting the guinea-pig vas deferens data.

Both EP_2- and EP_4-receptors induce relaxation of vascular smooth muscle, but little is known of their interactions with prostacyclin analogues in functional assays. On mouse cloned EP_2-receptors, iloprost, carbacyclin, isocarbacyclin and cicaprost competed poorly for [^{3}H]-PGE_2 binding (K_i 1600, 1600, 1000, 1300 nM respectively, PGE_2 12 nM) and a similar picture was found for iloprost and carbacyclin on the mouse cloned EP_4-receptor (K_i 2300 nM, PGE_2 1.9 nM).[96] 7,7-Difluoro-16S,20-dimethy1-18,19-tetra-dehydro prostacyclin (AFP-07), although a weak competitor for mouse cloned EP_1, EP_2 and EP_3-receptors, had a K_i of about 10 nM for the corresponding EP_4-receptor.[19]

Table 1. Potencies of PGE and prostacyclin analogues on EP_1- and EP_3-receptor preparations

Prostanoid	*Equi-effective molar ratios*			
	Guinea-pig trachea (EP_1)	*Mouse cloned EP_1-receptor*	*Guinea-pig vas deferens (EP_3)*	*Mouse cloned EP_3-receptor*
17-Phenyl-ω-trinor PGE_2	1.0 (EC_{50} = 2.2 nM)	1.0 (K_i = 14 nM)	54	6.2
Sulprostone	3.2	1.5	1.0 (IC_{50} = 0.2 nM)	1.0 (K_i = 0.6 nM)
PGE_2	bell-shaped*	1.4	7.2	1.4
TEI-9063	1.8			
Iloprost	2.7	2.1	(50 nM)†	37
Carbacyclin	13.5		(30 nM)†	52
Isocarbacyclin	bell-shaped*			52
TEI-3356	72		48	
Prostacyclin	160			
Taprostene	>400			
Benzodioxane prostacyclin	>400		(30 nM) †	
Cicaprost	>400		(300 nM) †	280

17-Phenyl -ω- trinor PGE_2 is a standard EP_1 agonist and sulprostone is a standard EP_3 agonist. Guinea-pig trachea: contractile activity; data from Lawrence et al.,[90] Jones et al.[91] and R.L. Jones, unpublished observations; *EP_1 contraction overcome by EP_2 relaxation. Guinea-pig vas deferens: inhibition of field stimulation responses;[90,91] †EP_3 inhibitory action seen at this and higher concentrations, but potency ratio not calculated due to opposing IP agonist action. Mouse EP_1- and EP_3-receptors: competition for [^{3}H]-PGE_2 binding.[96]

8.3 TP-receptors

Finally, we come to the potential activation of TP-receptors by prostacyclin analogues. Isocarbacyclin at concentrations of 1 - 10 μM produced transient contractions of monkey mesenteric, renal, cerebral, coronary and popliteal arteries, which were inhibited by the TP antagonist S 145.[101] U-46619 produced contraction at nanomolar concentrations, but it

was difficult to determine the relative TP potency of isocarbacyclin due to its concomitant IP relaxant action.

The 6α and 6β epimers of 16-phenoxy-ω-tetranor PGI_1 stimulate rather than inhibit aggregation of human platelets.[102] The direct aggregation seen with the 6a isomer may be due to activation of TP-receptors since 16-phenoxy (and 16-*p*-halophenoxy) substitution increases TP agonist potency in a range of prostanoid structures.[103,104] In contrast, the 6β isomer potentiated aggregation (aggregating agent not specified); this may involve a different mechanism since 16-phenoxy PGE analogues such as sulprostone enhance reversible aggregation waves induced by ADP or U-46619 by activating EP_3-receptors.[105]

9. CONCLUDING REMARKS

Many more prostacyclin analogues than discussed here have been synthesised and examined biologically, but what has been written so far illustrates the importance of the correct spatial orientation of the 1-carboxyl and 11- and 15-hydroxyls for high agonist potency at IP-receptors. With the continuous development of more powerful computing methods and the emergence of data on single-point mutated and chimeric IP-receptors (see Chapter 4), the time is approaching when more sophisticated molecular modelling may reveal the atomic interactions that lead to activation of IP-receptors.

From a pharmacological standpoint, there is still a need for more potent and selective IP agonists to characterise IP-receptors. So far, cicaprost clearly outshines other analogues in relation to the classical IP_1-receptor, but with the inevitable (and welcome) emergence of competitive IP-receptor antagonists it may fall short of the ideal. In addition, the identification of agonists besides TIC analogues that preferentially activate the newly-discovered IP_2-receptor in the rat brain is essential. Cicaprost is almost inactive on this receptor and it is possible that we should be looking for quite different types of molecules than have been developed so far.

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Chapter 3

Nonprostanoid prostacyclin mimetics

1. INTRODUCTION

Pharmacologists have always been intrigued by a compound that mimicks the action of a natural transmitter or hormone, but has a dissimilar chemical structure. Examples include morphine acting as an opioid receptor agonist and bromocriptine acting as a dopamine D_2 agonist.[1,2] In the prostanoid field, there exists a series of diarylalkanoic acids that activate IP-receptors whilst showing little structural resemblance to prostacyclin, apart from a carboxylic acid group and a similar molecular size. They have been termed nonprostanoid prostacyclin mimetics. Their development by groups at Edinburgh University and the Bristol-Myers Squibb and ONO pharmaceutical companies, together with their biological properties, are the subject of this chapter.

In the figures, the unfilled arrows represent development sequences for the nonprostanoid prostacyclin mimetics, and biological activities adjacent to chemical structures relate to inhibition of adenosine diphosphate (ADP)-induced aggregation in human platelet-rich plasma (PRP). The nonprostanoids are much more lipophilic than prostacyclin analogues such as iloprost and cicaprost, and this means that they show greater binding to plasma proteins. As a consequence, inhibitory potencies obtained in PRP may be considerably underestimated.

2. EMERGENCE OF THE NONPROSTANOID PROSTACYCLIN MIMETICS

2.1 PGH_2 analogues with modified ω-chains

The labile endoperoxide ring system in PGH_2 can be stabilised by replacement of its 9a-oxygen by carbon.[3] The product 11,9-epoxymethano PGH_2 (U-46619) is a specific and potent full agonist for thromboxane (TP-) receptors (Chapter 1, Fig. 8). However, replacement of both ring oxygens by carbon, as in 9,11-ethano PGH_2 and 9,11-etheno PGH_2 (Fig. 21), results in partial agonism at TP-receptors.[4]

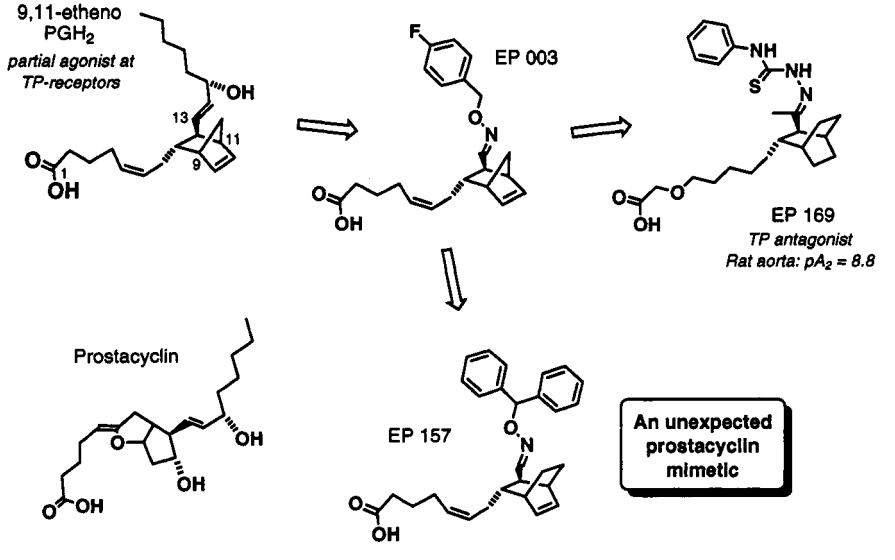

Figure 21, Early steps in the evolution of nonprostanoid prostacyclin mimetics. Modification of the ω-chain of 9,1 1-etheno PGH_2 (upper left) led to the TP antagonist EP 003 and then to more potent antagonists such as EP 169. However, the introduction of a diphenylmethoxime unit as in EP 157 led to prostacyclin-like activity.

The above findings led N.H. Wilson and R.L. Jones in the Pharmacology Department of Edinburgh University to make a series of 13-imino (-CH=N-R) analogues of 9,11 -etheno PGH_2, in which the structure of the ω-chain was radically different to that of a natural prostanoid. The objective was to obtain a specific TP-receptor antagonist, and indeed one of the first compounds synthesised, the *p*-fluorobenzyloxime EP 003 (Fig. 2 1), showed

weak blocking activity. It inhibited aggregation of human platelets induced by U-46619 in a surmountable manner, without affecting primary wave responses to other agonists such as ADP and platelet-activating factor (PAF). Introduction of other imino groups, such as acylhydrazones and semicarbazones, yielded more potent TP-receptor antagonists (e.g. EP 045)[5] and after some chemical fine-tuning high affinity specific blockers were obtained (e.g. EP 169, Fig. 21).[6]

Early in the Edinburgh research programme, it was observed that a diphenylmethoxime ω-chain (present in EP 035, Fig. 29) conferred an inhibitory profile quite different to that of the benzyloxime analogue EP 003. EP 035 inhibited aggregation responses induced by all aggregating agents tested, including U-46619, ADP, PAF, adrenaline and collagen. A modest increase in inhibitory potency was achieved by introducing an 8-membered bicyclic ring system as in EP 157 (Fig. 21).[7] The inhibitory actions of EP 035 and EP 157 were reminiscent of an agent that activates adenylate cyclase in the human platelet, such as prostacyclin or PGD_2, rather than a TP-receptor antagonist. The evidence for mimicry of prostacyclin is summarised below.

2.2 Evidence for activation of platelet IP-receptors by EP 035 and EP 157

The actions of EP 035 and EP 157 were compared with those of prostacyclin (or one of its stable analogues) and PGD_2 on human platelets in functional, second messenger and ligand binding experiments.[7]

(a) *Aggregation studies:* In human PRP, all of the agents could completely suppress aggregation induced by high concentrations of U-46619, ADP and PAF. The adenylate cyclase inhibitor SQ 22536 partially reversed the inhibitions, whereas it did not affect block of U-46619 aggregation by the specific TP-receptor antagonist EP 045. Complete inhibition of adenylate cyclase is difficult to achieve with SQ 22536, perhaps due to negative co-operativity.[8] Inhibition of aggregation by PGI_2, EP 035 and EP 157 was also unaffected or slightly enhanced by the DP-receptor antagonist AH 6809 (Chapter 1, Fig. 7), whereas PGD_2-induced inhibition was blocked.

(b) *Adenylate cyclase studies:* Iloprost, EP 035 and EP 157 markedly increased cyclic AMP levels in PRP. Adenylate cyclase activity in platelet homogenates, measured by the [^{32}P]-ATP method, was also elevated by EP 035 and EP 157, achieving the same V_{max} as the prostacyclin analogues.

(c) *Binding studies:* Iloprost, carbacyclin, EP 035 and EP 157 competed for [^{3}H]-iloprost binding to human platelet membranes.

The potency ranking for all three types of measurement was iloprost > carbacyclin > EP 157 > EP 035. The experimental findings were consistent with EP 035 and EP 157 binding to IP-receptors, but not DP-receptors on human platelets, with consequent activation of adenylate cyclase and inhibition of platelet aggregation.[7]

2.3 From octimibate to BMY 42393

In 1990, octimibate (Fig. 22) was reported to be an inhibitor of human platelet aggregation in vitro, and studies similar to those described for EP 035 and EP 157 established it as an IP-receptor agonist.[9,10] Octimibate was one of a series of compounds being investigated at Bristol-Myers Squibb for cholesterol lowering activity through inhibition of HMG-CoA reductase. Since octimibate is devoid of a ring system typical of the prostanoids, the term "nonprostanoid prostacyclin mimetic" was coined (see 1994 review by Meanwell et al[11]).

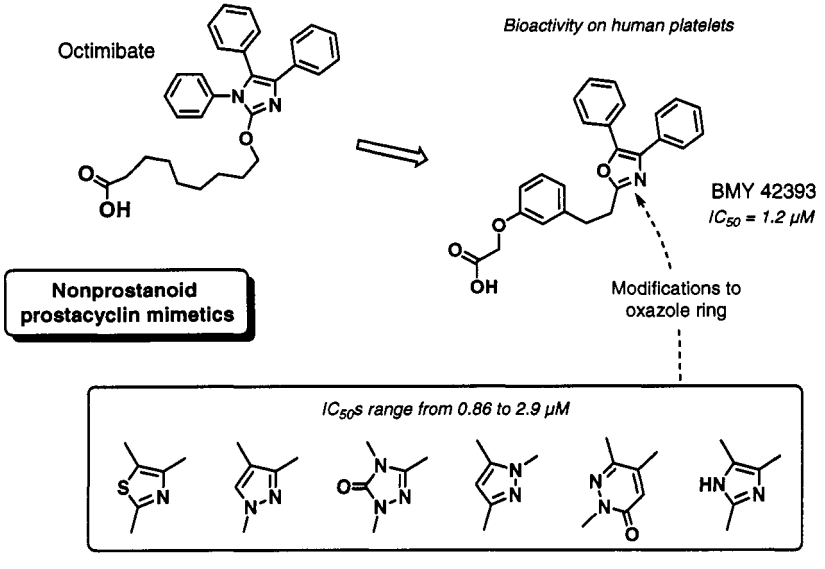

Figure 22. Octimibate, as the first true nonprostanoid prostacyclin mimetic, leading to BMY 42393. The lower structures show that the nature of the heterocycle in BMY 42393 can vary considerably without greatly changing IP agonist potency.

Octimibate has three phenyl groups attached to adjacent positions of an imidazole ring. However, it soon became obvious that only two phenyls, which must be adjacent, are required on the heterocycle for IP agonist

activity and this led to the development of BMY 42393 (Fig. 22).[12] There exists a clear structural similarity between EP 035 / EP 157 and octimibate / BMY 42393, and although the former are strictly prostanoids, it is more convenient for discussion to include these EP analogues under the heading of nonprostanoid prostacyclin mimetics.

2.4 The ONO hydronaphthalene series

The third avenue to nonprostanoid prostacyclin mimetics started with an attempt to ascertain whether the cyclopentane ring in the PGE analogue, 3,7-*m*-interphenylene-3-oxa PGE_1 (Fig. 23), is absolutely essential for IP agonist activity on human platelets. It seemed not, since the acyclic analogue shown upper right in Figure 23 displaced [^{3}H]-iloprost binding to human platelet membranes (IC_{50} 23 μM).[13]

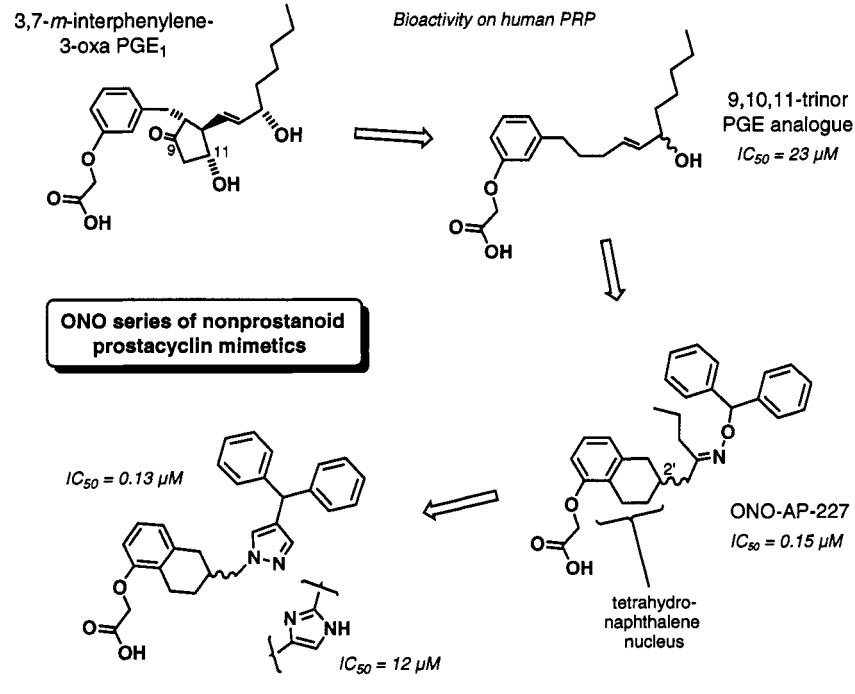

Figure 23. Development of the tetrahydronaphthalene group of nonprostanoid prostacyclin mimetics from the PGE analogue, 3,7-*m*-interphenylene-3-oxa PGE_1. The 2'-carbon in the tetrahydronaphthalene ring system is asymmetric. As shown lower left, the nature of the heterocycle can be quite critical for IP agonist potency in this series.

After preparation of many analogues, it became clear that high binding affinity for the IP-receptor required the presence of an ω-diphenylmethyl group, and that a diphenylmethoxime (as present in EP 035 and EP 157) and a tetrahydronaphthalene nucleus towards the 1-carboxylate were a particularly effective combination, as in ONO-AP-227 (Fig. 23, lower right).[13,14]

3. OPTIMISING AGONIST POTENCY

3.1 Orientation of the diphenyl unit

In all three series, analogues with a single aromatic ring at the ω-terminus are either very weak IP agonists or inactive. Also, replacement of one of the benzene rings by a cyclohexyl group abolishes activity as in EP 182, one of a group of 13-azine (-CH=N-N=CR_1R_2) analogues (Fig. 24).[15] These findings point to the essential nature of the diphenyl unit. For optimal binding to the IP-receptor, the evidence points to the phenyl rings being out-of-plane, as shown for the potent azine analogue EP 098 in Figure 24. Planarity due to conjugation is overridden by the need for rotation of the phenyl rings to prevent overlap of their *ortho* hydrogens. EP 079, in which the *ortho* carbons are joined by a single bond to create a planar tricyclic structure, is an order of magnitude less potent than EP 098 (Fig. 24). In the BMY series, the effect of inducing planarity is even is more dramatic; a single bond inserted between the ortho carbons of BMY 42393 to create a phenanthrene-type ring completely destroys IP agonist activity.[11]

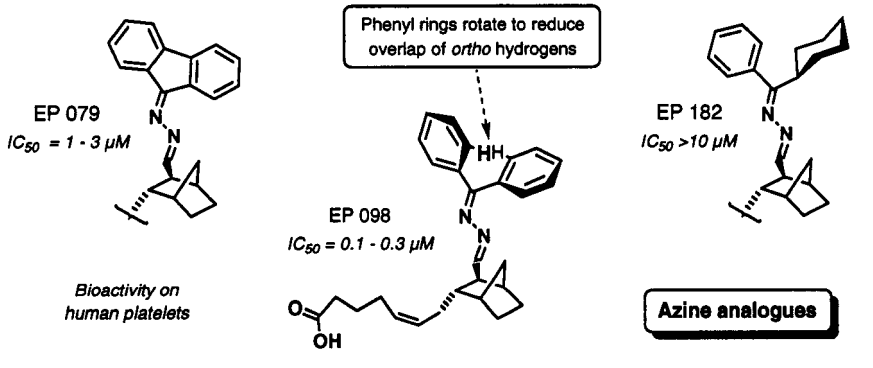

Figure 24. Azine analogues in the EP series demonstrate the importance of the 1,1-diphenyl unit and the out-of-plane orientation of the phenyl rings for IP agonist activity.

3.2 Varying the hetero unit

In 1,1-diphenyl analogues, high IP agonist potency is associated with an unsaturated linear heteroatomic unit such as an oxime; a simple diphenylmethyl ether at the ω-terminus confers low IP agonist potency.[15] In EP 157, there is a preponderance of one of the geometric oxime isomers, probably the *anti* isomer as drawn in Figure 21. However in the ONO series, the groups adjacent to the ketoxime carbon are similar in bulk and the isomers are present in more equal proportions. Careful HPLC separation of the readily interconvertible *syn* and *anti* isomers of the methyl analogue of ONO-AP-227 (Fig. 23) revealed that the *anti* isomer was more potent than the syn isomer.[16] Substituting the oxime unit with a heterocycle can maintain but also reduce IP agonist activity (Fig. 23, lower left).[17] In contrast, in 1,2-diphenyl substituted BMY analogues, considerable variation in the structure of the heterocycle is tolerated (Fig. 22, inset).[12]

3.3 Restricting conformational mobility: BMY 45778

The two-carbon link between the phenyl and oxazole rings of BMY 42393 confers considerable flexibility on the molecule. The Bristol-Myers Squibb chemists have attempted to enhance potency by locking this region in different ways (Fig. 25).

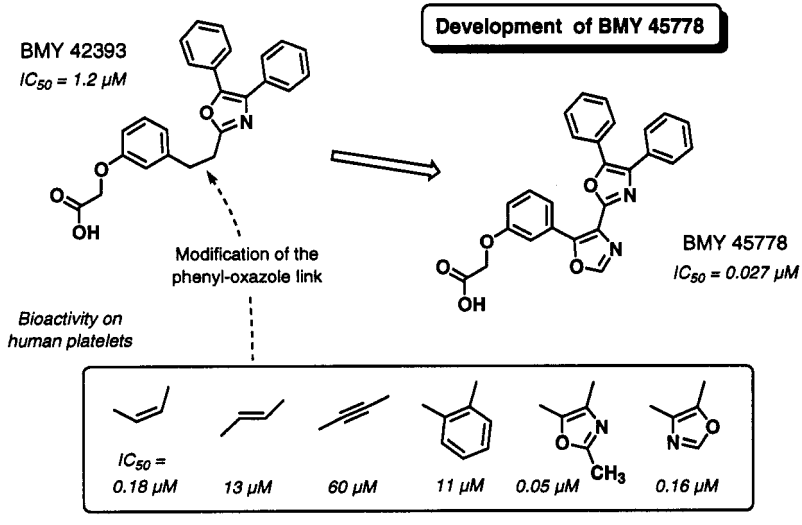

Figure 25. Development of the potent nonprostanoid prostacyclin mimetic BMY 45778. The nature and the stereochemistry of the link between the phenyl and oxazole rings has an important bearing on IP agonist potency.

The much greater potency of the *cis*-ethene analogue (Fig. 25 inset, extreme left) compared to the *trans* ethene and ethyne analogues clearly points to the conformation in which BMY 42393 is drawn in Figure 25.[18] This conformation can be maintained by expanding the *cis* -ethene linkage into a second oxazole ring as in BMY 45778.[19] Indeed, BMY 45778 is one of the most potent of the nonprostanoid prostacyclin mimetics synthesised so far.

3.4 Pharmacophores and receptor-bound conformations

Studies in the BMY series show that the highest IP agonist potency is associated with 7 or 8 methylene units between the heterocycle and the 1-carboxylate.[19,20] Thus from the findings discussed so far, two closely related nonprostanoid pharmacophores imparting moderate to high agonist activity at IP-receptors can be inferred (Fig. 26).

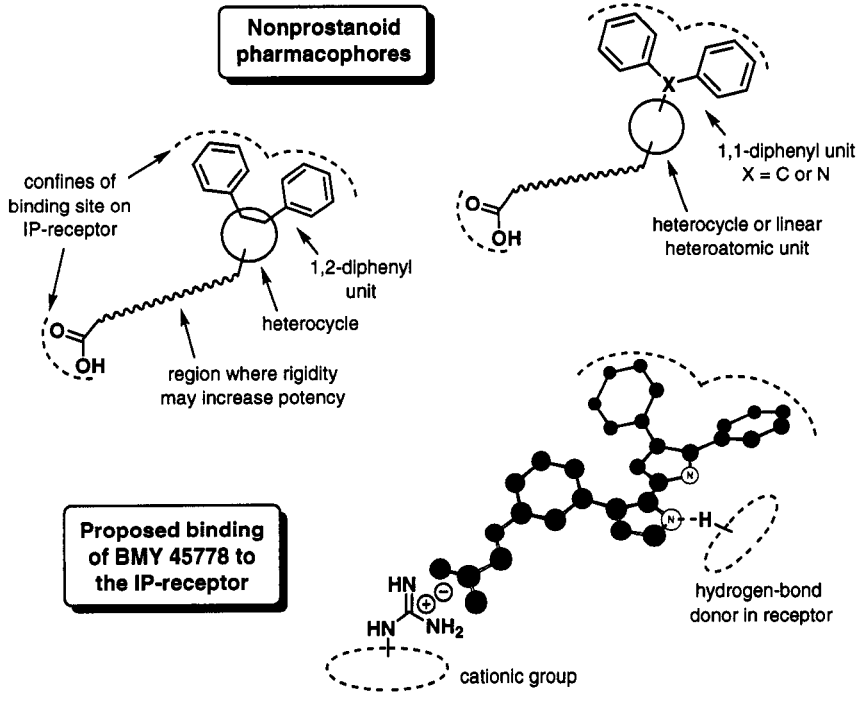

Figure 26. Upper section: pharmacophores that confer IP agonist activity in the nonprostanoid prostacyclin mimetic series. Lower section: proposed binding of BMY 45778 to the IP-receptor (after Meanwell et al.[11] with permission). Oxygen atoms are black; hydrogen atoms are not shown.

In Figure 26, the spatial limits of the diphenyl and carboxylate units are represented by arcs. On the left, the two phenyl groups are attached to adjacent atoms of a heterocycle; this is the 1,2-diphenyl substitution pattern typical of the BMY series. On the right, the two phenyl groups are attached to a single atom, which may be either carbon or nitrogen; this is the 1,1-diphenyl substitution pattern. The single atom is linked to a linear heteroatomic group (e.g. an oxime) in the EP series and to a heterocycle in some of the later ONO and BMY analogues.

Molecular modelling of BMY 45778 based on energy minimisation gives the conformation shown in Figure 26. Again, conjugation does not guarantee planarity: the two oxazole rings are roughly in the same plane but the phenoxy ring is not. The Bristol-Myers Squibb group have proposed that the second oxazole ring in BMY 45778 is more than a locking device and is involved in hydrogen bonding with a specific group in the IP-receptor.[11,19] The reasoning for this is based on the lower potency of an exact isostere of BMY 45778 in which the oxygen and nitrogen of the second oxazole ring are interchanged (Fig. 25 inset, extreme right), and the greater hydrogen bond acceptor capability of oxazolyl nitrogen compared to oxazolyl oxygen. The hydrogen-bond donor in the IP-receptor is thought to be the same group that interacts with the 1 I-hydroxyl of prostacyclin. Structure is indeed critical in this region: a methyl group on the second oxazole ring can be tolerated (Fig. 25 inset), but a corresponding phenyl group cannot (IC_{50} >60 μM). A phenyl ring in place of the second oxazole also produces a weak IP agonist (Fig. 25 inset).

Unlike the prostacyclin analogue area (see Chapter 2), the effect of chirality on IP agonist potency in the nonprostanoid series has received little attention. This is mainly due to the fact that most of the BMY analogues are achiral. However, in the ONO series, attachment at the 2'-position of the tetrahydronaphthalene nucleus produces an asymmetric centre; the S enantiomer (2'H down) of the methyl analogue of ONO-AP-227 (Fig. 23) was about 10 times more potent than the R enantiomer (2'H up).[16]

4. BIOLOGICAL ACTIONS

4.1 Receptor subtypes and species differences

Although in vitro aggregation of platelets is inhibited by the nonprostanoid prostacyclin mimetics in all species studied, there are considerable differences in potencies relative to iloprost.[10,21] Thus human, monkey (cynomolgus), pig and horse platelets comprise a high potency

group, whereas rabbit, rat, guinea-pig, cat, dog and cow platelets form a low potency group (see Chapter 5, Fig. 36). It is well recognised that for a single receptor system the potency of a low efficacy agonist relative to a higher efficacy agonist often decreases as the sensitivity of the preparations to the higher efficacy agonist decreases.[22] However, this is unlikely to be the major cause of the species differences observed. For example, on human and horse washed platelets where the IC_{50} values for iloprost (0.15 nM and 23 nM respectively) differ considerably, EP 157 has similar equi-effective molar ratios (EMR) of 54 and 98 respectively; rat washed platelets show an intermediate sensitivity to iloprost (IC_{50} = 4.7 nM) but the relative potency of EP 157 is much less (EMR = 3500).[21] In addition, there were differences between the kinetics of [^{3}H]-iloprost binding to human/horse/pig platelet membranes compared to rabbit/rat platelet membranes in that satisfactory binding assays could only be performed on the latter group when the filtration temperature was reduced from 30°C to 4°C, suggesting a much more rapid dissociation of radioligand-IP-receptor complexes.[21] Recently, Seiler et al.[23] have reported that IC_{50} values of iloprost for competition with [^{3}H]-iloprost in human and rabbit platelet membranes at 0 - 4°C were similar (61 and 63 nM), whereas the corresponding IC_{50} values of BMY 45778 were 49 and 578 nM. Whether these relative potency differences signify a common difference in the receptor structure of the IP-receptors in the two groups of species is not yet clear.

4.2 Partial agonism at IP-receptors

In spite of what has been discussed in Section 4.1, reduced efficacy does appear to be a characteristic of many of the nonprostanoid prostacyclin mimetics, but it does not always display as a lower functional maximum response. For example, an analogue of EP 157 with a 6-oxabicyclo[3.2.1]octane ring could completely inhibit aggregation in human PRP induced by U-46619, PAF and ADP (IC_{50} range 0.8 - 4.0 μM). However, it would only raise cyclic AMP levels in human washed platelets by 1.5-fold at 1 μM and 3.0-fold at 10 μM; the log concentration-response curves for cicaprost and EP 157 itself were much steeper with 10 - 15-fold increases in cyclic AMP obtainable at 0.1 and 1 μM respectively.[24] Similarly, octimibate had a lower maximum (~60%) for activation of both adenylate cyclase and cyclic AMP-dependent protein kinase compared to iloprost in human washed platelets.[10] In addition, the maximum activity of human platelet membrane GTPase was less for BMY 45778 than for iloprost, and BMY 45778 opposed the action of iloprost in raising GTPase activity (Fig. 27).[23] However, all the nonprostanoids studied so far have produced complete inhibition of human platelet aggregation. The

explanation lies with the exquisite sensitivity of the human platelet to prostacyclin and its close analogues, which may be the result of two factors: a high catalytic efficiency of the IP-receptor/G_s/adenylate cyclase system and a high responsiveness of the protein kinase A system to the generated cyclic AMP (either through a genuinely high molecular sensitivity or through compartmentalisation of the cyclic AMP). Thus, iloprost at about 0.2 nM raises the apparent level of cyclic AMP in human washed platelets by about 50% and this is sufficient to abolish aggregation;[21] the IP-receptor occupancy at this concentration is about 1% assuming a K_d of 20 nM (see Chapter 5). Since many nonprostanoids can more than double cyclic AMP levels in human platelets, they behave functionally as full agonists.

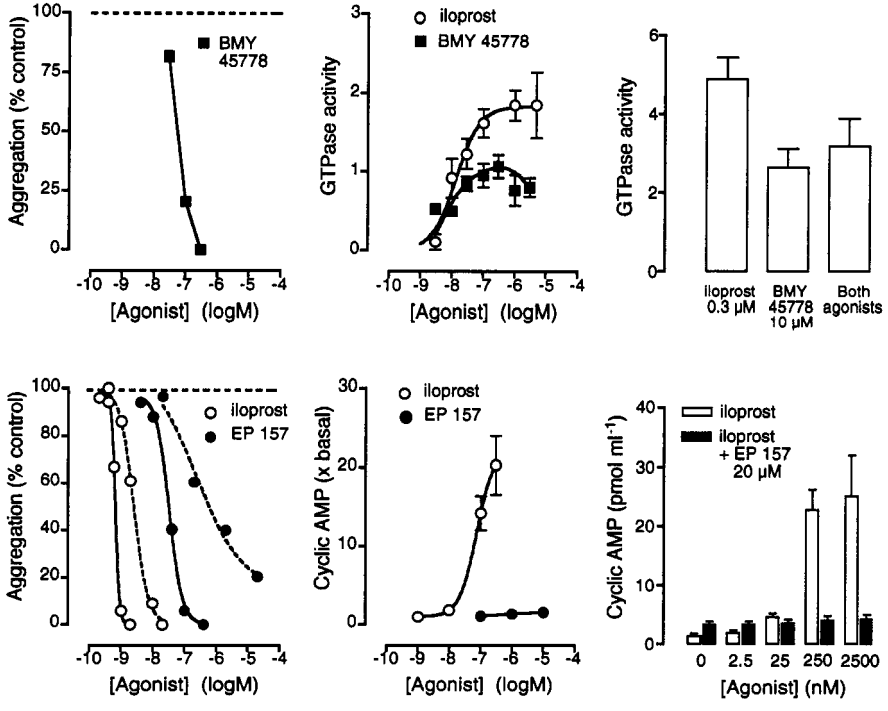

Figure 27. Agonist profiles of some nonprostanoid prostacyclin mimetics. Upper panels: BMY 45778 on human platelets. Left: inhibition of ADP-induced aggregation in platelet-rich plasma (data derived from an experimental tracing). BMY 45778 is highly bound to plasma proteins and the true inhibition curve probably lies at least one log unit to the left of that shown. Centre and right: stimulation of platelet membrane GTPase (pmol mid^{-1} mg $protein^{-1}$ above basal; plasma-free). Data from Seiler et al.,[9] with permission. Lower panels: EP 157 on pig washed platelets. Left: inhibition of PAF-induced aggregation on preparations with higher (solid line) and lower (broken line) sensitivity to iloprost. Centre and right: elevation of cyclic AMP levels. Data from Armstrong et al.,[21] with permission.

There are however several situations where a nonprostanoid has clearly shown a lower functional maximum compared to iloprost. In pig washed platelets, the threshold concentration of iloprost for inhibition of aggregation was always similar (~ 0.5 nM), but the slope of the log concentration-response curve varied somewhat (Fig. 27, lower left).[21] On the "steeper preparations", EP 157 completely inhibited aggregation, whereas on the "shallower preparations", inhibition was incomplete even at 20 µM. In the same study, iloprost raised cyclic AMP levels by at least 20-fold, whereas the maximum elevation to EP 157 was only 1.5 - 2.0-fold (Fig. 27, lower centre), and EP 157 suppressed iloprost's effect on cyclic AMP (lower right). It is possible that in some of the pig platelet preparations more cyclic AMP is required for maximal inhibition of aggregation and when this exceeds twice the resting level, EP 157 behaves as a partial agonist.

Turning to vascular smooth muscle, Figure 28 shows results obtained on human artery ring preparations from different locations. Iloprost showed high relaxant potency on the human pulmonary artery precontracted with phenylephrine (right panel) and BMY 45778, BMY 42393 (not shown) and octimibate behaved as full agonists.[25] CU 602, which is related to BMY 42393, showed variable results. On human coronary artery contracted with 45 mM K^+ (centre panel), iloprost showed moderate relaxant sensitivity and only induced a maximum relaxation of about 60% (the limited relaxant action of IP agonists on K^+-depolarised vascular smooth muscle is discussed in Chapter 6). Under these conditions, octimibate behaved as a partial agonist, surmountably antagonising the action of iloprost.[26]

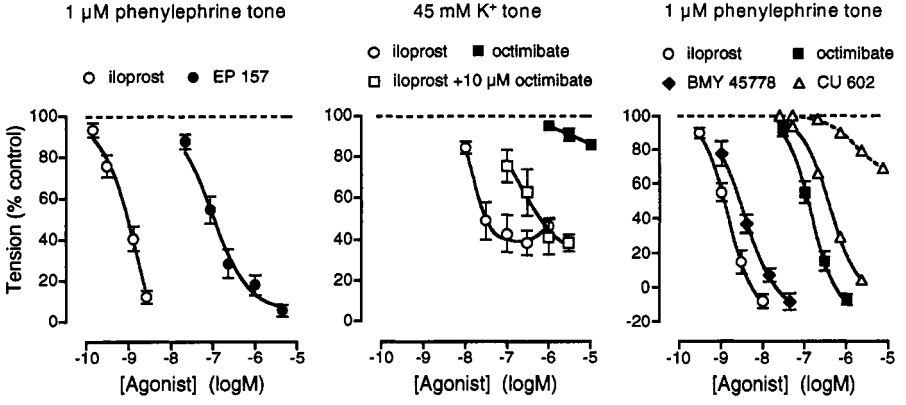

Figure 28. Relaxant profiles on human vascular smooth muscle of nonprostanoid prostacyclin mimetics compared to iloprost. Left: EP 157 on mesenteric artery; right: BMY 45778, octimibate and CU 602 on pulmonary artery. The CU 602 curves are from two separate experiments. Data from Jones et al.,[25] Merritt et al.,[26] and Armstrong et al.,[21] with permission.

It should be noted that EP 157 maximally activated adenylate cyclase in human platelet homogenates[7] and was close to a full agonist on human mesenteric artery (Fig. 28, left panel).[21] One needs to be careful not to read too much into small differences in maximal response either of a functional or a second messenger nature, since interference from several sources is possible when the nonprostanoid is present at high concentrations (1 - 30 μM). Firstly, compared to a typical prostacyclin analogue with its polar 11- and 15-hydroxyls, all the nonprostanoids are highly lipophilic and may exert physiochemical effects on the cell membrane and its integral proteins. Secondly, the 1,1-diphenyl and 1,2-diphenyl units present in the nonprostanoids are commonly found in many other classes of pharmacologically-active molecules such as the α-adrenoceptor antagonists and various morphine analogues.[1,2] Pertinent to these notions is the observation that in rat neutrophils the azine analogue EP 185 at 10 μM induced a greater maximum inhibition of FMLP-induced aggregation than either cicaprost or BMY 45778.27 Thirdly, the EP_3 agonist activity of a nonprostanoid may be sufficient to oppose its IP agonist activity (see Section 4.5).

4.3 In vivo actions

From the discussion so far, it appears that the nonprostanoid prostacyclin mimetics, like analogues of prostacyclin (see Chapter 2), do not radically discriminate between IP-receptors in platelets and blood vessels of a single species. However, could the "efficacy/post-receptor gain" element generate enough of a difference to allow a nonprostanoid to inhibit platelet aggregation without causing marked peripheral vasodilatation and depression of blood pressure? Published data on this point is rather sparse, but the answer appears to be no. ONO-AP-227 at an intraduodenal dose of 0.3 mg kg^{-1} in the anaesthetised dog inhibited collagen-induced platelet aggregation (ex vivo), and at 3 mg kg^{-1} the effect lasted for at least 8 hours.[14] The same doses also caused falls in blood pressure with similar durations of action; a peripheral vasodilator action was confirmed by the increase in femoral artery blood flow following close-intra-arterial injection of 3 - 100 μg kg^{-1} ONO-AP-227. A similar conclusion can be drawn from experiments in the rat where systemic blood pressure and *ex vivo* inhibition of ADP-induced platelet aggregation were measured after administration of 3 - 30 mg kg^{-1} ONO-1301 (see Fig. 29 for structure).[14] The long duration of the ONO analogues is partly due to the presence of the phenoxy unit which blocks β-oxidation (see Chapter 2 for metabolism of prostacyclin analogues).

4.4 TP antagonism

Since EP 157 evolved from a TP antagonist development project, it was of interest to know whether a 1,1-diphenyl unit at the ω-terminus affords TP-receptor blocking activity. EP 157 did indeed displace [^{3}H]-9, 11-epoxymethano PGH_2 binding to human washed platelets with an IC_{50} of 3.5 μM, indicating a low affinity for the TP-receptor.[21] On human platelets, this activity of EP 157 is unlikely to contribute to inhibition of U-46619-induced aggregation, since its IP agonist activity occurs in the low- to mid-nanomolar concentration range. EP 157 does inhibit U-46619-induced aggregation at slightly lower concentrations than are required against PAF- and ADP-induced aggregation. However, this probably reflects differences in the activation mechanisms of the aggregating agents rather than TP antagonism, since a similar inhibitory profile is seen with selective IP agonists such as cicaprost and iloprost.

TP-receptor antagonism by EP 157 can be observed in smooth muscle preparations lacking IP relaxant systems. Thus, contractile responses of rabbit aorta, dog saphenous vein and guinea-pig trachea to U-46619 were completely blocked by 10 μM EP 157, whereas contractions to noradrenaline on the aorta, clonidine (postsynaptic α_2-adrenoceptor agonist) on the saphenous vein, and histamine and 16,16-dimethyl PGE_2 (EP_1 agonist) on the trachea were unaffected.[21] Both the rates of attainment and reversal of TP block by EP 157 were considerably slower than for specific TP antagonists of the EP series (e.g. EP 045). This profile is probably unrelated to the kinetics of binding to the TP-receptor, since (in agreement with the human platelet binding data) the affinities of EP 157 for smooth muscle TP-receptors are only low to moderate; approximate pA_2 values calculated from dose ratios quoted by Armstrong et al.[21] are 6.1 for rabbit aorta, 7.0 for dog saphenous vein and 6.3 for guinea-pig trachea. A more likely explanation is that EP 157 being highly lipophilic partitions extensively into fatty regions of the smooth muscle cells; the rise to equilibrium concentration in the extracellular fluid in the centre of the muscle mass is slow and on washout this concentration is maintained by agonist released from the cellular reservoir.

TP antagonism by 1,1-diphenyl EP analogues can be maintained or enhanced at the expense of IP agonism by increasing the apparent distance between the diphenyl unit and the 1-carboxylate (Fig. 29). EP 043 and EP 076 have pA_2 values on guinea-pig trachea of 7.5 and 7.8 respectively, while EP 043 has weak IP agonist activity and EP 076 is devoid of IP agonism on human platelets. Conversely, in ridogrel the 1,1-diphenylmethyl/carboxylate distance is much shorter (Fig. 29). This simple azine is a potent TXA synthase inhibitor (see Section 4.5, and a TP antagonist (rat caudal artery

pA_2 = 5.47), but not an IP agonist as judged by its inability to raise cyclic AMP levels in human PRP at 100 μM.[28-30]

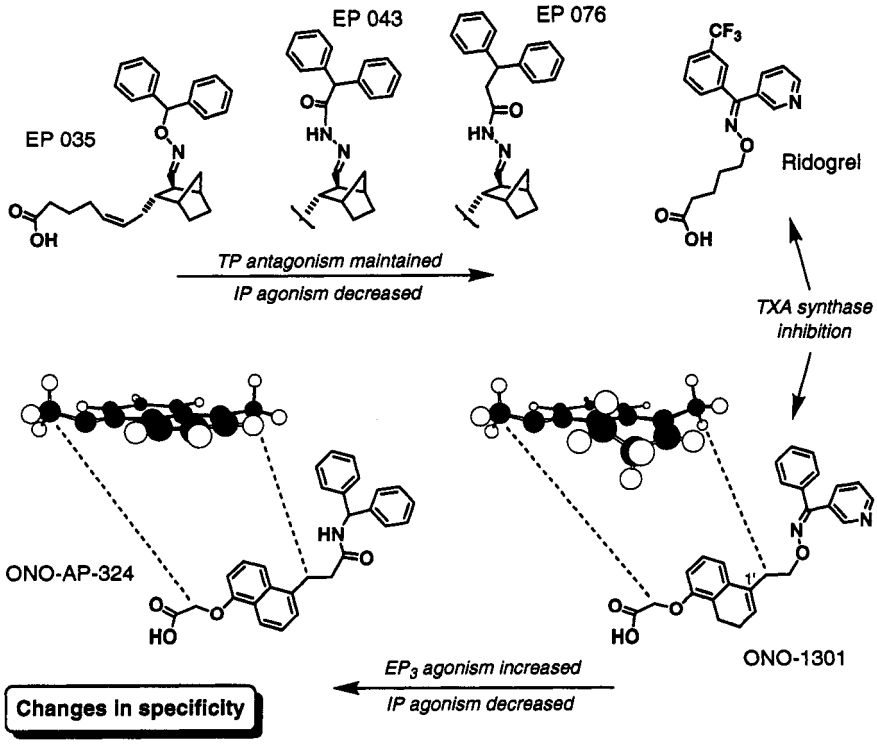

Figure 29. Changing prostanoid receptor specificity from IP agonism to TP antagonism and to EP_3 agonism. The 3-D fragments show that the 1',5'-disubstituted-dihydronaphthalene nucleus (right) is nonplanar compared to the corresponding naphthalene nucleus (left). In ridogrel and ONO-1301, the presence of the *m*-pyridyl unit confers TXA synthase inhibitory activity.

What of the TP blocking potencies of the BMY and ONO types of nonprostanoid? BMY 45778, BMY 42393, octimibate and CU 602 inhibited U-46619-induced contraction of guinea-pig trachea with IC_{50} values lying between 1.4 and 3.0 μM,[25] rather weak activity relative to the IC_{50} of 1.8 nM for the specific TP antagonist GR32191.[31] ONO-Ap-227 (Fig. 23) and ONO-1301 (Fig. 29) appear to show high selectivity for IP-receptors over TP-receptors on human platelets, having K_i values of 130 and 190 nM for competition for [^{3}H]-iloprost binding and >10 μM for competition for [^{3}H]-SQ29548 binding.[14]

4.5 The switch to EP_3 agonism

In ONO-1301 and ONO-AP-324 (Fig. 29), the central linkage is attached to the 1'-position of the naphthyl unit; the double bond at the 1',2'-position in ONO-1301 confers additional planarity and this is complete in the fully aromatic naphthalene unit of ONO-AP-324. Both ONO-1301 and ONO-AP-234 activate EP_3-receptors and in the case of ONO-AP-324 specificity is high. Its K_i value for the cloned mouse $EP_{3\alpha}$-receptor expressed in CHO cells is 11 nM, compared to >10 μM for mouse EP_2-, EP_4- and FP-receptors and human IP- and TP-receptors, and 4.6 μM for the mouse EP_1-receptor (K. Kondo, personal communication).

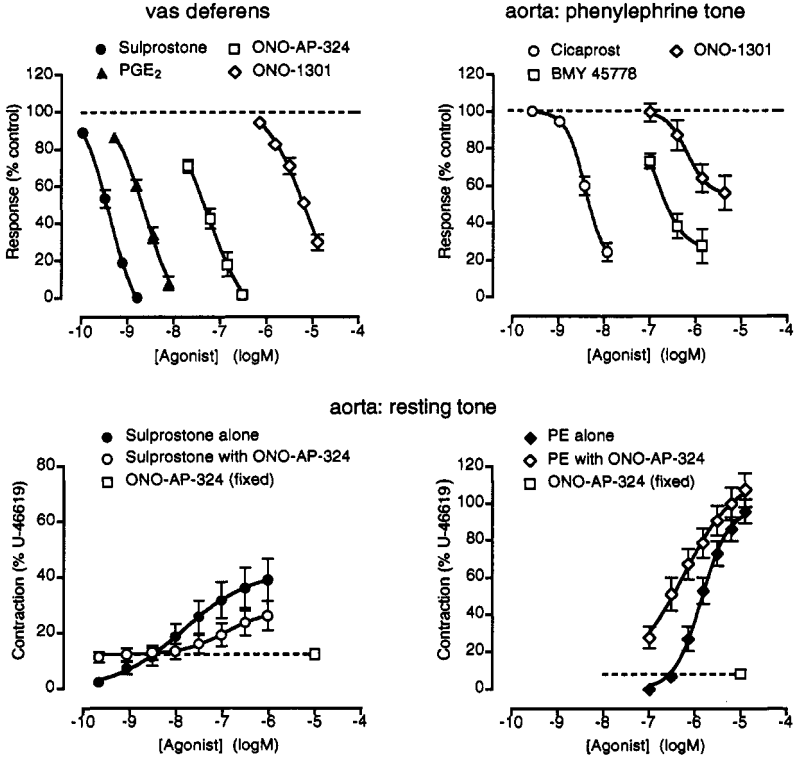

Figure 30. Biological activity of nonprostanoid EP_3 agonists on guinea-pig isolated tissues. Top left: inhibition of twitch responses of vas deferens to electrical field stimulation. Top right: relaxant activity against phenylephrine-induced tone on the aorta. Bottom left: ONO-AP-324 contracts the aorta and opposes the action of sulprostone. Bottom right: ONO-AP-324 acts synergistically with phenylephrine on the aorta. Data from Jones et al.,[32] with permission.

Activation of EP_3-receptors on postganglionic sympathetic nerve endings in the guinea-pig vas deferens (the archetypical EP_3 preparation) suppresses transmitter release and this is represented functionally by inhibition of twitch responses to electrical field stimulation.[33] ONO-AP-324 behaves as a full agonist in this system and is about 125 and 20 times less active than sulprostone and PGE_2 respectively (Fig. 30).[32] ONO-1301 showed weaker EP_3 agonism. Some arterial vessels such as rabbit renal artery,[34] human pulmonary artery[35] and guinea-pig aorta[32] contain contractile EP_3-receptors. ONO-AP-324 contracted human pulmonary artery in the presence of the TP antagonist GR-32191 and was about 125 times less active than the EP_3 standard agonist sulprostone.[32] On guinea-pig aorta ONO-AP-324 behaved as a partial agonist, antagonising the action of sulprostone and synergising with phenylephrine (Fig. 30). In contrast, the IP agonist activity of ONO-1301 was dominant on the aorta (Fig. 30).

4.6 Inhibition of thromboxane synthase

Some of the first and most potent TXA synthase inhibitors (e.g. ONO-1581, human platelet microsomes IC_{50} = 3 nM), contained a 1-phenyl-1-*m*-pyridyl-methyl moiety.[36] The ONO prostaglandin team have substituted this unit for the 1,1-diphenylmethyl unit present in ONO-AP-227 and obtained both IP agonism (human PRP: IC_{50} = 320 nM) and inhibition of TXA synthase (human platelet microsomes: IC_{50} = 85 nM) in the same molecule.[37] Enzyme inhibition is greatest when the nitrogen is in the *meta* as opposed to the *ortho* or *para* positions of the pyridine ring, whereas IP agonist potency is little affected. ONO- 130 1, which also contains a 1 -phenyl- 1 -*m*-pyridyl-methyl unit (Fig. 29), is a more potent inhibitor (IC_{50} = 26 nM).[14] However, this additional property, which was evident in vivo in the rat, was not sufficient to create a non-vasodepressor antithrombotic (see Section 4.3).

5. CONCLUDING REMARKS

The nonprostanoid prostacyclin mimetics offer the potential for more than one prostanoid action of varying intensity within the same molecule, coupled with relative ease of synthesis compared to analogues of prostacyclin. There is also the possibility of obtaining nonprostanoid agonists and antagonists for other prostanoid receptors, such as DP- and TP-receptors and the EP subtypes. Combinatorial chemistry coupled with large scale screening using radioligand binding has the ability to efficiently explore many different functional group combinations. However, the conventional synthetic methods which have been used so far may be more

suited to the generation of fused ring systems that explore the rather subtle changes in molecular shape associated with for example the switch from IP to EP_3 agonism in the ONO series.

The K_d of 6 - 10 nM for BMY 45778 in the human platelet [^{3}H]iloprost binding assay[23,25] demonstrates that nonprostanoid molecules can bind with high affinity to the IP-receptor. Whether one can proceed to a pure IP-receptor antagonist by structural modification within the EP, BMY or ONO series is not known; certainly a blocker has not simply "popped out of the hat" so far

Finally, the nature of the molecular interaction of the nonprostanoids with the IP-receptor could be investigated by the use of chimeric or point-mutated receptors expressed in suitable cell lines (see Chapter 4). One aspect is clear: the diphenyl moiety in the nonprostanoids is not a simple replacement for the ω-chain of prostacyclin, since a hydrid of carbacyclin (α-chain and ring system) and EP 157 (ω-chain) is only a weak IP-receptor agonist.[38]

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Chapter 4

Isolation, cloning and characterisation of IP-receptors

1. INTRODUCTION

One of the principal obstacles to the study of IP-receptors has been the lack of highly specific ligands with which to distinguish IP-receptor mediated responses from those due to activation of other prostanoid receptors. Thus, a considerable advantage gained from the cloning and expression of IP-receptors in transfection systems has been the ability to assess the actual potency of nonselective ligands in systems devoid of complications of mixed receptors, as found in most biological systems. The recent comparative ligand binding data of Kiriyama et al.,[1] adapted in Table 2, is invaluable in this respect and should help us to interpret better the data implicating IP-receptor subtypes *versus* multiple G-protein coupling. This chapter aims to discuss the recent data derived from IP-receptor cloning studies in relation to older data from more classical pharmacological approaches. Much of the confusion concerning the existence of IP-receptor subtypes can now be put in perspective given our more recent knowledge on prostanoid receptor classification, prostanoid drug specificities and the influence of receptor-effector coupling in determining the agonist or antagonist property of many of the prostanoid mimetics.

Although authors have been quick to label their proposed IP-receptor subtypes as IP_1, IP_2 and IP_3 (see Hébert et al.[2] and Takechi et al.[3]), this may lead to confusion with the more well recognised use of IP3 to describe inositol 1,4,5-trisphosphate. The International Union of Pharmacology Committee on Drug Classification and Receptor Nomenclature recognises the possible existence of IP-receptor heterogeneity, but notes the lack of

unequivical experimental evidence. We have decided in this book to refer to the only cloned IP-receptor as the IP_1-receptor. As we shall see in the following sections, the cloned IP_1-receptor has all the properties associated with the well-established antiplatelet and vasorelaxant properties of prostacyclin. For ease of discussion, the novel IP-receptor identified in the rat central nervous system will be referred to here as the IP_2-receptor (see Chapter 11).

Table 2. K_i values of prostanoids for mouse cloned prostanoid receptors

Drug	*IP*	*EP_1*	*EP_2*	*EP_3*	*EP_4*
Cicaprost	10	*	1300	170	*
Iloprost	11	21	1600	22	2300
Carbacyclin	110	*	1600	31	2300
Isocarbacyclin	15	*	1000	31	*
Beraprost	16	*	*	110	*
ONO-1301	47	*	*	740	*
PGE_1	33	36	10	1.1	2.1
PGE_2	*	20	12	0.85	1.9

Data are K_i values (nM) from ligand binding experiments. *K_i value greater than 10 μM. Reprinted from Kiriyama et al.,[1] with permission.

The mouse and human IP_1-receptor genes have been localised on chromosomes 7 and 19 respectively,[4] and are phylogenetically related more to EP_2, EP_4, and DP-receptors than to EP_1, EP_3, FP and TP-receptors,[5] as would be expected for G_s-coupled receptors. Indeed, a single point mutation in the seventh transmembrane domain (TMVII) of the EP_2-receptor (leucine to tyrosine at position 304) results in a 100-fold increase in the activity of iloprost, suggesting that the IP-receptor evolved from the EP_2-receptor.[6] A significant breakthrough in our understanding of the function of the IP_1-receptor, and the possible existence of IP-receptor subtypes, has come from the production of IP_1-receptor knockout mice.[7] The gene for the IP_1-receptor was disrupted in mice by using homologous recombination. The targeting vector was constructed by replacing a 2.4 kb fragment containing parts of the putative exons II and III, which encode the TMVI domain of this seven TM-domain receptor, with the neomycin-resistance gene; this disruption of the gene sequence then prevents gene transcription. Although there was some prenatal mortality in the IP_1-receptor-deficient mice, especially in males, homozygous mice that survived to birth grew normally, lived for more than one year and were fertile. Most importantly when considering IP-receptor subtypes, there was evidence that the IP-receptors in platelets and vascular smooth muscle derive from the same gene. Therefore the search for the Holy Grail of a nonvasodilating, platelet inhibiting IP agonist must be over; the combination is impossible unless one can utilise the tissue-specific nature of partial agonists.

2. IP-RECEPTOR ISOLATION

2.1 Native IP-receptors

Before the successful use of molecular biology techniques to clone and express the IP1-receptor, attempts were made to isolate and characterise IP-receptors using conventional biochemical approaches. Leigh et al.[8] used radiation inactivation of NCB-20 neuroblastoma cells to determine a molecular weight of approximately 83 kDa for the IP-receptor, and Tsai et al.[9] used gel filtration to give an estimate of 150 kDa for the IP-receptor solubilised from human platelets. Ito et al.[10] prepared an irreversible photoaffinity probe [15-^{3}H]-19-(3-azidopheny1)-20-nor isocarbacyclin ([^{3}H]-APNIC) to isolate the IP-receptor from the mouse mastocytoma cell line P-815 and from porcine platelet membranes. [^{3}H]-APNIC specifically labelled a protein of 43 - 45 kDa, and this binding was modulated by GTPγS. A lower molecular weight is apparently often seen using photoaffinity labelling and SDS-PAGE compared with these other methods which appear to measure the association of the receptor with other molecules such as G-proteins.[10] The data obtained from photoaffinity labelling and SDS-PAGE analysis agrees much better with data on the cloned IP_1-receptor;[11-16] see Table 3. A broad band around 43 - 45 kDa was observed by fluorography and suggested that the IP-receptor is a glycoprotein;[10] a conclusion that has been substantiated now using the cloned IP1-receptor.[16]

2.2 Cloned IP-receptors

Because the IP-receptor was one of the last of the prostanoid receptor family to be cloned, the screening strategy could make use of the observation that there are highly conserved amino acid sequences in the second and seventh transmembrane spanning domains of EP and TP-receptors.[17-19] By using primers corresponding to these conserved sequences, Namba et al.[14] were the first to isolate the mouse IP_1-receptor cDNA using the reverse transcription polymerase chain reaction and hybridisation screening of a cDNA library prepared from P-815 mouse mastocytoma cells. Thereafter, the cDNA for the human and rat IP_1-receptors was rapidly isolated by the same team using cross-hybridisation of cDNA libraries with a probe prepared from the mouse IP1-receptor cDNA.[12,13,15] Boie et al.[11] took a similar approach to Namba et al.[14] to isolate the human IP_1-receptor cDNA; in this case they prepared a [^{32}P]-labelled probe based on the 9 amino acids (NPILDPWIY) in TMVII of the mouse EP_2-receptor and used this to screen human lung, thymus, and small intestine cDNA libraries. The general

properties of the resulting IP_1-receptor cDNA expressed in COS and CHO cells are summarised in Table 3. It is interesting to note that although the first IP-receptor to be cloned was isolated from a mouse mastocytoma cell line, there is no evidence for a functional role for IP-receptors in mast cells in general. High concentrations of cicaprost can inhibit histamine release from activated rat peritoneal mast cells, but this is unlikely to be due to activation of IP_1-receptors.[20]

Table 3. Summary data for recombinant IP_1-receptors

	Human[12]	*Human*[13]	*Human*[11]	*Cow*[21]	*Mouse*[14]	*Rat*[15]
Source of cDNA	CMK cells	lung	lung	liver	P-815 cells	lung
Number of amino acids	386	386	386	385	417	416
Deduced Mol. Wt.	40,956	40,956	40,961	41,247	44,722	44,662
Cell line for expression	CHO	COS-7	COS-M6	nd	CHO	COS-7
K_d (nM)*	3.3	24	1 and 44	nd	4.5	1.3
B_{max} (pmol mg protein-1)*	3.2	5.7	2.0 and 2.3	nd	4.6	1.2

CMK cells: megakaryocytic leukaemia cells. P-815 cells: mastocytoma cells.
*[^{3}H]-iloprost binding data. nd; not determined.

The rat IP_1-receptor clone has an N-terminus with 68% amino acid identity to the corresponding region of the mouse IP1-receptor,[22] whereas the cow and human homologues have an N-terminus which is 30 amino acids shorter than that of the mouse and rat IP_1-receptors; see Figure 31. The N-terminus may therefore not be important for IP_1-receptor function.[18] From Met-31 to the C-terminal tail, mouse and rat clones have very high sequence homology (98%), whereas the human clone is only 79% identical to mouse and rat; most of this difference lies in the C-terminal tail. The second cytoplasmic loop is completely conserved in cow, human, mouse and rat IP_1-receptors which suggests that this region is important for coupling to G_s-proteins. In contrast to the cow, mouse and rat IP_1-receptors, the human IP_1-receptor has no potential phosphorylation site for cyclic AMP-dependent protein kinase (PKA), although all four clones have consensus sequences for phosphorylation by protein kinase C (PKC). Therefore, the deduced amino acid sequences of these cloned receptors infer membership of the G-protein-

coupled receptor superfamily and, in general, the clones from the four species are very similar in their primary structure (Fig. 32), especially in the second cytoplasmic loop. The human IP1-receptor has two potential *N*-glycosylation sites at Asn-7 (in the N-terminal tail) and Asn-78 (in the first extracellular loop); see Figure 32. Western blotting of membranes prepared from HEK 293 cells expressing an epitope-tagged human IP_1-receptor resolved as a broad complex with a molecular mass ranging from 44 to 62 kDa.[16] When treated with PNGase F to deglycosylate the receptor, the major band then appeared at 39 kDa.

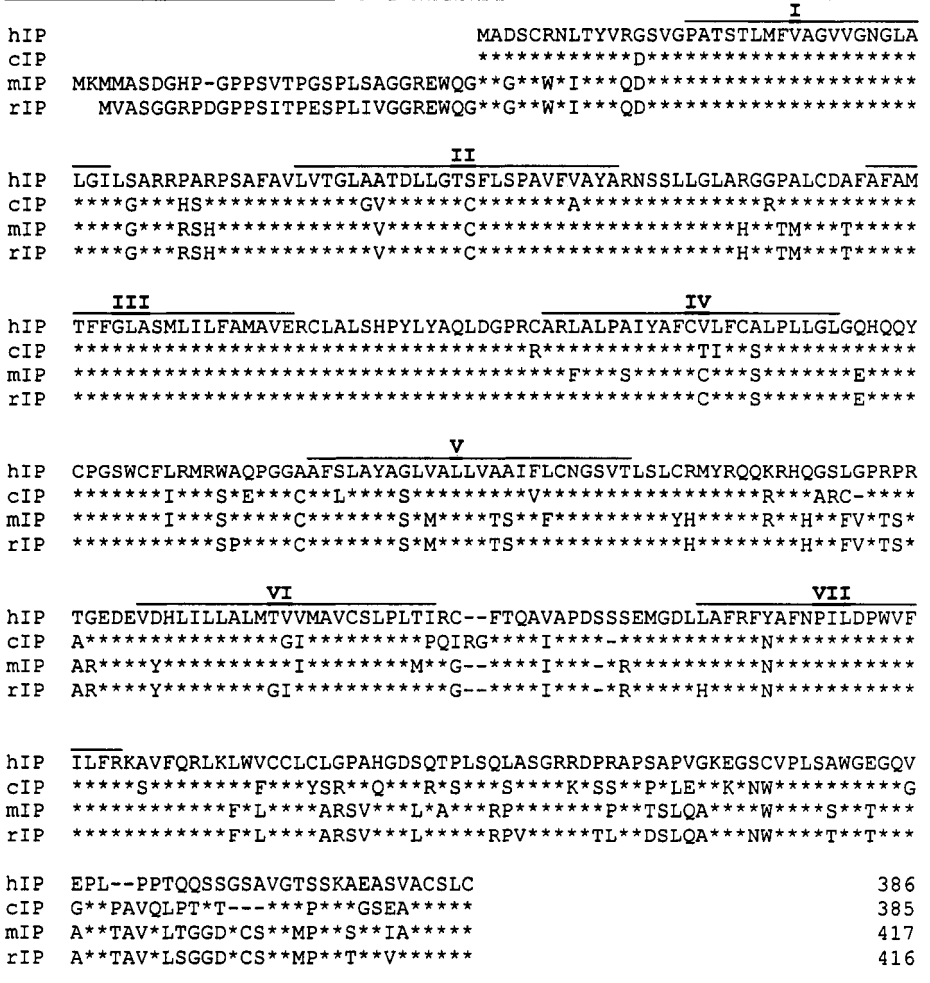

Figure 31. Comparison of amino acid sequence of cow (c), human (h), mouse (m) and rat (r) IP_1-receptors. Identical amino acid residues are indicated by asterisks. Positions of the putative transmembrane domains I to VII are indicated above the sequences. Reprinted from Katsuyama et al.[12] and Sasaki et al.,[15] with permission, and data from Hasse et al.[21]

Although the overall homology of prostanoid receptors with other rhodopsin-type receptors is not high (about 10 - 20% homology with adrenergic and muscarinic receptors), they have several features in common:[19] (1) asparagine residue(s), which are putative *N*-glycosylation sites, are conserved in the N-terminus of the extracellular portion of all prostanoid receptors, (2) there are two cysteine residues in the first and second extracellular loops which are presumably required for maintenance of appropriate structure, and (3) there are serine and threonine, i.e. putative phosphorylation sites, widely distributed in the cytoplasmic portion of prostanoid receptors. The second extracellular loop and TMVII of all prostanoid receptors have 22 highly conserved amino acid residues, of which eight are nonvariable.[17] Using the human IP_1-receptor as an example, the important region of the second extracellular loop has PGS**WCF**L, and TMVII has **R**FYAFNP**IL****DPW**VFI (the bold letters represent the invariable amino acids). Comparing the transmembrane segments of TP, EP_3 and β_2 receptors, it seems that the transmembrane segments of prostanoid receptors are more hydrophobic than the adrenergic receptor, which is consistent with the hydrophobic nature of prostanoid molecules, and suggests that their alkyl chains interact with these regions.[23]

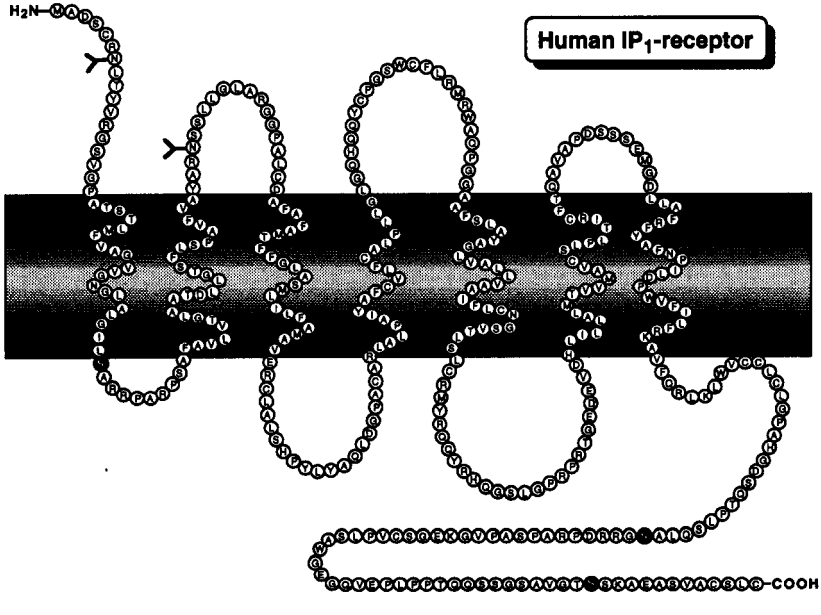

Figure 32. Diagram showing the predicted configuration of the human IP_1-receptor. Putative membrane spanning regions are shown in the shaded region. Potential PKC phosphorylation sites are shown in black. *N*-Linked glycosylation sites in the N-terminus and the first extracellular loop are indicated with a *Y*. Reprinted from Smyth et al.,[16] with permission.

3. [^{3}H]-ILOPROST BINDING

3.1 Native IP-receptors

[^{3}H]-Iloprost is the most frequently used radioligand in IP-receptor studies, due to its increased specificity towards IP-receptors, its increased stability and reduced level of nonspecific binding compared with [^{3}H]-PGI_2. However despite this, there are problems when using [^{3}H]-iloprost at concentrations greater than 30 nM for Scatchard plot analysis. At these high concentrations, an appreciable component of the displaceable [^{3}H]-iloprost binding has been identified as due to a very high capacity, low affinity, nonreceptor site in neuroblastoma cells.[24,25] The association of [^{3}H]-PGI_2 and [^{3}H]-iloprost to binding sites in vitro is rapid. Even at 0°C almost complete saturation of [^{3}H]-PGI_2 binding to bovine platelet membranes is reached after only 10 min,[26] and 30 sec is sufficient for [^{3}H]-iloprost binding to pig aorta membranes at 37ºC.[27]

Prior to the cloning of the IP_1-receptor, human platelet membranes served as the most popular source of IP-receptors for binding studies, with [^{3}H]-iloprost having typical equilibrium dissociation constants (K_d) of 8 - 20 nM (see Table 4). Occasionally both [^{3}H]-iloprost and [^{3}H]-PGI_2 can recognise two binding sites (Table 4) and in human platelets, PGE_1 and 6β-PGI_1 can interact with both.[28] In this latter case, there is some evidence that there is a biological response (increased cyclic AMP) associated with this low affinity site in platelets. In contrast, prostacyclin activates guinea-pig lung adenylate cyclase, probably in small pulmonary blood vessels,[29] at concentrations appropriate to the high affinity [^{3}H]-PGI_2 binding site, and no biological role for the lower affinity species has been identified.[30] It is important to remember that [^{3}H]-iloprost can also bind with moderately high affinity to EP_1 and EP_3-receptors (Table 2). Therefore, although specific [^{3}H]-iloprost binding has been observed in the membrane fraction from bovine heart,[31] this may not necessarily represent binding to an IP-receptor. For example, this [^{3}H]-iloprost binding was displaced with higher potency by PGE_1 and PGE_2 than by iloprost, therefore it could conceivably represent [^{3}H]-iloprost binding to the EP_3-receptor as there is evidence of EP_3-receptor mRNA in human,[32,33] mouse[34] and rabbit hearts.[35]

Table 4. A selection of IP-receptor binding data from various tissues

Species	*Tissue preparation*	*Ligand*	*K_d (nM)*	*Ref. No.*
Human	cloned in CHO cells	[^{3}H] -iloprost	3.3	12
	cloned in COS-7 cells	[^{3}H] -iloprost	24	13
	cloned in COS-M6 cells	[^{3}H] -iloprost	1& 44	11
	cloned HA-IP-receptor in HEK 293 cells	[^{3}H] -iloprost	0.4& 75	16
	platelets	[^{3}H]-iloprost	8.2, 9.7, 12.9,14, 16.6,19.6	9,36-40
	platelets	[^{3}H]-PGI$_2$	6.5 & 400, 63	26,28
	platelets*	[^{3}H]-PGI$_2$	12.1 & 909	41
	MEG-01 cells (megakaryocyte)	[^{3}H]-iloprost	1.2 & 950	37
	CML basophils*	[^{3}H]-iloprost	0.5 & 27	42
Rat	cloned in COS-7 cells	[^{3}H]-iloprost	1.3	15
	platelets	[^{3}H]-PGI$_2$	55	26
	CNS; nucleus tractus solitarius†	[^{3}H]-iloprost	6.8	3
	CNS; nucleus tractus solitarius†	[^{3}H]-isocarb.	3.9	3
	CNS; thalamus?	[^{3}H]-iloprost	159	3
	CNS; thalamus?	[^{3}H]-isocarb.	7.8	3
	CNS; rostral cortex†	[^{3}H]-l5R-TIC	<1 & ~30	43
Mouse	cloned in CHO cells	[^{3}H]-iloprost	4.5	14
	cloned HA-IP-receptors in COS-7 cells	[^{3}H]-iloprost	13	44
	P-815 cells (mastocytoma)	[^{3}H]-APNIC	4.7	10
Rat/ Mouse	NG108-15 neuroblastoma cells	[^{3}H]-iloprost	10*	24
Mouse/ Hamster	NCB-20 neuroblastoma cells	[^{3}H]-iloprost	5.1, 10;* 10;* 29.9	8,24,25,36
	NCB neuroblastoma cells	[^{3}H]-PGI$_2$	16.6, 18.1	45,46
Guinea-pig	lung	[^{3}H]- PGI$_2$	16 & 258	30
Rabbit	oxyntic mucosa cells*	[^{3}H]-iloprost	0.11 & 70	47
Dog	platelets	[^{3}H]-PGI$_2$	42	26
Pig	aorta smooth muscle	[^{3}H]-iloprost	22.4	27
Cow	coronary artery	[^{3}H]-iloprost	21 & 750	48
	platelets	[^{3}H]-PGI$_2$	7 & 320	26

All data are from membrane preparations, except those marked (*)which are from whole cells, and (†)slice preparation for autoradiography. **Additional low affinity, high capacity, binding site. HA-humadmouse IP_1-receptors have been constructed with the tag of a haemagglutinin epitope from the human influenza virus. [^{3}H]-APNIC is [15-^{3}H]-19-(3-azidophenyl)-20-nor isocarbacyclin. [^{3}H] -isocarb. is [^{3}H] -isocarbacyclin. [^{3}H]-15R-TIC is [^{3}H]-15R-16-*m*-tolylisocarbacyclin.

In view of the finding that the cloned human IP_1-receptor can also display two binding components for [^{3}H]-iloprost, [11,16] it is unlikely that the lower affinity component of the native receptor represents a second distinct IP-receptor or an EP-receptor. Instead, it is feasible that we are looking at the consequence of using an agonist as the radioligand in these IP-receptor binding studies. For G-protein coupled receptors, the equilibrium between low and high affinity conformations of the receptor is regulated by the activation state of the G-protein. High concentrations of the GTP-bound form of the G-protein will reduce the affinity of the receptor for the agonist, with a resultant decrease in [^{3}H]-agonist binding. For example, in IC2 mast cell membranes, the binding of [^{3}H]-iloprost (10 nM) decreased from 357 fmol mg protein^{-1} to 69 fmol mg protein^{-1} in the presence of GTPγS.[49] This loss of [^{3}H]-iloprost binding is most likely due to a decrease in binding affinity as in MEG-01 cells, which display two binding sites for [^{3}H]-iloprost, the high affinity site is lost in the presence of GTPγS.[37]

Eggerman et al.[50] have suggested that the variability in reporting one or two affinity binding sites for [^{3}H]-PGI_2 binding to human platelets may depend both on their method of isolation, and the manner in which the nonspecific component of binding has been accounted for. Another matter to consider is the possible presence of an anti-IP-receptor antibody in the circulation of the blood donors. For example, Kahn et al.[51] have demonstrated the presence of such an antibody in the serum of patients with chronic spinal cord injury, and this antibody preferentially binds to the high affinity binding site for [^{3}H]-PGE_1. Furthermore, it seems that activation of the high affinity state of the IP-receptor is responsible for the inhibition of platelet-stimulated thrombin generation, while activation of the low affinity site results in cyclic AMP accumulation (see Chapter 5, Section 3.1, for further details).

The hydrolysis of prostacyclin to 6-oxo $PGF_{1\alpha}$ results in almost complete loss of binding activity in guinea-pig lung,[30] along with the inability to stimulate adenylate cyclase in guinea-pig lung[30] and NCB-20 neuroblastoma cells.[52] The inactivity of 6-oxo $PGF_{1\alpha}$ illustrates the importance of the enol ether moiety of prostacyclin for receptor binding, as discussed more fully in Chapter 2. In this context, reduction of the 5,6-double bond in prostacyclin to give 6p-PGI_1 destroys the unique planar stereochemistry of the C4-C7 region and also results in significant loss of binding affinity to guinea-pig lung membranes.[30] However, some PGI_1 analogues, such as SM-10906, show high affinity binding (IC_{50} 20 nM) to the IP-receptor in mouse mastocytoma P-815 cells.[53] Interestingly, the methyl ester of SM-10906 (SM-10902) behaves as a partial agonist; the esterification not only reduces the binding affinity (IC_{50} 2000 nM), but appears to reduce its efficacy as well. Esterification also reduces the binding affinity of SM-10902 by 7 - 20-

fold in human platelets, and umbilical vein and arterial endothelial cells.[54] The methyl ester analogue (TEI-9090) of isocarbacyclin (TEI-7165) shows a 40-fold loss in binding affinity in P-815 cell membranes, providing further evidence that a free carboxyl group on the prostanoid a-chain is essential for efficient binding to the IP-receptor.[55]

The higher affinity of PGE_1 relative to PGE_2[50] probably means that the highly mobile α -side chain of PGE_1 can adopt a conformation that is unobtainable by the more conformationally-restricted PGE_2, and more closely resembles prostacyclin. Similar structure-activity-relationships have been reported for activation of adenylate cyclase in NCB-20 neuroblastoma cells.[52]

3.2 Cloned IP-receptors

The binding properties of the cloned IP_1-receptors have been studied using [^{3}H]iloprost, and are summarised in Tables 3 and 5. The cloned mouse and rat IP_1-receptors display a single class of [^{3}H]-iloprost binding site with K_d values of 4.5 and 1.3 nM, respectively.[14,15] Although both Katsuyama et al.[12] and Nakagawa et al.[13] also found a single class of [^{3}H]-iloprost binding site for the human IP_1-receptor (K_d values of 3.3 and 24 nM, respectively), Boie et al.[11] reported that the binding profile could be best fitted with a two-site model yielding K_d values of 1 and 44 nM. Two populations of iloprost binding sites with different affinities have been reported elsewhere but in one of these studies,[16] the same human IP_1-receptor cDNA was used as described by Boie et al.[11] As noted in Section 3.1 above, the full significance of this second [^{3}H]iloprost binding site remains to be clarified.

The binding affinities of the major prostanoid ligands are similar for human, mouse and rat IP_1-receptors expressed in COS-M6, COS-7 or CHO cells; they compete with [^{3}H]iloprost binding in the following order: cicaprost $\geq$ iloprost > PGE_1 = carbacyclin >> PGD_2 = PGE_2 = STA_2 or U-46619 (TP-receptor agonists) > $PGF_{2\alpha}$. Although the overall pattern of responses to prostanoid ligands is similar, the data in Table 5 would indicate the existence of species differences with regard to the affinity of PGE_1 and carbacyclin for IP_1-receptors. Thus, both PGE_1 and carbacyclin compete with higher potency for [^{3}H]iloprost binding to the cloned mouse and rat IP_1-receptors when compared with the human IP_1-receptor. Similar species differences can be seen when looking at the ability of IP agonists to inhibit platelet aggregation, where carbacyclin is clearly more potent in inhibiting rat platelet aggregation compared with human platelet aggregation;[56] see Chapter 5, Section 3.3 for further discussion. Furthermore, iloprost and carbacyclin showed similar potencies in competition for [^{3}H]-iloprost

binding[8,36] and for stimulation of cyclic AMP production[36] in NCB-20 neuroblastoma cells (mouse/hamster hybrid). However, carbacyclin was at least 10-fold less potent than iloprost in both competition for [^{3}H]-iloprost binding and stimulation of adenylate cyclase activity in human platelets.[36] In contrast to the relatively higher potency of PGE_1 and carbacyclin in rodents, octimibate and related compounds are 10 - 50-fold less potent in inhibiting rat and rabbit platelets compared with human platelets.[40,57-60] Our own preliminary studies indicate that in COS-7 and CHO cells transiently expressing the mouse IP_1-receptor, cicaprost and iloprost have EC_{50} values of approximately 9 nM in stimulating adenylate cyclase activity, but the nonprostanoid prostacyclin mimetic BMY 45778 is 35-fold less potent. At the present time, we have no comparable data for the potency of non-prostanoid prostacyclin mimetics on the human IP_1-receptor.

Table 5. Competition binding data for recombinant IP_1-receptors; IC_{50} values for prostanoid agonists

	Human[12]	*Human*[13]	*Human*[11]	*Mouse*[14]	*Rat*[15]
[^{3}H]-iioprost concentration	~20 nM	~20 nM	4nM	20 nM	20 nM
Iloprost	20	60	4 ± 0.14	30	20
Cicaprost	30	60		20	20
Carbacyclin	2,000		431 ±71	600	
PGE_1	3,000	2,000		200	300
PGE_2	>10,000	> 10,000	– 10,000	>10,000	8,000
PGD_2	> 10,000	>10,000	>30,000	> 10,000	> 10,000
STA_2	9,000	> 10,000		> 10,000	8,000
U-466 19			>30,000		
$PGF_{2\alpha}$	> 10,000	>10,000	>30,000	>10,000	>10,000

Except for data from Boie et al.,[11] the IC_{50} values (nM) have been estimated directly from graphs of the original data.

3.3 Mutant prostanoid receptors

All prostanoid receptors have arginine residues at identical positions in TMVII.[23] In rhodopsin, a lysine residue is present at an analogous position and makes a Schiff base with its ligand, all-*trans*-retinal. Based on this analogy and the fact that the α-carboxyl group is essential for the biological activities of most prostanoids, it has been suggested that this arginine residue in TMVII serves as the binding site for the α-carboxyl group of prostanoids. No site-directed mutagenesis studies have been reported yet for the IP_1-receptor, but we may infer some properties of this receptor from information on other prostanoid receptors. Substitution of arginine 302 in TMVII of the

EP_2-receptor with a neutral residue (glutamine) or a negatively charged residue (glutamate) resulted in a loss of activity for this receptor.[6] When the positively charged Arg-309 (TMVII) of the mouse $EP_{3\alpha}$-receptor was replaced with glutamate (neutral), valine (neutral) or lysine (positively charged), only the lysine mutant maintained PGE_2 binding.[61] Similar modifications of the bovine EP3D-receptor also highlight the important role for a charged residue at Arg-332 (TMVII) in relation to the selectivity of G-protein coupling. The interaction between the α-caboxylic acid of the prostanoid agonist and the Arg-332 of EP3D-receptor was not essential for G_i coupling, but was necessary for coupling to G_s and for changes in Ca^{2+} signalling.[62,63]

The mouse IP_1 and DP-receptors show relatively high homology, with 58% identity in the transmembrane domains, therefore chimeric IP_1/DP-receptors have been made in order to study the domains conferring ligand binding specificity.[44] [^{3}H]- PGE_1, but not [^{3}H]-PGE_2, bound with high affinity (K_d = 27 nM) to the IP $_1$-receptor. When the region from TMVI to the C-terminus of the IP_1-receptor was replaced with that of the DP-receptor, then [^{3}H]-PGE $_2$gained the ability to bind with high affinity (K_d = 40 nM); together the results suggest that the region from TMVI to TMVII confers the specificity of this IP_1-receptor to bind PGE_1 and not PGE_2, i.e. it recognises the a-chain configuration. The other distinguishing feature of prostanoids is their cyclopentane ring structure, and from the IP_1/DP-chimeric receptor studies, it seems that the DP-receptor is much stricter in its recognition of the cyclopentane ring than is the IP_1-receptor. In addition, the binding pocket of the IP_1-receptor for the cyclopentane ring of prostanoid molecules is localised in a region containing the first to the third TM domains. Another striking result from these chimeric receptor studies was the influence of the C-terminal tail.. Thus, when the C-terminal tail of the IP_1-receptor was replaced with that of the DP-receptor, then we see a 10-fold increase in binding affinity for both [^{3}H]-iloprost and [^{3}H]-PGE $_1$. Although several residues of TMVII were also replaced, seven of these nine residues remained unchanged with the majority of changes being in the C-terminal tail itself. The influence of the DP-receptor C-terminal tail on the binding affinity of the IP_1-receptor was lost when TMVI and TMVII of the IP_1-receptor was replaced with the corresponding regions of the DP-receptor.

4. G-PROTEIN COUPLING

4.1 Native IP-receptors

Multiple signalling pathways for IP-receptors have been noted in a variety of normal and transformed cell lines, providing constant speculation to the existence of IP-receptor subtypes. It is notable that in normal cells, e.g. rat dorsal root ganglion (DRG) cells in vitro,[64] and in transformed cell lines.[37,65,66] IP agonist potencies for stimulating cyclic AMP and IP_3 production and increasing Ca^{2+} mobilisation are similar, but the sensitivity of the adenylate cyclase response is a 1000-fold higher than phospholipase C (PLC) activation in transfected cells;[12,14,16] see Figure 33.

In isolated perfused rabbit cortical collecting ducts, iloprost and carbacyclin inhibit vasopressin-stimulated water conductivity (AVP-*L*p).[2] In an attempt to identify the receptor mediating this action of these IP agonists, Hébert et al.[2] proposed the existence of three subtypes of IP-receptor: IP_1-receptor coupled to increases in IP_3 and $[Ca^{2+}]_i$, presumably via PLC; IP_2-receptor coupled to G, and cyclic AMP production; while the IP_3-receptor coupled to decreases in cyclic AMP, and was responsible for the inhibition of AVP-*L*p. Given the lack of suitable receptor antagonists to help identify the site of action of iloprost and carbacyclin, evidence from cross-desensitisation studies with PGE_2 was used as evidence that the IP agonists were not acting on EP-receptors. However, it is still possible that we are looking at the multiple G-protein coupling of IP-receptors, in this case the IP-receptor could be coupling to G_q to cause an increase in $[Ca^{2+}]_i$. Furthermore, the existence of a novel IP_3-receptor mediating the inhibition of AVP-*L*p may also be premature because this is a well known property of EP_3-receptor activation, and as shown in Table 2, both iloprost and carbacyclin have relatively high affinity for EP_3-receptors.

HEL cells (human erythroleukaemia cells) are often used as a model of platelet/megakaryocyte function. In these cells, IP agonists can increase both cyclic AMP and $[Ca^{2+}]_i$.[67] In fact, one group have reported that iloprost and cicaprost increase $[Ca^{2+}]_i$ with high potency in HEL cells (EC_{50} values of 40 nM).[68,69] The increase in $[Ca^{2+}]_i$ is due to mobilisation from internal stores and influx from the extracellular space, is not mimicked by dibutyryl cyclic AMP and is pertussis toxin-insensitive, Schwaner et al.[68] concluded that the IP agonist effects were due either to the presence of IP-receptor subtypes, or to a tumour cell-associated aberration in receptor-effector coupling. Current evidence from the cloned IP_1-receptors would suggest that we are looking here again at multiple G-protein coupling by an IP-receptor, i.e. coupling to both G_s and G_q. In other human megakaryocyte leukaemia

cell lines (CMK and MEG-0l), PGE_1 and iloprost also increase $[Ca^{2+}]_i$ by a mechanism independent of cyclic AMP and which is also pertussis toxin-insensitive,[37,70] but it is important to note that in platelets themselves, there is no evidence for IP-receptor mediated increases in $[Ca^{2+}]_i$.[37] More recent evidence suggests that in some HEL cell lines, the Ca^{2+} mobilisation response to IP agonists does not occur at the same concentrations needed to stimulate adenylate cyclase.[71] To complicate matters further, iloprost appears to activate a G_s-coupled receptor to cause Ca^{2+} mobilisation, whereas PGE_1 can activate both this receptor (presumably the IP_1-receptor) and another (novel) G_i-coupled prostanoid receptor to increase $[Ca^{2+}]_i$; see Chapter 5, Section 3.6, for further details.

The effectiveness of prostacyclin as an adipogenic agent is related to its ability to induce an increase in both intracellular cyclic AMP and $[Ca^{2+}]_i$ in preadipocytes (rat adipose precursor cells, mouse Ob177 1 and 3T3-F442A cells).[72] The carbacyclin-induced increase in $[Ca^{2+}]_i$ in Ob1771 cells was independent of changes in cyclic AMP, yet did not involve IP_3 or DAG, and occurred over a similar concentration range (EC_{50} values of 200 nM and 300 nM for stimulation of cyclic AMP and $[Ca^{2+}]_i$, respectively).[73] Differentiated 3T3-F442A cells lose their ability to produce cyclic AMP in response to carbacyclin,[73] so that while in rat, mouse and man, prostacyclin affects adipose precursor cells, it has no effect on adipocytes. In contrast, PGE_2 exclusively affects adipocytes. Prostacyclin therefore may play a specific role in the development of adipose tissue in vivo.

IP agonists act on preadipose cells (Ob1771 and 3T3-F442A cells) by increasing cyclic AMP and increasing $[Ca^{2+}]_i$, but carbacyclin additionally acts independently of cell surface receptors, possibly by activating a member of the peroxisome proliferator-activated receptor (PPAR) family.[74] Carbacyclin appears unique among IP agonists in its ability to regulate the expression of two differentiation-dependent genes in preadipose and adipose cells.[74] Prostacyclin and prostaglandins also have a cytoprotective action in the central nervous system which does not appear to be mediated by cell surface receptors[75] or by cyclic AMP[76]

To date therefore, there is no consistent pattern describing the IP_3 and Ca^{2+} elevating properties and pertussis toxin-sensitivities of IP-receptors expressed in normal, transformed or transfected cells. The agonist action of PGE_1 at a novel prostanoid receptor in HEL cells,[71] and the possible agonist action of carbacyclin at PPARs mentioned above, only serves to highlight the need for caution in interpreting agonist-based data.

4.2 Cloned IP-receptors

Namba et al.[14] clearly demonstrated that the cloned IP_1-receptor couples to multiple G-proteins (G_s and G_q), rather than using G_s or its $\beta\gamma$ subunits to generate the inositol phosphate response. However, the affinity and efficacy of coupling to G_q is relatively weak,[12,14] see Figure 33.

Given the promiscuous nature of receptor-G-protein coupling,[77] one could simply conclude that the IP_1-receptor activates phosphatidylinositol phosphate turnover due to the relatively high expression level of IP_1-receptors in transfection experiments (typically 2 - 6 pmol mg protein^{-1}). In spite of this, the G_q-coupled pathway could have a role in normal cells. For example, it could be responsible for the agonist-dependent PKC-mediated phosphorylation of the human IP_1-receptor, especially as higher concentrations of IP agonist are also required for this process.[16] Iloprost can produce a rapid phosphorylation of the human IP_1-receptor, this being evident within 15 sec with an EC_{50} of 27 nM, and this agonist-dependent phosphorylation is dependent on PKC rather than PKA activity. G-protein coupled receptor phosphorylation is thought to play a key role in receptor desensitisation and in crosstalk between signalling pathways.[78] The C-terminal tail may be the site of phosphorylation since conserved potential PKC phosphorylation sites are found in this region of the cow, human, mouse and rat IP_1-receptors at Ser-328.22 Indeed, Ser-328 appears to be the locus of iloprost-induced PKC-mediated rapid phosphorylation of the human IP1-receptor.[79] Intriguingly, mutation of Ser-328 to Ala-328 (thus preventing PKC-mediated phosphorylation) drastically reduces coupling to PLC activation while leaving coupling to G_s intact.

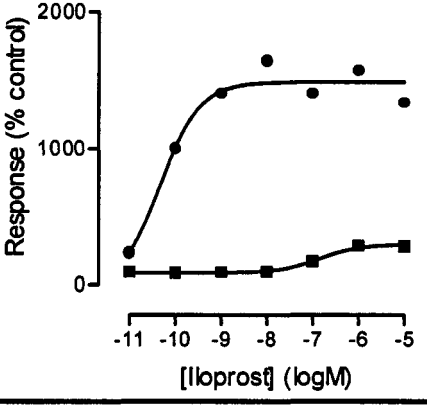

Figure 33. The response to iloprost of mouse IP_1-receptors expressed in CHO cells. Cyclic AMP (●) production; EC_{50} 60 pM. IP_3 content (■); EC50 150 nM. Adapted from Namba et al.,[14] with permission.

Both TXA_2 and phorbol myristate acetate (PMA)-treated platelets show an attenuated cyclic AMP response to iloprost, and desensitisation of TP-receptors (which couple to PLC) potentiates the cyclic AMP response to iloprost,[80] which suggests that phosphorylation normally desensitises the IP_1-receptor. It is possible therefore that the IP_1-receptor could utilise G_s-coupled pathways for normal cell signalling, but under abnormally high tissue concentrations of prostacyclin, could down-regulate its responsivity via activation of the G_q-coupled pathway leading to agonist-dependent PKC-mediated phosphorylation of the IP_1-receptor. It is becoming apparent that IP_1-receptors expressed in transformed cell lines do not always behave the same as those in normal cells. Thus, in cultured mast cells (IC2 cells) and MEG-01 cells,[81] PKC activation enhances, rather than attenuates, IP-receptor-mediated activation of adenylate cyclase via a calmodulin/MARCKS (myristoylated alanine-rich C kinase substrate) system.[49]

Given that splice variants of the cytoplasmic tail have been described for other prostanoid receptors, it has been suggested that such variation in the IP_1-receptor may account for its interaction with multiple G-proteins, but to date there is no evidence for splice variants of IP_1-receptors. Furthermore, the single variant of the human[12,16] and mouse[14] IP_1-receptors expressed in CHO cells or HEK 293 cells was capable of interacting with more than one signalling pathway, with apparent differential affinity for the G-proteins involved. It is unlikely therefore that this multiple coupling is simply an artefact of the transfection systems, as IP-receptor coupling to increases in cyclic AMP, IP_3 and $[Ca^{2+}]_i$ have been reported in normal cells[2,64,73] and transformed cells.[37,6.5,67-71,73]

By comparison with cloned EP-receptors, we see that the TMVII region rather than the C-terminal tail may be more important in determining selectivity of G-protein coupling, as suggested from data using a chimeric hybrid of rat EP_3 and human EP_4-receptor. In this example, the C-terminal tail of the rat EP_3-receptor was replaced with the C-terminal tail of the human EP_4-receptor, and the resulting receptor behaved like the native EP_3-receptor; thus the C-terminal tail did not confer G-protein coupling specificity.[82] Furthermore, a charged residue at Arg-332 (TMVII) in cow EP3D-receptor is clearly important for selectivity of G-protein coupling. Although the interaction between the α-carboxylic acid of the prostanoid agonist and the arginine residue of the EP3D-receptor was not essential for G_i coupling, it was necessary for coupling to G_s and for changes in Ca^{2+} signalling.[62,63] A similar result is seen for the mouse EP_3-receptor where Arg-338 is critical for G_i activation and substitution of Arg-329 (TMVII) with alanine or glutamate resulted in complete loss of PGE_2 binding and receptor activation.[83] Given that the second cytoplasmic loop is highly

conserved in cow, human, mouse and rat IP_1-receptors, it would be interesting to determine whether this region provides for the specificity of coupling to G-proteins.

5. EVIDENCE FOR IP-RECEPTOR SUBTYPES

To date there is no evidence from IP-receptor cloning studies to indicate the existence of IP-receptor subtypes, so we eagerly await further studies of the IP_1-receptor knockout mice which should clarify this statement. Evidence from classical pharmacological studies has always been complicated by the lack of suitably selective ligands and difficulties in establishing the validity of cross-species comparisons. For example, the 5E and 5Z geometric isomers of carbacyclin showed equal efficacy for increasing cyclic AMP and inhibiting aggregation in human platelets, yet the unnatural 5Z-carbacyclin had lower efficacy for increasing cyclic AMP and inducing relaxation in rabbit mesenteric artery.[84] It was concluded that different IP-receptors coupled to adenylate cyclase in platelets and vascular smooth muscle, because SZ-carbacyclin could discriminate between them, being a partial agonist at the myocyte but not on the platelet system. However, the human platelet is much more sensitive to IP agonists than the rabbit mesenteric artery; in the carbacyclin study, K_{act} for cyclic AMP production by carbacyclin in the two systems was 0.5 and 8 μM respectively, and in our hands, functional IC_{50} values for cicaprost were about 0.2 and 10 nM respectively. Thus, instead of truly different receptor subtypes, we may simply be looking at the consequences of different receptor-effector coupling efficiencies of the same receptor, and/or species differences between IP-receptors. In addition, in the rabbit myocyte inhibition of PGE_1-induced activation of adenylate cyclase required 1 - 2 mM 5Z-carbacyclin and one very much doubts the specificity of any antagonism at these very high concentrations.

Similar arguments relating to coupling efficiency and species differences may be applied to another carbacyclin MM-706 (6a-carba-15-cyclohexyl-13,14-didehydro-ω-pentanor PGI_2), which is claimed to discriminate between platelet and vascular IP-receptors.[85] MM-706 was a full agonist on human platelets (IC_{50} 250 - 500 nM) yet was completely inactive (at concentrations up to 10 pM) at relaxing the rabbit femoral artery. However, we have found that on the much more sensitive human pulmonary artery preparation (cicaprost IC_{50} ~1 nM), MM-706 can produce complete relaxation with an IC_{50} of 700 nM.[86]

Finally, FCE-22176, the 5Z (unnaturally configured) isomer of 6a-carba-13,14-didehydro-20-methyl PGI_2, has been claimed to be a potent IP-

receptor antagonist in tissues where prostacyclin has a contractile effect.[87] Thus on guinea-pig trachea, FCE-22176 (100 - 1000 nM) blocked the contractions of the circular muscle induced by prostacyclin (pA_2 ~7, our calculation), but not those induced by histamine. In contrast, the 5E isomer FCE-22177 contracted the trachea with a potency similar to prostacyclin (EC_{50} 850 nM). The same authors also showed that both isomers behave as conventional IP agonists on guinea-pig platelets,[88] with the 5E isomer having the expected greater potency than the 5Z isomer (IC_{50} 8.6 and 276 nM) (see Chapter 2). Subsequent studies confirmed the IP agonist action of FCE-22 176: [^{3}H]-iloprost binding to human platelet membranes and NCB-20 neuroblastoma cell membranes was inhibited with K_i values of 400 and 280 nM respectively, and adenylate cyclase activity was correspondingly increased with K_{act} values of 174 and 193 nM respectively.[89] So, is there a distinct IP-receptor in guinea-pig trachea? The answer is probably not. First, the potent and specific IP agonist cicaprost has minimal contractile activity up to concentrations of 10 μM. Secondly, the moderately potent contractile actions of other prostacyclin analogues, such as iloprost (EC_{50} 27 nM) and isocarbacyclin (EC_{50} 66 nM), are due to activation of the highly sensitive EP_1 contractile system in the trachea. Matching contractions to 16,16-dimethyl PGE_2 (standard EP_1 agonist, EC_{50} 3 nM), iloprost and isocarbacyclin were blocked to similar extents by the EP_1-receptor antagonists SC 19220 and SC 25 191 , whereas responses to histamine and the TP agonist U-46619 were unaffected.[90] Thus, an alternative explanation is that the FCE isomers and prostacyclin are interacting with EP_1-receptors on the guinea-pig trachea. The missing piece of information is the effect of FCE-22176 on the contractile activity of 16,16-dimethyl PGE_2 or the more selective EP_1 agonist 17-phenyl-ω-trinor PGE_2 (EC_{50} 2.2 nM).[91]

Octimibate, BMY 42393 and BMY 45778 aroused much interest because of their high affinity for platelet IP-receptors and their novel chemical structures.[92] Furthermore when compared with iloprost, octimibate had only weak vascular relaxant activity when tested in monkey aorta, and human coronary and mesenteric arteries.[93] Unfortunately, when tested in a more appropriate vascular system, i.e. human peripheral resistance vessels, octimibate showed a degree of relaxant activity expected from the platelet data thus helping to dismiss the idea that platelet and vascular IP-receptors were different.[93]

The methyl ester derivative of PGI_1 analogue SM-10906, i.e. SM-10902, is another example of an IP agonist which is a partial agonist for stimulating cyclic AMP in cell membranes (P-815 cells) yet acts as a full agonist for inhibiting human platelet aggregation.[53] There are now an increasing number of IP agonists which are partial agonists for stimulating adenylate cyclase, yet act as full functional agonists for inhibition of platelet

aggregation, for example, EP 157[94] and those compounds related to octimibate,[40,57] i.e. BMY 42393[58] and BMY 45778.[60] In addition, these compounds consistently behave as partial agonists with regard to stimulation of adenylate cyclase in a variety of tissues, e.g. rat peritoneal neutrophils,[95] SK-N-SH neuroblastoma cells,[96] and human lung.[93]

This long lasting determination to prove that platelet and vascular IP-receptors are examples of IP-receptor subtypes was encouraged by the pharmaceutical desire for a platelet-specific antithrombotic drug. Now that the IP_1-receptor knockout mice of Murata et al.[7] have clearly shown that the platelet and vascular IP-receptors derive from the same gene, are we left with any other evidence for receptor subtypes? In 1994, Wise et al.[97] showed that BMY 45778 could distinguish between the IP-receptor on rat neutrophils with that in the rat enteric nervous system (see Chapter 10), and in 1995, Hébert et al.[2] proposed the existence of three IP-receptor subtypes to explain the complex effects of iloprost in rabbit cortical collecting ducts (see Section 4.1). In 1996, Takechi et al.[3] identified a novel binding site for [^{3}H]-isocarbacyclin in rat brain with characteristics quite different from the binding site recognised by [^{3}H]-iloprost. Whether any of these proposed IP-receptor subtypes survive the test of time is a prospect which keeps the field of IP-receptor pharmacology alive.

6. IP-RECEPTOR REGULATION

Prolonged activation of IP-receptors on NG108-15 neuroblastoma cells leads to a heterologous form of desensitisation, where there is loss of responsiveness not only to IP agonists, but also to adenosine A_2 agonists and sodium fluoride; the latter directly activating adenylate cyclase.[24] It was suggested that this homologous and heterologous desensitisation was most likely mediated by a single process such as co-internalisation of receptors and G-proteins.[98] Kelly et al.[24] proposed that the loss of responsiveness in NG108-15 cells is probably related to the functional loss of $G_{s\alpha}$ in these cells, and in this respect the characteristics of desensitisation resemble those for human platelets following iloprost pretreatment. Early studies suggested that the decreased potency of IP agonists to stimulate adenylate cyclase following prolonged incubation of NCB-20 neuroblastoma cells with carbacyclin was due to a change in IP-receptor binding affinity.[46] However in these and other studies,[45] [^{3}H]-PGI_2 was used which exhibits a high level of nonspecific binding and thus can complicate data interpretation. Later studies with the more useful radioligand [^{3}H]-iloprost found that prolonged incubation (16 h) of NCB-20 cells with carbacyclin,[25] actually decreased the B_{max} value for [^{3}H]-iloprost binding sites, but had no affect on binding

affinity. The consequence of this change in the number of [^{3}H]-iloprost binding sites was a decrease in cyclic AMP production in response to IP agonists.

A more recent study of the internalisation and downregulation of the IP-receptor in human platelets confirms that preincubation with iloprost (100 nM for 18 h) decreases the number of [^{3}H]iloprost binding sites, in particular one sees the loss of the high affinity binding component.[99] This was reflected by a dramatic reduction in the maximal level of cyclic AMP production in response to iloprost, and a reduction in the potency of iloprost as an inhibitor of collagen-induced aggregation. Thus, at 37°C, [^{3}H]-iloprost is internalised by a time-dependent, temperature-dependent and energy-dependent process in human platelets, reflecting the loss of specific binding sites from the cell surface.[99] This study concluded that each IP_1-receptor shuttles between the plasma membrane and another compartment transferring some 25 ligand molecules per receptor molecule during the 2 h incubation period. It is likely therefore that IP-receptor internalisation underlies the desensitisation process. Resensitisation of neuroblastoma cells can occur in the absence of IP agonist and this is dependent on *de novo* protein synthesis.[25]

The pattern of IP agonist-induced receptor desensitisation very much depends on the cell system being investigated. In both NCB-20 and NG108-15 neuroblastoma cell lines, as seen predominantly in platelets, the loss of [^{3}H]-iloprost binding is due to a decrease in B_{max} rather than any change in K_d values.[24,25,98] However, only homologous desensitisation is seen in NCB-20 cells,[24] whereas heterologous desensitisation is found in NG108-15 cells,[24] and platelets.[100,101] In NG108-15 cells, IP agonists induce a biphasic desensitisation of IP-receptor-stimulated adenylate cyclase with an initial phase coinciding temporally with loss of receptors and $G_{s\alpha}$, whereas the second component appears to occur independently of $G_{s\alpha}$ loss.[102] The role of IP-receptor internalisation in the process of IP-receptor downregulation is still far from clear,[99,103-105] although a major component may be due to loss of IP-receptors and $G_{s\alpha}$[102] and the increased breakdown of existing receptors.[106] In fact in HEL cells, iloprost-induced desensitisation of the cyclic AMP response is abolished by pertussis toxin treatment, suggesting an involvement of G_i.[107] IP agonist-induced receptor desensitisation also depends on the IP agonist itself. For example, both iloprost and PGE_1 show antimitogenic effects in bovine coronary smooth muscle cells, yet only the response to iloprost shows desensitisation.[108] These results provide yet another example of complications associated with the use of PGE_1 as an IP agonist (see Chapter 5, Section 3.6).

Iloprost can produce a rapid PKC-dependent phosphorylation of the human IP1-receptor which is mirrored by rapid desensitisation of iloprost-

stimulated cyclic AMP and inositol phosphate production.[79] Truncation of the C-terminal tail of the human IP1-receptor completely prevented this iloprost-induced desensitisation. Similarly, mutant human EP_4-receptors lacking the long C-terminal tail, and therefore also lacking 36 of the 38 serines and threonines which are targets for phosphorylation, also cannot undergo agonist-induced desensitisation.[109] Although PKC appears to be the major phosphorylating and desensitising kinase in human IP_1-receptors overexpressed in HEK293 cells, this may not be the case in cells that normally express these receptors. Smyth et al.[79] suggest that it remains possible that GPCR kinase (GRK)-mediated phosphorylation of IP_1-receptors is important but presently no data exist on this subject.

In a variety of tissues, activation of TP-receptors has the opposite or antagonistic effect to activation of IP-receptors. The crosstalk between TP-receptor-associated phosphatidylinositol metabolism and IP-receptor-associated adenylate cyclase activation has been studied in several cell types. For example, TP-receptors in platelets can undergo rapid desensitisation in response to TP agonists and in human platelets this results in an enhanced stimulation of the cyclic AMP response to iloprost,[80] thus potentiating the turn-off signal of the platelet. Surprisingly, although this TP-receptor is linked to PLC, the sensitisation process was not mediated by PKC. In contrast, activation of TP-receptors in the human megakaryocytic cell line MEG-01 augments iloprost-induced cyclic AMP formation, an effect which does appear to be mediated by PKC.[81] The enhanced cyclic AMP formation in turn suppresses TXA_2-stimulated increases in $[Ca^{2+}]_i$which in turn will lead to increased cyclic AMP levels. In MEG-01 cells and IC_2 cells (mouse mast cell line), the addition of TPA (12-O-tetradecanoylphorbol- 13-acetate) markedly enhanced cyclic AMP production in response to carbacyclin.[49] Since cow, human, mouse and rat cloned IP_1-receptors have at least two potential PKC-phosphorylation sites (Fig. 32), these might be the target of TP agonist action in IC_2 and MEG-01 cells. However, Watanabe et al.[81] suggest that adenylate cyclase, and not the IP-receptor, is a more plausible target because the TP agonist U-466 19 also enhanced forskolin-induced cyclic AMP accumulation. This crosstalk could therefore work as a negative feedback system for $[Ca^{2+}]_i$ mobilisation induced by PLC-coupled agonists. Alternatively, there is evidence for a role for PKC in MEG-01 and IC_2 cells, its target being a component(s) other than those involved in the sequential coupling of IP-receptors, G_s and adenylate cyclase; this component could be MARCKS, a calmodulin-binding protein which releases calmodulin when phosphorylated by PKC, resulting in activation of adenylate cyclase.[49]

7. IP_1-RECEPTOR mRNA DISTRIBUTION

One exciting option available once the IP_1-receptor had been cloned was to look at the distribution of IP_1-receptor mRNA in various tissues, and this is summarised in Table 6. Although the apparent absence of IP_1-receptor mRNA may simply reflect the limitations of the assay system, the presence of IP_1-receptor mRNA in unexpected places was a valuable surprise. Apparent differences exist between IP_1-receptor mRNA distribution in the different species, but it is too early to assess the full significance of these observations. For example, according to Hirata et al.[22] mRNA for the IP_1-receptor is most abundant in the mouse thymus and spleen, but is undetectable in the human thymus. One can also detect IP_1-receptor mRNA in rat thymus, but it is far more abundant in rat aorta and lung.[110] Differences in IP_1-receptor mRNA levels might just reflect different stages of maturation of thymocytes in lymphatic organs of different species. High level expression of IP_1-receptor mRNA is seen in human liver and kidney, but this time not in mouse tissues.[22] The different pattern of IP_1-receptor mRNA expression seen in human and mouse kidney may reflect the relative abundance of prostacyclin synthase expression in human glomeruli or glomerular cells.[111] Furthermore, prostacyclin is the main prostanoid in human glomeruli, whereas in rodents, PGE_2 appears to be the main prostanoid (see Kömhoff et al.[111] for further details).

Table 6. Distribution of IP_1-receptor mRNA in various tissues

	Human[13]	*Human*[11]	*Mouse*[22]	*Rat*[22]
Brain	-	-	-	nd
Thymus	-	nd	+++	+
Lung	+++	++	++	+++
Heart	++	+	+	+
Aorta	+++	nd	nd	++
Spleen	+	nd	+++	+++
Stomach	nd	nd	-	+
Liver	±	++	-	nd
Pancreas	nd	+	nd	++
Intestine	+	+	+	+
Kidney	++	+++	-	nd
Testis	nd	nd	-	nd
Adrenal	+	nd	nd	nd
Placenta	+	±	nd	nd
Skeletal muscle	nd	+	nd	nd

nd; not determined. Reprinted from Hirata et al.,[22] with permission.

Human IP_1-receptor mRNA has been detected in a wide range of megakaryocytic (e.g. MEG-01) and megakaryocytic/erythroid (e.g. HEL) cell lines, and in human cord blood megakaryocytes.[112] In addition, this IP_1-

receptor mRNA can be upregulated by cytokines involved in megakaryocytopoiesis (see Chapter 5, Section 3 .6).

More detailed cellular localisation studies highlighted the presence of IP_1-receptor mRNA, as expected, in the smooth muscle of a variety of mouse arterial tissues,[113] see Table 7. More importantly, these studies furthered our appreciation of the role of IP-receptors by detecting IP_1-receptor mRNA in the mouse[14] and rat spleen and thymus.[15] These experiments, using in situ hybridisation techniques, demonstrated that hybridisation signals were visible only in the medulla and not the cortex of rat thymus, and were predominantly in thymocytes and not stromal cells.[113] The IP_1-receptor mRNA is therefore exclusively present in the thymic medulla where $CD4^+8^-$ and $CD4^-8^+$ thymocytes are harboured. Since prostacyclin is produced by thymic stromal cells and IP_1-receptor mRNA is localised to thymocytes and is distributed in lymphocytes in the white pulp of the spleen,[113] then we should look further now for the functional significance of these unexpected observations (see Chapter 8).

Other unexpected results came with the evidence of very high levels of expression of mouse IP_1-receptor mRNA in the neurones of the DRG.[113] Approximately 40% of neurones in mouse DRG expressed IP_1-receptor mRNA, and about 70% of the substance P-containing cells co-expressed IP_1-receptor mRNA. This evidence therefore suggests a role for these IP_1-receptors in the transmission of sensory signals, as discussed in Chapter 9.

Table 7. Cellular localisation of IP_1-receptor mRNA in the mouse

System	*Labelled cells*	*Relative grain densities on cell bodies*
Cardiovascular	Smooth muscle cells in:	
	aorta	++
	coronary artery	++
	pulmonary artery	++
	cerebral artery	++
	renal artery	++
	renal interlobular artery	++
	renal afferent arteriole	++
Nervous	Dorsal root ganglion neurone	++++
	Spinal cord	+
Respiratory	Trachea	+
Reticulo-endothelial	Mature thymocyte	+
	Spleen lymphocyte	+
	Megakaryocyte	+++

Relative grain densities: ++++, very high; +++, high; ++, moderate; +, low; -, background level. Reprinted from Oida et al.,[113] with permission.

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Chapter 5

IP-receptors on platelets

1. INTRODUCTION

Platelets contribute to maintaining the normal circulation of blood through the preservation of vascular integrity and the control of haemorrhage after injury. Thrombosis is the unwanted formation of a haemostatic plug or thrombus within the blood vessels or heart, and most often manifests itself as myocardial infarction, cerebral infarction or venous thromboembolism, i.e. deep-vein thrombosis and pulmonary embolism.[1] Acute thrombotic arterial occlusion is the leading cause of morbidity and mortality in industrial societies,[2] and therefore the search for effective antithrombotic drugs has been extensive. Platelet adhesion and aggregation are the primary events in the formation of coronary and cerebrovascular thrombi, and together with platelet-dependent vasoconstriction are responsible for the primary flow obstruction within a ruptured atherosclerotic vessel.[3]

The high potency of prostacyclin as an inhibitor of platelet aggregation has made IP agonists prime targets as antithrombotic drugs, and the ready availability of human platelets has made the IP-receptor in human platelets the most well studied of all the prostacyclin receptor systems. Indeed, the reader will already have noticed the use of the platelet aggregation response as the primary screening model to detect IP agonist activity (see Figures in Chapters 2 and 3). The human platelet aggregation model however is less useful for detecting partial agonism, and potential leads to the design of IP-receptor antagonists may have been lost. This is because there is a high degree of receptor-effector coupling in platelets, such that IP agonists can produce complete inhibition of platelet aggregation despite being low

efficacy agonists for stimulating adenylate cyclase, as discussed in more detail in Section 3.

In this chapter, we aim to identify the mechanism of prostacyclin's antiplatelet action with particular reference to the use of prostacyclin mimetics as antithrombotic drugs. Where the antithrombotic action of prostacyclin impinges on other disorders, e.g. atherosclerosis, these concerns will be dealt with more fully in the following chapter.

2. PROSTACYCLIN AS AN ENDOGENOUS ANTITHROMBOTIC AGENT

2.1 Platelet thrombus formation

Under normal circumstances, platelets circulate in close contact to the endothelial cell lining of the vessel wall without adhering to it, but they respond rapidly to alterations of endothelial cells and to exposure of subendothelial structures by attaching firmly to the site of the lesion. The first layer of platelets is connected with the thrombogenic surface, whereas subsequent growth of the haemostatic plug depends primarily on platelet-platelet interactions; these two stages represent "adhesion" and "aggregation", respectively. Therefore, although platelet adhesion to exposed reactive surfaces is the necessary initial response to vascular injury, essentially all the platelets recruited into the mass of a haemostatic or pathological thrombus are interacting with one another, not with matrix components. The important ligands for platelet binding to the subendothelial surface are von Willibrand factor (vWF) and platelet glycoprotein GP1 b. Subsequent platelet aggregation is mediated primarily by fibrinogen binding to platelet glycoprotein GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$) complexes. For a more detailed description of platelet function, see reviews by MacIntyre,[4] Ruggeri,[2] Seiss,[5] Hawiger,[6] and Body.[7]

Platelets respond to rupture of an atherosclerotic plaque as they would to traumatic damage to a normal blood vessel, with platelet deposition and aggregation, helped by the fact that virtually all the known platelet agonists are released or generated at sites of injury.[8] For example, activation by thrombin, collagen or vWF involves shape change to allow platelets to flatten and cover the lesion in the endothelium. This is rapidly followed by the release from platelets of activators such as ADP, fibrinogen, serotonin, platelet-activating factor (PAF) and TXA_2.

Thrombosis may occur in any part of the circulatory tree and develop silently until either a local obstruction or a distant obstruction by embolisation produces clinical symptomatology. Arterial thrombosis, which is usually associated with atherosclerosis, is a dynamic process which tends to develop in areas of medium-high flow and shear stress, usually with a triggering factor of vascular origin. Its main effect is to retard or interrupt blood flow, causing ischaemia or actual death (infarction) of the tissue beyond. In contrast, overt vessel wall disease does not appear to be an important factor in venous thrombosis, which tends to occur in areas of relative stasis, and therefore under different conditions and triggering factors, either vascular or flow-related.[9] In addition, venous thrombi are composed predominantly of fibrin and red blood cells, with a more variable platelet and leukocyte component compared with arterial thrombi. If a portion of a venous thrombus should break away, it can form an embolus which may block blood vessels, again causing damage to the tissues supplied.

The endothelium has a major role to play in limiting/preventing thrombus formation, and this is discussed in greater detail in the following chapter. Not only does prostacyclin decrease platelet activation, adhesion and aggregation, but its additional vasodilator activity also helps prevent thrombi formation. For example, the vasodilator action would favour anticoagulation because of dilution or washout of procoagulant substances, reduced shear stress, and decreased proximity of vascular elements to platelets.[7]

2.2 A role for endogenous prostacyclin

Prostacyclin was discovered in the mid-1970's when its potent antiaggregatory activity clearly distinguished it from other prostanoids known at that time.[10] It was soon realised that arterial walls are protected against deposition of platelet thrombi by producing prostacyclin, and the concept that a balance between the amount of TXA_2 formed by platelets and prostacyclin formed by blood vessel walls might be critical for thrombus formation, was developed.[11] Thus, prostacyclin generated by vascular endothelium could contribute to the thromboresistance of this tissue when intact. The ability of the arterial wall to generate prostacyclin is highest in the intimal surface and progressively decreases to the adventitial surface, accompanied by an increase in the pro-aggregatory activity of each layer.[12] These observations help to explain the lack of thrombus formation on normal vascular endothelium.

In 1978, Higgs et al.[13] demonstrated for the first time the inhibitory effect of prostacyclin on platelet aggregation in vitro, ex vivo and in vivo in the same species (hamster), and in particular, demonstrated the antithrombotic

nature of prostacyclin in an in vivo model of thrombosis. Furthermore, they showed that rings of hamster aorta generated a substance which prevented ADP-induced aggregation of human platelets, and whose production was inhibited by aspirin. These results therefore supported the concept that generation of prostacyclin by vascular tissue is an important natural defence against intravascular thrombus formation.

Originally, it was suggested that blood vessels could use endoperoxides generated by platelets to increase their production of prostacyclin,[10] but this does not appear to be the case under normal circumstances.[14] However, if TXA_2 synthesis in platelets is inhibited, then PGH_2 may become available for conversion to prostacyclin by endothelial cells;[15] this mechanism may contribute to the antithrombotic efficacy of TXA synthase inhibitor / TP antagonist compounds (e.g. ridogrel).[16] Interestingly, human lymphocytes possess prostacyclin synthase activity which is capable of converting platelet-derived PGH_2 into prostacyclin, and this prostacyclin is sufficient to inhibit platelet function.[17] Importantly, the platelets have to be activated in order to donate PGH_2 to the lymphocytes.

At one stage it was thought that prostacyclin was a circulating factor, released continuously from the lungs,[18,19] but this view was quickly countered.[15,20] In fact there is no detectable 6-oxo $PGF_{1\alpha}$ in human blood except under conditions of severe infection or endotoxin shock.[12] The implication that prostacyclin deficiency is a cause of thrombotic events was suggested by the successful use of prostacyclin (epoprostenol) in the treatment of recurrent arterial and venous thromboses.[21] Prostacyclin is the most potent endogenous inhibitor of platelet aggregation yet discovered and IP agonists not only inhibit thrombus formation, but also disperse platelet aggregates.[22] Prostacyclin and its mimetics inhibit aggregation (platelet-platelet interactions) at much lower concentrations than those needed to inhibit adhesion (platelet-collagen interactions).[23-25] This is thought to be beneficial since limited production of prostacyclin by the endothelium would allow platelet adhesion for repair purposes, while at the same time preventing or limiting thrombus formation.[26]

In mice lacking the IP_1-receptor gene, bleeding time was normal.[27] The absence of IP_1-receptors was confirmed by beraprost failing to prolong bleeding time and cicaprost failing to inhibit ADP-induced platelet aggregation in vitro. However, when the vascular endothelium in the carotid arteries of these IP_1-receptor knockout mice was damaged, there was an increased incidence of obstructive thrombi formation resulting in death due to bilateral occlusion of the carotid arteries and/or embolic stroke. These results would suggest that while prostacyclin is not involved in the regulation of bleeding under basal conditions, it is crucial to prevent thrombus formation arising from damaged vascular endothelium.

Altered sensitivity of platelets to prostaglandins has been observed in a variety of conditions, For example, in coronary heart disease and angina pectoris, there is a significant decrease in sensitivity to prostacyclin and PGE_1 (with no change for PGD_2).[28,29] Furthermore, those patients with type 2 diabetes in addition to coronary heart disease had even lower sensitivity than those with coronary heart disease alone. Platelet aggregability was also significantly higher in patients with cardiac ischaemia and prostacyclin was less effective in inhibiting platelet aggregation.[30] It was rapidly recognised that intravenous infusion of prostacyclin could have transient beneficial effects in coronary artery disease, peripheral arterial disorders, and thrombotic thrombocytopenic purpura, but its vasodilator effects and extreme lability would always be limiting factors in its usefulness. We have seen in the preceeding chapters the results of extensive chemical efforts to produce more platelet-selective and stable prostacyclin mimetics, and we have seen that the advent of cloned IP_1-receptors has enabled us to appreciate the innate flaw in this proposition, i.e. one cannot hope for antiplatelet activity in the absence of vasodilator activity. However, it is still possible that by more fully understanding the function of platelet IP_1-receptors, and the differing receptor-effector coupling mechanisms in platelets and vascular smooth muscle cells, we may be able to use the newer agents for well-defined tasks, being thoroughly aware of the nature of the expected side effects.

3. CHARACTERISATION OF PLATELET IP_1-RECEPTORS

Our primary concern here is to consider what we know about the IP-receptors which regulate platelet function. Receptors of four of the five major classes of prostanoids are represented on platelets (i.e. DP, EP, IP and TP) and the pharmacology of these receptors has been reviewed by Armstrong.[31] Platelets can be activated by a considerable variety of agonists, each with its own profile of intracellular signalling pathways, It would be beyond the scope of this chapter to deal with each platelet agonist in detail and therefore the reader is referred elsewhere to discover more of the complexity of platelet activation.[5,7,32] What is clear is that IP agonists can inhibit platelet function independent of the platelet agonist used, for example, octimibate inhibits human platelet aggregation stimulated by ADP, U-466 19, serotonin, thrombin and vasopressin.[33] In addition, the platelet IP-receptor and the cloned IP_1-receptor appear to be one and the same. In this section we will consider the evidence for species-specific IP_1-receptors, concentrating on the results of binding assays, adenylate cyclase assays and

functional assays such as inhibition of platelet aggregation. We will also look at the mechanism of action of IP agonists and the properties of IP-receptors in platelet-like cells.

3.1 IP_1-receptor binding studies

Classically, prostaglandins compete for [^{3}H]-iloprost binding to human platelets with an order of potency PGI_2 > PGE_1 >> PGE_2 > 6-oxo $PGF_{1\alpha}$; [34] typical K_d values for [^{3}H]-IP agonist binding to platelets can be found in Chapter 4, Table 4. Photoaffinity labelling of porcine platelet membranes with [^{3}H]-APNIC ([15-^{3}H] 19 -(3-azidophenyl)-20-nor isocarbacyclin) suggested that the iloprost binding site was a protein of 43 - 45 kDa,[35] which agrees well with the deduced molecular weight of the cloned IP_1-receptors from cow, human, mouse and rat (Chapter 4, Table 3).

In 1986, Eggerman et al.[36] used binding assays to determine if prostacyclin and prostaglandins of the E series shared a common receptor. Their results are shown in Figure 34 and clearly demonstrate that prostacyclin and PGE_1 share a different binding site to PGE_2, and that PGE_1 can recognise both the prostacyclin and the PGE_2 binding sites. They also found only one affinity binding site for [^{3}H]-PGI_2, in contrast to other reports of two sites.

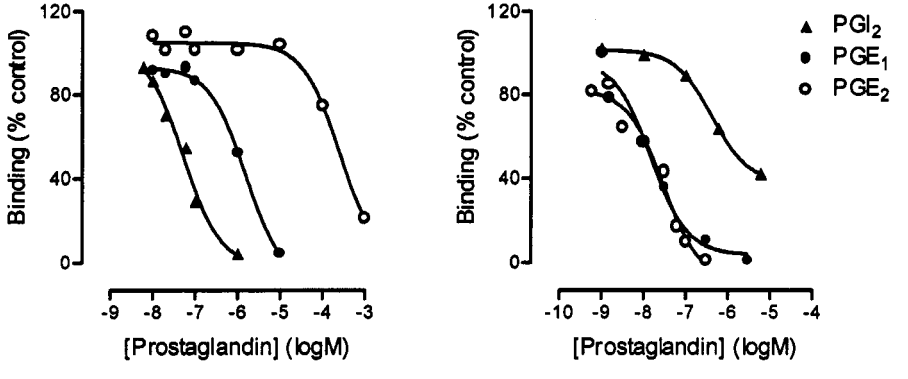

Figure34. Competition for specific binding of [^{3}H]-PGI_2 (left) and [^{3}H]-PGE_2 (right) to human gel-filtered platelets. Reprinted from Eggerman et al.,[36] with permission.

From our own studies of [^{3}H]-iloprost binding to human platelet membranes we also detected only one affinity binding site with a K_d = 12.9 ± 1.7 nM and B_{max} = 943 ± 49 fmol mg protein^{-1}.[37] We also tested an extensive range of prostacyclin mimetics in competition binding assays with [^{3}H] -iloprost (see Fig. 35) and noted that the order of potency of these

compounds to relax human pulmonary artery and their affinity for the platelet [^{3}H]-iloprost binding site was similar and provides good evidence that we are looking at the same IP-receptor in vascular tissue and platelets, i.e. the IP_1-receptor. In general there is also a good correlation between competition binding data and inhibition of human platelet aggregation when looking at the commonly used IP agonists prostacyclin, iloprost, carbacyclin and PGE_1.[38] However, if we look at data from nonprostanoid prostacyclin mimetics belonging to the BMY and EP series (Table 8), the correlation is less good. For these nonprostanoid prostacyclin mimetics, it is difficult to predict their potency in inhibiting human platelet aggregation, based solely on competition binding data. The apparent functional potency of these compounds in vitro is heavily influenced by their relatively high affinity for plasma proteins present in PRP (platelet-rich plasma), and their efficacy for activating IP_1-receptors, see Sections 3.2 and 3.3.

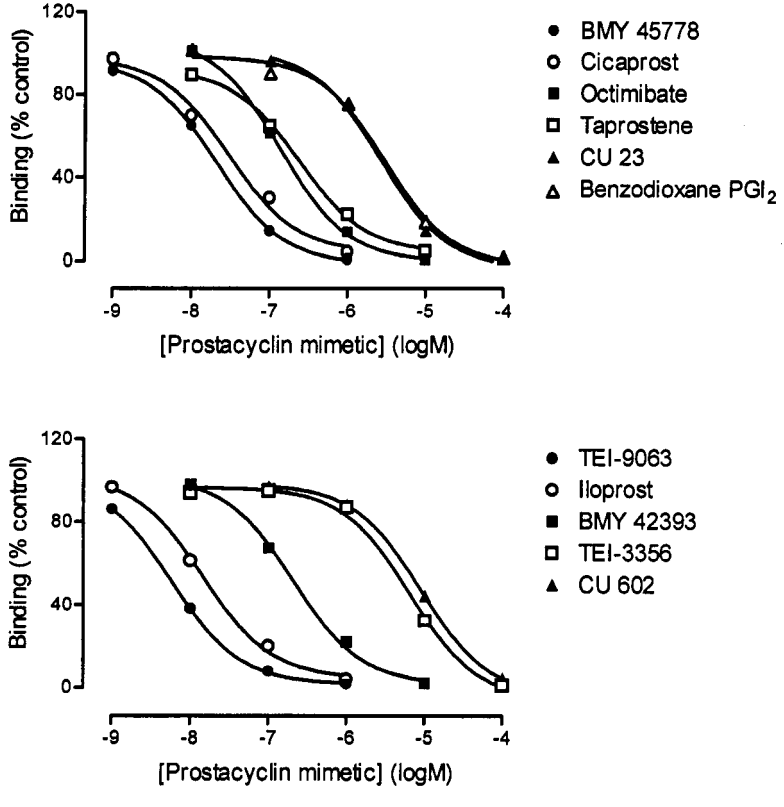

Figure 35. Competition for specific [^{3}H]-iloprost binding to human platelet membranes. Raw data for IC_{50} values quoted in Jones et al.[37]

Table 8. A comparison of the potency of IP agonists in human platelets, expressed as equi-effective molar ratios compared to iloprost

	Qui-effective molar ratios (iloprost = 1.0)			
	[^{3}H]-iloprost binding	*Cyclic AMP production*	*Inhibition of aggregation (PRP)*	*(washed platelets)*
BMY 45778	0.2 - 0.3	0.9 - 2	25	
BMY 42393	5–8	4	879	
Octimibate	8 –17	4 - 7	161 - 729	
EP 157	18	121		49
EP 035	41	438		276

For BMY 45778, BMY 42393 and octimibate, data are from Ref. No. 33,37,39-41. Control values for iloprost are: [^{3}H]-iloprost binding, IC_{50} = 29 nM; cyclic AMP production, EC_{50} = 6.8 nM; inhibition of aggregation in human PRP, IC_{50} = 1.4 nM. For EP 157 and EP 035, data are from Ref. No. 42. Control values for iloprost are: [^{3}H]-iloprost binding, IC_{50} = 110 nM; cyclic AMP production, EC_{20} = 2.4 nM; inhibition of aggregation in human washed platelets, IC_{50} = 0.06 - 0.1 8 nM.

[^{3}H]-PGI_2 binding to bovine platelet membranes is extremely rapid; even at 0°C almost complete saturation occurred after only 10 min, but minute changes during the isolation procedure of the platelets can prevent binding of [^{3}H]-PGI_2 to the high affinity site.[43] Although the K_d values remained fairly constant at 35 - 63 nM for incubations performed at 0°C the number of binding sites varies considerably between human, cat, cow and rat platelets. Results at slight variance to these have also been reported, with rat and rabbit platelet membranes having considerably lower affinity for [^{3}H]-iloprost than human, pig and horse platelets.[44] Indeed, to detect specific binding of [^{3}H]-iloprost to rat and rabbit platelet membranes, it is essential to perform the assays at 4°C, although identical binding constants can be obtained with pig platelet membranes when incubated at either 4°C or 30°C.[44] Temperature-dependent effects can also be specific to the competing compound under test. For example, BMY 45778 competes for [^{3}H]-iloprost binding to human platelet membranes with an IC_{50} value of 7 nM at 37°C, but this value increases to 49 nM when assayed at 4°C.[40] This temperature-dependent affinity is not seen with either iloprost itself or BMY 42393 and it has been suggested that BMY 45778 is a much more conformationally-restricted molecule than either of the other IP agonists tested.

Lombroso et al.[45] noted two classes of binding site and two components for adenylate cyclase activation in human platelets, and suggested that these could be located on different platelet populations. In Chapter 4 we noted the variable reporting of two affinity binding sites for [^{3}H]-PGI_2 and [^{3}H]-iloprost in a variety of tissues (see Chapter 4, Table 4), including platelets. One interesting explanation comes from the work of Kahn et al.[46,47] who have presented evidence that a novel circulating anti-IP_1-receptor antibody preferentially binds to the high affinity state of the platelet IP_1-receptor, and

have identified a specific function associated with this site. These conclusions came from a rather unexpected source, i.e. a study of the relatively high degree of coronary artery disease (CAD) occuring in patients with chronic spinal cord injury (SCI). Incubation of normal platelets with SCI plasma resulted in the loss of high affinity binding of [^{3}H]-PGE_1 without affecting either the number of low affinity binding sites, or the binding affinity of these two sites (normal platelets: K_{d1} = 9.1 nM, B, = 170 sites per cell; K_{d1} = 1.9 μM, B_{max} = 1832 sites per cell. SCI-treated platelets: K_{d1} = 7.2 nM, B_{max} = 23 sites per cell; K_{d1} = 1.6 μM, B_{max} = 1740 sites per cell). Furthermore, incubation of platelets with 100 nM prostacyclin produced similar increases in cyclic AMP formation in both normal and SCI-treated platelets, but this same concentration of prostacyclin failed to inhibit platelet-stimulated thrombin generation in platelets treated with the IgG-like protein from SCI patients. One conclusion from this study therefore is that activation of the high affinity state of the IP_1-receptor is responsible for the inhibition of platelet-stimulated thrombin generation, yet activation of cyclic AMP production results from the stimulation of the low affinity state. Since cyclic AMP generation is unaffected by the presence of SCI plasma, then presumably cyclic AMP does not mediate the antithrombin generation activity of prostacyclin. The loss of control of platelet-stimulated thrombin generation would be expected to have significant consequences for the pathogenesis of atherosclerosis and CAD. Thus, prostacyclin binding to the high affinity site would prevent atherogenesis, while binding to the low affinity site would prevent thrombosis. We look forward to hearing if this antibody can help further dissect the nature of the interaction of prostacyclin with its platelet receptors.

[^{3}H]-PGE_1 binding sites on platelets can be increased by insulin pretreatment, and it is thought that this may relate to the hyperactivity of platelets isolated from diabetic patients lacking insulin.[48] The loss of high affinity [^{3}H]-PGE_1 binding in platelets from SCI patients was indeed restored with insulin preincubation, which also restored the inhibitory effect of prostacyclin on platelet-stimulated thrombin generation.[46]

3.2 Adenylate cyclase activation

Traditionally, agents that increase cyclic AMP levels in platelets, such as prostacyclin, are described as having receptors that are coupled to G_s and capable of activating adenylate cyclase, (see Brass et al.[32] for further details of platelet G-proteins). Relatively little information is available about the forms of adenylate cyclase present in platelets, beyond the absence of type I enzyme.[49] Types III and IV have been detected by polymerase chain

reaction (PCR) in the megakaryocytic HEL cell line,[50] but as we shall see in Section 3.6, the HEL cell line is not necessarily representative of platelets.

Prostacyclin and PGE_1 stimulate adenylate cyclase in human platelets with K_{app} values of 52 nM and 140 nM, respectively.[45] Octimibate, BMY 42393 and BMY 45778 clearly have lower efficacy for stimulating platelet adenylate cyclase when compared with prostacyclin mimetics such as iloprost and cicaprost (also seen in lung membranes,[51] neutrophils (see Chapter 7) and neuroblastoma cells (see Chapter 11)). For example, octimibate stimulated platelet cyclic AMP production to a level of 57 - 72% of the maximum response to full agonists such as iloprost and PGE_1.[33,39] Furthermore, BMY 42393 and BMY 45778 gave 75 - 80%[41] and 80 - 100%[40] maximum responses, respectively. Despite this, these compounds can still act as full functional agonists for inhibition of platelet aggregation.

It has been known for some time that PGE_1 inhibits platelet aggregation at concentrations where there was no measurable increase in cyclic AMP.[52] Seiler et al.[40] remind us that even small increases of platelet cyclic AMP levels can translate into a large degree of protein kinase activation in the whole platelet. This disparity has been explained by the contention that physiologically important pools of cyclic AMP cause dramatic activation of the cyclic AMP-dependent protein kinase (PKA) yet represent only a small change in the total cellular cyclic AMP concentration. In addition, these very small changes in cyclic AMP may still be sufficient because most of the basal cyclic AMP in platelets is already bound to the tight-binding sites on PKA, with the result that a doubling of platelet cyclic AMP can cause almost full activation of these enzymes.[53]

In general, stimulation of adenylate cyclase correlates well with the functional response of platelets. For example, for human platelets the antiaggregatory potencies of iloprost, PGE_1 and OP-1206 (a PGE_1 analogue with IP and EP_2 agonism) were positively correlated with increases in cyclic AMP production.[52] The equi-effective molar ratios for iloprost, cicaprost, carbacyclin and EP 157 matched well in assays of cyclic AMP production and inhibition of platelet aggregation,[44] and parallel effects of prostacyclin, OP-41483 (prostacyclin analogue), carbacyclin and PGE_1 were observed for stimulation of adenylate cyclase activity and inhibition of human platelet aggregation;[54] see also Table 8. Evidence linking IP agonist-mediated increase in platelet cyclic AMP levels to inhibition of platelet aggregation will be presented in Section 3.4.

3.3 Inhibition of platelet activation

The inhibitory activity of natural prostaglandins on human platelet aggregation is similar, regardless of the platelet activator used, and follows

the order of potency: PGI_2, 6-oxo PGE_1, PGD_2, 6-oxo $PGF_{1\alpha}$, 13,14-dihydro PGE_2 (a pulmonary metabolite of PGE_2), PGE_2 and $PGF_{2\alpha}$.[4] It was also quickly recognised that PGE_1 and prostacyclin share the same receptor which is different from that used by PGD_2 although both seemed to function by increasing platelet cyclic AMP to inhibit aggregation.[55] To complicate matters a little, it was found that PGE_2 could potentiate rather than inhibit platelet aggregation and subsequent studies clearly showed that EP_3 agonists could potentiate the primary aggregation wave in human platelets.[56] However, iloprost is considerably more selective for IP_1-receptors compared to EP_3-receptors, and its antiplatelet activity dominates. For less selective agonists though, interpretation of data may be more complicated under conditions where the EP_3 response dominates over the IP response.[57,58]

Because of the relative ease of using platelet aggregation as the primary screen for prostacyclin-like activity (see Chapters 2 and 3), an overwhelming amount of data exists for human platelets. However, when we try to look for evidence of species specificity in IP_1-receptors, we encounter the problem of comparing data from functional experiments where the inhibitory agonist's profile is dependent on the strength of the aggregation stimulus used and on the degree of protein binding when assays are performed in PRP, diluted PRP or plasma-free solution. The potential interference of all these factors can be demonstrated with BMY 45778. For example, BMY 45778 inhibits human platelet aggregation in PRP with an IC_{50} value of 35 nM, compared with 1.4 nM for iloprost.[33,40] In all other respects though, the platelet activity of BMY 45778 when tested in plasma-free conditions is very similar to that of iloprost: BMY 45778 stimulates cyclic AMP production with an EC_{50} value of 6 - 10 nM; it stimulates GTPase activity (EC_{50} 10 nM) and competes with [^{3}H]-iloprost binding (IC_{50} 7 nM).

These data have been drawn together in Figure 36 and we can see the expected pattern of a classical IP_1-receptor mediated response, i.e. that prostacyclin, iloprost and cicaprost have comparable potency for inhibiting human platelet aggregation, whereas PGE_1 and carbacyclin are less potent. Beraprost is intermediate in activity between these two groups, and both EP 157 and octimibate are the least potent. There is one trend that stands out in Figure 36; the two nonprostanoids (octimibate and EP 157) are relatively more potent in the upper four species. than in the lower six (sheep data unavailable for these two IP agonists), and this is not correlated with absolute sensitivity to the standard agonist iloprost. In addition, carbacyclin is relatively less potent in human, pig and horse platelets compared to rat and rabbit platelets, and a similar picture is seen for cyclic AMP production and inhibition of [^{3}H]-iloprost binding.[43] However, another study shows consistent potency ratios for prostacyclin and carbacyclin between the two groups of species.[54] There is also some evidence that PGE_1 is a relatively

more potent inhibitor in rat platelets compared to human platelets,[55] and this is supported by data on cyclic AMP production, inhibition of thrombin-stimulated increases in IP_3 and $[Ca^{2+}]_i$, and [^{3}H]-iloprost binding.[59,60] Again, not all studies agree.[54]

Now does any of this mean that we are looking at different receptors in different species? As suggested above, and from the cloned IP_1-receptor data, we see that PGE_1 and carbacyclin have higher affinity for rat and mouse IP_1-receptors compared with human IP_1-receptors. Furthermore, our data[61] looking at cyclic AMP production in COS-7 cells transfected with the mouse IP_1-receptor also finds PGE_1 merely 10-fold less potent than iloprost and cicaprost, and BMY 45778 is 40-fold less potent which is lower than one would expect if this was the human IP_1-receptor. Indeed, the family of related compounds octimibate,[33,39] BMY 42393 [41,62] and BMY 45778 [40,63] are consistently less potent than iloprost and cicaprost in inhibiting rat platelets compared with human platelets (Table 9), even when problems of protein binding are accounted for. So for the moment, we conclude that rat platelets are different from human platelets, but the question remains why PGE_1 and carbacyclin are relatively more potent in rat than human platelets, yet octimibate, BMY 42393 and BMY 45778 are relatively more potent in human compared with rat platelets.

Table 9. A comparison of the potency of octimibate and related compounds in platelets from different species

IP agonist	*Inhibition of ADP-stimulated platelet aggregation in PRP (IC_{50} values, nM)*			
	Human	*Monkey*	*Rat*	*Rabbit*
BMY 45778	35		1,300	126
BMY 42393	1,230	83	7,300	27,500
Octimibate	1,020		28,800	21,600

Data taken from Seiler et al.[39-41]

3.4 Mechanism of antiplatelet action

Most aggregating agonists activate phospholipase C (PLC) and elevate $[Ca^{2+}]_i$ by an IP^3-dependent release of Ca^{2+} from intracellular stores, as well as stimulation of the entry of extracellular calcium.[5] Therefore, since IP agonists inhibit platelet activation independent of the activator used, then it is probable that they interfere in some way with this pathway. Indeed, prostacyclin has several inhibitory mechanisms. It inhibits the release of Ca^{2+} from the dense tubular system, resulting in decreased TXA_2 production and decreased actin-myosin interactions, i.e. platelet contraction.[64] Moreover, prostacyclin can potentiate Ca^{2+} reuptake into platelet stores by inhibiting the inositol 1,4,5-trisphosphate ($InsP_3$)-receptor.[65]

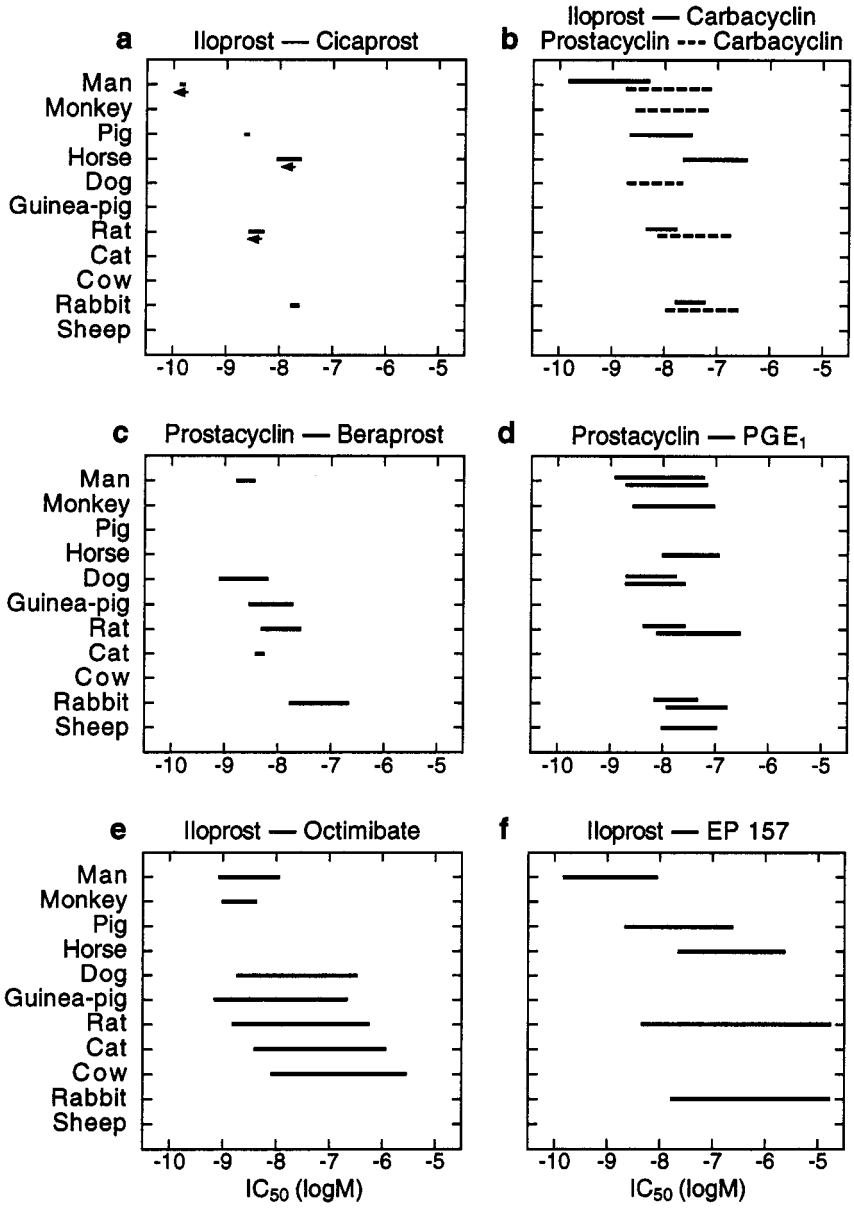

Figure 36. Potencies of IP agonists to inhibit platelet aggregation in different species. In each panel, horizontal bars link the IC_{50} values of the pair of IP agonists; arrows in (a) indicate that cicaprost is more potent than iloprost; dual bars in (d) indicate separate studies. Data in (a), (b, iloprost - carbacyclin), (e) and (f) were obtained on washed platelet suspensions,[33,44] and in (b, prostacyclin - - - carbacyclin), (c) and (d) on platelet-rich plasma.[51,66,67]

Fibrinogen binding to its platelet receptor (GPIIb-IIIa) plays a pivotal role in the formation of platelet thrombi, and the negative regulation of the fibrinogen receptor involves cyclic AMP or cyclic GMP-mediated reactions.[6] Certainly prostacyclin-stimulated increase in cyclic AMP correlates with the inhibition of binding of fibrinogen to activated platelets, which is paralleled by inhibition of platelet aggregation. In platelets and vascular smooth muscle cells, cyclic AMP and cyclic GMP are synergistic, but their mechanisms are not identical;[7] see Chapter 6, Figure 46. Cyclic GMP inhibits cyclic AMP-dependent phosphodiesterase (PDE-III) activity, thereby increasing platelet cyclic AMP.[68] The effect of cyclic AMP on inhibiting activation occurs at its physiological concentration in the platelet, suggesting that cyclic AMP is the primary controller of platelet reactivity. In contrast, one needs 10-fold the basal level of cyclic GMP to observe any effect on platelet function.[53] In addition, cyclic GMP can also inhibit cyclic AMP accumulation by activating cyclic GMP-stimulated PDE-II.[69] Provided cyclic GMP is present, PDE-II plays a major role in the hydrolysis of low concentrations of cyclic AMP, and restricts any increase in cyclic AMP and inhibition of platelet aggregation caused by inhibition of PDE-III. But at high cyclic AMP concentrations, PDE-II plays the major role in cyclic AMP breakdown, whether cyclic GMP is present or not. A functional synergism has been noted between iloprost and the nitric oxide donor SIN-1 in human platelets (probably due to the cyclic GMP-mediated inhibition of PDE-III), and between iloprost and aspirin, but the interaction between these three drugs is less predictable.[3]

Cyclic AMP not only inhibits platelet fibrinogen receptor exposure and PLC activity, it also inhibits secretion and aggregation independently of Ca^{2+} mobilisation by decreasing activation of protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinases.[70] These actions suppress the excitatory effects of phorbol esters and the Ca^{2+} ionophore ionomycin (Fig. 37). Finally, it has been shown that prostacyclin or PGE_1-induced inhibition of aggregation is associated with activation of PKA and phosphorylation of proteins with molecular masses ranging from 22 - >400 kDa.[5]

Evidence that IP_1-receptor activation leads to inhibition of platelet function via stimulation of adenylate cyclase comes not only from positive correlations between the potency of IP agonists to increase cyclic AMP levels and to inhibit platelet aggregation. For example, as predicted, inhibition of PDE activity potentiated prostacyclin and PGE_1-mediated inhibition of human platelet aggregation in vitro,[55,71] and in vivo.[72] Moreover, the ability of prostacyclin, PGE_1 and the prostacyclin mimetic SM- 10902 to inhibit thrombin-stimulated increase in IP_3 production, $[Ca^{2+}]_i$ and $^{45}Ca^{2+}$ influx in rat platelets was reversed by pretreatment with the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA) at concentrations

shown to inhibit cyclic AMP production.[59] The adenylate cyclase inhibitor DDA also inhibited the antiaggregation activity of the prostacyclin analogue OP-41483 in guinea pigs,[54] as did the adenylate cyclase inhibitor SQ 22536 for prostacyclin, iloprost, PGE_1 and EP 157-mediated inhibition of human platelet aggregation.[42,44] Furthermore, octimibate and its related nonprostanoid prostacyclin mimetics clearly stimulate cyclic AMP production and activate PKA in human platelets.[39-41]

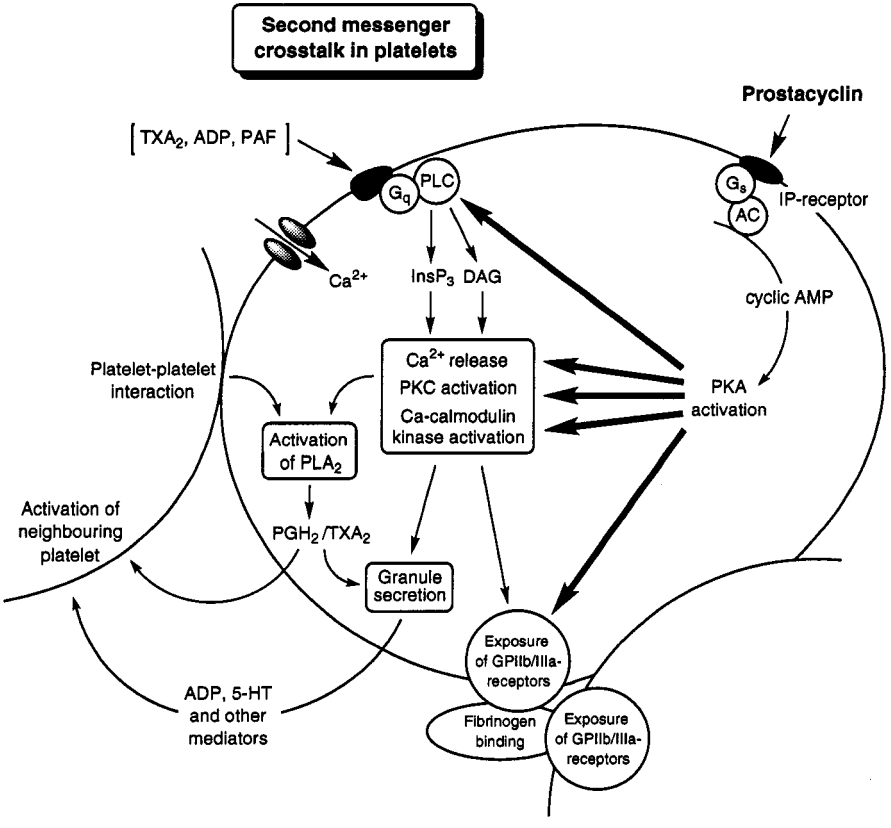

Figure 37. Pathways involved in platelet aggregation. The bold arrows indicate inhibitory mechanisms following PKA activation by prostacyclin.

3.5 Regulation of platelet IP_1-receptors

Incubation of human platelets with iloprost (1 00 nM - 1 μM) for periods of 12 - 24 h decreases the amount of [^{3}H]-iloprost binding due to a decrease

in B_{max} rather than changes in K_d values.[73,74] This loss of [^{3}H]-iloprost binding is matched by a decreased cyclic AMP response to iloprost.[73] Iloprost preincubation of human platelets also leads to heterologous desensitisation due to a decrease in $G_{s\alpha}$.[75]

In a study where two affinity binding sites for [^{3}H] -iloprost could be detected, it appeared that the iloprost preincubation-induced decrease in B_{max} value was due to the loss of the high affinity binding site.[76] It would appear that incubating platelets at 37°C leads to internalisation of the high affinity binding site by a process which is energy-dependent, and this process could presumably subserve desensitisation. IP_1-receptors are therefore internalised during short-term desensitisation (3 h with 100 nM iloprost), and they are not degraded but can be recycled rapidly to the platelet surface in a functionally active form after withdrawal of the agonist.[77] Giovanazzi et al.[76] proposed that each IP_1-receptor shuttles between the platelet plasma membrane and another compartment transferring some 25 ligand molecules per receptor molecule during a 2 h incubation period.

Platelet IP_1-receptor mediated responses can be indirectly regulated as a result of TP-receptor desensitisation. When human platelets are incubated with a TP agonist, this leads to rapid desensitisation of the TP-receptor-associated responses and causes an increase in the cyclic AMP response to iloprost (and forskolin), i.e. sensitisation of the platelet adenylate cyclase system which would function to potentiate the turn-off signal of the platelet.[78] In addition, exogenous alkaline phosphatase (a dephosphorylating enzyme) also enhances the cyclic AMP response to prostacyclin.[79]

The clinical relevance of IP_1-receptor desensitisation/downregulation observed in vitro has been the subject of much debate, see Gryglewski & Stock.[80] For example, patients with peripheral vascular disease, when infused with prostacyclin (5 ng kg^{-1} min^{-1}), rapidly developed (24 - 48 h) a decrease in sensitivity of their platelets to prostacyclin, returning to normal within a few days of cessation of the infusion.[81] This is known as the rebound phenomenon, seen as increased susceptibility of platelets to the proaggregatory action of platelet agonists. However, not all prostacyclin mimetics behave the same way, and the desensitisation of platelets against taprostene[82] appears to be much less than that for iloprost.[81] Infusion of rabbits with carbacyclin (5 µg kg^{-1} min^{-1}) caused a 70 - 80% inhibition of ADP-aggregation ex vivo, with no apparent desensitisation after 5 h infusion.[83] As seen with prostacyclin and beraprost,[64] the antiaggregation action of carbacyclin was short-lived once the infusion was terminated. The extent of loss in platelet responsiveness to prostacyclin mimetics is greater than would be expected from a consideration of the plasma concentration of drug, and MacDermot et al.[84] suggested that this was due to a high

concentration of agonist at the site of injection causing desensitisation of platelets at that site.

Patients with acute myocardial infarction or with unstable angina showed decreased [^{3}H]-PGE $_1$ binding, due to a significant decrease in B_{max}, which normalised within 2 - 8 weeks and but was not due to any changes in endogenous prostacyclin.[29] Since platelets do not synthesise new protein macromolecules (i.e. receptors), it was suggested that the transient decrease in IP_1-receptors could be related to a temporal derangement of protein synthesis in megakaryocytes, the precursor cells of platelets.

3.6 IP-receptors in platelet-like cells

CMK and MEG-01 cells are human megakaryocytic (the progenitor of platelets) leukaemia cells, and HEL is a pluripotent human leukaemia cell line which possesses erythrocytic, megakaryocytic and macrophage-like properties; both of these cell types are frequently used as models for human platelets. As already well established for platelets, IP agonists also increase cyclic AMP levels in MEG-01,[85] CMK[86] and HEL cells.[87,88] but in marked contrast with platelets,[89] activation of these cells with IP agonists also increases [Ca^{2+}]$_i$.[86-91] Furthermore, additional discrepancies appear when comparing the responses to iloprost and PGE_1. For example, in MEG-01 cells, iloprost-stimulated increase in [Ca^{2+}]$_i$ is pertussis toxin (PT)-insensitive whereas the response to PGE_1 is attenuated by PT-treatment.[89] Schwaner et al.[90] reported similar observations in HEL cells and also suggested that PGE_1 acted on both IP and EP-receptors, although the dependence on extracellular Ca^{2+} was not consistent between these two cell types. It is possible that we can now consider resolving this issue with data from Feoktistov et al.[87] using HEL cells. Here they observed that iloprost increased[Ca^{2+}]$_i$ only at concentrations greater than 1 μM, but could potentiate thrombin-stimulated increase in [Ca^{2+}]$_i$ with an EC_{50} value of 0.6 nM (comparable with an EC_{50} value of 3.3 nM for stimulation of cyclic AMP). The Ca^{2+} response to iloprost was PT-insensitive, involving an increase in IP_3 only at high concentrations, and involved facilitating Ca^{2+} influx. In contrast, the dominant Ca^{2+} response to PGE_1 was PT-sensitive, was independent of extracellular calcium and involved an increase in IP_3. More significantly, 11-deoxy-16,16-dimethyl PGE_2 was the most potent of the prostanoids able to mimic the profile of Ca^{2+} responses to PGE_1. We would expect this analogue to have little agonist activity at IP-receptors, at least at IP_1-receptors. Thus, there appear to be at least two prostanoid receptors functionally coupled to changes in intracellular Ca^{2+} in HEL cells: a putative IP-receptor coupled to G_s proteins that enhances Ca^{2+} influx

independently of cyclic AMP, and a novel prostanoid receptor that evokes Ca^{2+}mobilisation through stimulation of PLC by a PT-sensitive pathway.

In all cases where IP agonists such as cicaprost or iloprost increase $[Ca^{2+}]_i$ in these platelet-like cells, the response is always independent of cyclic AMP production.[86,87,89,90] and therefore could be due to IP_1-receptor coupling to more than one G-protein, as observed for the cloned IP_1-receptors,[92-94] or could be mediated by G_s coupling directly to a Ca^{2+} channel.[87] Now that the hazards associated with using PGE_1 as an IP agonist are more obvious (e.g. its ability to stimulate this novel prostanoid receptor in HEL cells and its potent agonist activity on all EP-receptor subtypes), further characterisation of IP_1-receptors in these platelet-like cells should concentrate more on responses to cicaprost as the most specific of the available IP_1-receptor agonists. When HEL cells are differentiated with DMSO, their time-course profile of cyclic AMP response to iloprost becomes more similar to that seen in platelets, due to the induction of a G_i -coupled EP_3-receptor.[57] It was suggested therefore that IP agonist-mediated activation of the EP_3-receptor would serve to limit the antiplatelet activity of any nonselective agonist such as PGE_1.

Although [^{3}H]-iloprost binding studies and cyclic AMP responses to iloprost are comparable between MEG-01 cells and human platelets,[89] that appears to be where the similarity ends. In addition, there are differences in the regulation of adenylate cyclase by thrombin in HEL cells and platelets.[95] For example, in HEL cells but not in platelets, thrombin can increase cyclic AMP levels and act synergistically with forskolin and prostacyclin. Therefore, if we really want to study IP_1-receptors on platelets, then we must use platelets as our starting material.

On testing a wide range of megakaryocytic and megakaryocytic/erythroid cell lines (HEL, NS-Meg, CMK11-5, CMK, MEG-01 and K562), Sasaki et al.[96] found only one (K562) which failed to express mRNA for the human IP_1-receptor. Phorbol ester-induced maturation and a range of cytokines also increased human IP_1-receptor expression in these cells. In addition, this treatment also increased [^{3}H]-iloprost binding and iloprost-stimulated cyclic AMP production in HEL and NS-Meg cells, as reported reported earlier for HEL cells.[97] Furthermore, normal umbilical cord blood megakaryocytes also expressed IP_1-receptor mRNA with peak expression occurring before terminal differentiation. Platelets possess only a small amount of remnant RNA transcribed from the nucleus of the megakaryocyte, therefore the supply of new receptors on platelet membranes is trivial and the expressed receptors on platelets must have originated from the megakaryocyte. Sasaki et al.[96] discussed the relevance of IP_1-receptor mRNA upregulation by cytokines involved in megakaryocytopoiesis, and concluded that since interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα) stimulate

coagulation activity in conditions such as endotoxemia, then their ability to induce expression of IP_1-receptors in megakaryocytes might act as a homeostatic mechanism to inhibit thrombosis.

4. CLINICAL USE OF PROSTACYCLIN AND ITS ANALOGUES

The major clinical goal in antiplatelet therapy has been the prevention of arterial thrombosis.[98] Arterial thrombi are composed predominantly of platelets formed under conditions of elevated shear stress at sites of atherosclerotic vascular injury and disturbed blood flow. In contrast, venous thrombi tend to form in the absence of underlying vascular damage and are composed predominantly of red cells enmeshed in fibrin. Although the clinical indications for antiplatelet agents are commonly coronary, cerebrovascular and peripheral arterial thrombosis, the pathophysiological and clinical distinctions between arterial and venous thrombosis are not absolute. The platelet GPIIb-IIIa receptor is the sole mediator of platelet aggregation, but the arachidonic acid pathway (i.e. increase in TXA_2) is not absolutely required for GPIIb-IIIa activation by platelet agonists.[8] It is for this reason that aspirin only partially inhibits platelet aggregation, and that the ability of IP agonists to suppress all modes of aggregation promises to be advantageous.

Initial animal studies were often promising; for example, clinprost (isocarbacyclin, TEI-9090) caused thrombus disaggregation and decreased thrombus growth rates subsequent to ADP-induced stimulation of platelet thrombi in venules of the microcirculation in a hamster cheek pouch model.[99] In addition, both iloprost and MM-706 (a carbacyclin analogue) prevented the reduction in circulating platelets induced by ADP in conscious rats, but only MM-706 produced this response in the absence of significant changes in blood pressure and heart rate.[100] Prostacyclin and carbacyclin infusion in rabbits produced similar cardiovascular changes in doses producing an equivalent degree of platelet inhibition. In rabbits, the prostacyclin analogue OP-4 1483 was antithrombotic; a property associated with its potent antiplatelet activities.[54] And, unlike prostacyclin, OP-4 1483 (i.v.) showed antiaggregation activity at doses which had no significant effect on blood pressure in monkeys. Together these results were sufficient to indicate that one might be able to use IP agonists as antithrombotic drugs without any cardiovascular side effects.

The clinical use of prostacyclin however is consistently hampered by its variable effect on bleeding time,[101] and the small margin between antiplatelet effects and undesirable side effects. Early evaluation of carbacyclin

infusions in man suggested that it could inhibit platelet aggregation ex vivo yet have no effect on blood pressure or heart rate.[102] Oral dosing with carbacyclin did though increase heart rate and it was clear that substantial inhibition of platelet aggregation ex vivo often required drug concentrations sufficient to give side effects of facial flushing and headache. Beraprost sodium is orally active as an antiplatelet drug in man, yet is only effective against ADP, and not collagen-induced aggregation, and needs several days of therapy to become effective.[64] In addition, the left ventricular pre-ejection period was significantly diminished and this needs further investigation. Unfortunately, the separation of antiplatelet and vasodilator properties of IP agonists in humans has been rare, and it is these haemodynamic factors which have severely restricted the clinical use of these compounds to date (see iloprost,[103] isocarbacyclin[99] and beraprost[67]).

Around 1985 it was still envisioned that the definitive separation of platelet and cardiovascular system properties of prostacyclin analogues, if demonstrated in man, would have extensive therapeutic possibilities. Ten years later, the value of IP agonists as antiplatelet compounds was reassessed by Schrör,[104] where he predicted major problems associated with the low selectivity for the platelet and the receptor-mediated nature of the response. To date, the occurrence and even relevance of IP_1-receptor desensitisation and rebound phenomena remain uncertain (see Section 5). Schrör noted that there were only a small number of large trials with prostacyclin-related drugs, and the results, in general, were not encouraging. He suggested that while prostacyclin mimetics with oral activity have an impressive therapeutic potential, their future may not be in the antiplatelet area. He felt that the receptor-mediated nature of their antiplatelet effects, i.e. dependence on the number of available functioning IP-receptors, limited selectivity for platelets, short duration of action, narrow therapeutic range and the availability of established pharmacological alternatives (e.g. aspirin), is a severe limitation. However, the advantage of IP agonists compared with current antithrombotic drugs such as aspirin, is that while aspirin irreversibly inhibits COX, this is only one component of platelet activation; it serves mainly as a positive feedback for secondary platelet aggregation and secretion induced by weak platelet stimuli such as ADP and adrenaline, but it is not essential for platelet adhesion, shape change, aggregation and secretion induced by strong physiological agonists such as collagen or thrombin. Therefore, it has been suggested that the combination of aspirin with antiplatelet drugs such as IP agonists or nitric oxide donors which have alternative mechanisms of action, should provide a more effective antiplatelet treatment.[3]

Despite these drawbacks, a useful clinical role for iloprost has been found in the management of thrombotic microangiopathy; in this case there appears

to be defective bioavailability of prostacyclin due to impaired vascular generation or accelerated degredation.[105] Low plasma 6-oxo $PGF_{1\alpha}$ levels are also found in the Upshaw-Schulman syndrome, and 10 days of continuous infusion of the prostacyclin analogue OP-4 1483 successfully treated the microangiopathic haemolytic process.[106]

Regional administration of prostacyclin prevents extracorporeal platelet consumption and reduces the risk of platelet embolisation and bleeding in cardiopulmonary bypass, charcoal haemoperfusion and haemodialysis,[98] especially when the use of heparin may be dangerous because of the risk of bleeding involved.[100] The potent short-lived platelet antiaggregatory action of iloprost has indeed been exploited with good effect during cardiac surgery in patients with heparin hypersensitivity.[103] Interestingly, prostacyclin prolongs the viability of washed human platelets used in the treatment of thrombocytopaenic patients,[107] and indeed helps in the production of washed platelets for experimental purposes by preventing any premature aggregation reactions.

One especially intriguing use of iloprost and platelets has recently been described, and although not yet perfected, has much potential; this process involves the use of autologous platelets, electroloaded with iloprost.[108] Thus, by exploiting the natural haemostatic properties of platelets to adhere to sites of endothelial denudation, drugs can be targeted to and retained at a vascular lesion. In this case, the vascular lesion would be one typically resulting from angioplasty, and it was hoped that reducing platelet accumulation on the damaged vascular tissue, one would see decreased smooth muscle cell migration and proliferation which commonly lead to restenosis. However, although studies in pigs found decreased platelet deposition, there was no inhibition of neointima formation.[108]

5. DISCUSSION

One of the prime clinical uses of IP agonists would be expected to be as antiplatelet drugs, targeted towards the management of arterial thrombosis and atherosclerosis. Yet, despite the synthesis and testing of numerous IP agonists, none have satisfactorily demonstrated antiplatelet activity at doses which did not produce side effects resulting from the vasodilator properties of these compounds. In fact, the discovery that IP_1-receptor knockout mice lacked a vasodilator response to cicaprost merely confirmed what had been suspected for so long, that IP-receptors on platelets and vascular smooth muscle were identical. The partial agonist properties associated with many of the nonprostanoid prostacyclin mimetics offered the scope for utilising the tissue-specific nature of agonist-mediated responses. Unfortunately, studies

of vascular IP1-receptors in human small resistance vessels (in vitro) and cardiovascular responses in cynomolgus monkeys (in vivo) both indicated a lack of platelet selectivity for, octimibate, despite its weak activity in human coronary and mesenteric arteries (in vitro).[51] Thus, the vascular IP_1-receptors responsible for the unwanted side effects of IP agonists are presumably as well-coupled as are platelet IP_1-receptors with regard to receptor-effector mechanisms. Furthermore, there is already a vast array of potential antithrombotic agents currently under clinical investigation (see Fareed[109]), such as heparin-related drugs, endothelial lining modulators and viscosity modulators, and it is against these drugs that IP agonists would have to compete in the market for antiplatelet drugs. Without sufficient separation of platelet and vascular responses, the clinical use of IP agonists may be relegated to more highly specialised applications.

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Chapter 6

IP-receptors in the vasculature

1. INTRODUCTION

Prostacyclin (IP-) receptors are distributed throughout the cardiovascular systems of many species and their activation leads to arteriolar dilatation and reduction in blood pressure. Many blood vessels also contain a prostacyclin-generating system in the endothelium. Indeed, prostacyclin is one of several endothelium-derived relaxing factors (EDRFs) that help to control the distribution of blood flow within the cardiovascular system. In this chapter we shall discuss some basic and clinical attributes of vascular IP systems: their agonist profiles, second messenger systems and interactions with other vasoactive agents; how endogenous prostacyclin may affect cardiovascular function in both health and disease; and the therapeutic uses of IP agonists in cardiovascular disease. With respect to the latter two areas, IP agonists have powerful inhibitory actions on platelets (Chapter 5) and modulatory actions on white cells (Chapter 8) that may well influence their vascular effects. The reader will find more detailed descriptions of studies performed prior to 1991 in the excellent review by Dusting & MacDonald.[1]

2. ACTIONS OF IP-RECEPTOR AGONISTS ON ISOLATED VESSEL PREPARATIONS

2.1 Distribution and cellular location of IP-receptors

In general, prostacyclin-sensitive relaxant systems are not limited to particular areas of the arterial system. Systemic arteries with conduit functions (e.g. guinea-pig thoracic aorta[2]), and resistance functions (human cerebral arteries of 0.5 mm diameter[3]), together with pulmonary arteries (intralobular arteries of rabbit, dog and man[4]) are all dilated by prostacyclin, and there is no evidence for gradation of sensitivity within a vascular tree. IP agonists tend to have weaker dilator actions on veins compared to arteries. For example, prostacyclin and its analogue iloprost relaxed bovine coronary artery at nanomolar concentrations, whereas on bovine coronary vein contractile and relaxant effects respectively were observed, but only at micromolar concentrations.[5] Also, prostacyclin was a much weaker relaxant agent than PGE_2 on rabbit saphenous vein, and its activity may be due to activation of EP_4-receptors.[6] A few vessels, for example rabbit aorta, are devoid of IP-receptors and are useful for studying other properties of IP agonists, such as the TP-receptor antagonism shown by some nonprostanoid prostacyclin mimetics (see Chapter 3).[7]

Relaxation induced by an IP agonist is usually unaffected by physical removal of the endothelium,[4,8] blockade of neuronal Na^+-channels with tetrodotoxin,[9] and pretreatment with antagonists for muscarinic, β-adrenergic, and histamine H_2-receptors;[10] these findings indicate that the IP agonist acts directly on the vascular smooth muscle cell.

2.2 Choice of vascular preparation

In deciding on a suitable vascular preparation for the study of IP agonists (and of course the elusive IP antagonist), ready availability of tissue and high sensitivity to prostacyclin are important considerations, but the presence of other prostanoid receptor systems can be a critical factor. For example, on rat aorta,[11] rat pulmonary artery[12] and monkey peripheral arteries,[8] submaximal relaxation progressed to contraction as the concentrations of prostacyclin and carbacyclin were increased. Inclusion of a TP-receptor antagonist showed that the contractions were due to activation of TP-receptors, and this was supported by binding studies on cultured smooth muscle cells from rat aorta, where both prostacyclin and carbacyclin

competed with [^{125}I]-BOP for TP-receptors (IC_{50} = 2.3 and 1.5 μM respectively).[11]

Certain human blood vessels are relatively easy to obtain from surgical procedures and of these, intrapulmonary,[4,9] uterine,[13] umbilical[14] and mesenteric[15] arteries relax well to IP agonists (cicaprost IC_{50}s 0.1 - 1 nM). While the contractile TP systems in these preparations can be suppressed with a TP antagonist, the presence of a contractile EP_3 system (for which no antagonist is available) may affect estimation of IP agonist potency. On human intrapulmonary artery for example, Walch et al.[16] concluded that only IP-receptors are involved in prostanoid-induced relaxation mainly on the basis that cicaprost and iloprost inhibited noradrenaline-induced tone, but PGE_2 did not. However, in an earlier study Qian et al.[17] showed that PGE_2 could induce relaxation in some pulmonary artery preparations, whilst in the remainder its $EP_{2?}$ relaxant action was obscured by an EP3 contractile action. The prostacyclin analogue TEI-3356 also activates this EP_3 system leading to underestimation of IP agonist potency (Fig. 38).[9]

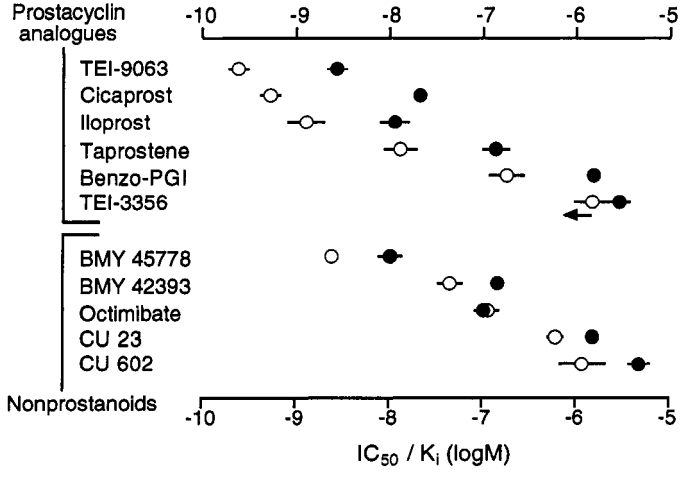

Figure 38. Comparison of relaxant potencies on human pulmonary artery (IC_{50}, unfilled circle) and binding affinity for human platelet IP_1-receptors (K_i, filled circles) for prostacyclin analogues and nonprostanoid prostacyclin mimetics. Prostacyclin has an IC_{50} for relaxation of about 4 nM. The horizontal arrow indicates that the IP agonist potency of TEI-3356 is underestimated due to its EP_3 contractile action. Benzo-PGI = benzodioxane prostacyclin. Data from Jones et al.,[9] with permission.

The presence of relaxant EP_2 and/or EP_4 systems (for which there are again no good antagonists) can also create difficulties, and this point is well illustrated by the studies of Hedlund & Andersson[18] on vascular tissue from

the human penis (Fig. 39). Under conditions of resting tone, corpus cavernosum preparations were potently contracted by the TP agonist U-466 19; $PGF_{2\alpha}$ (data not shown) and prostacyclin were moderately active contractile agents, whereas PGE_1 and PGE_2 showed no effect. When tone was raised with either $PGF_{2\alpha}$ or noradrenaline, relaxation was seen with PGE_1 and PGE_2 but not with prostacyclin. Thus the corpus cavernosum possesses a relaxant EP system, which undoubtedly contributes to the therapeutic efficacy of PGE_1 (Alprostadil) in erectile dysfunction in man when given by the intracavernosal or intraurethral routes.[19,20] However, it should not be concluded that prostacyclin is inactive at EP-receptors in the corpus cavernosum preparation, since its contractile activity (at TP-receptors?) may have counteracted its relaxant activity. Relevant to this suggestion is the finding that cicaprost, iloprost, carbacyclin and isocarbacyclin all compete with $[^3H]$-PGE_2 for mouse EP_2-receptors expressed in CHO cells with K_i values between 1 .0 and 1.6 μM.[21]

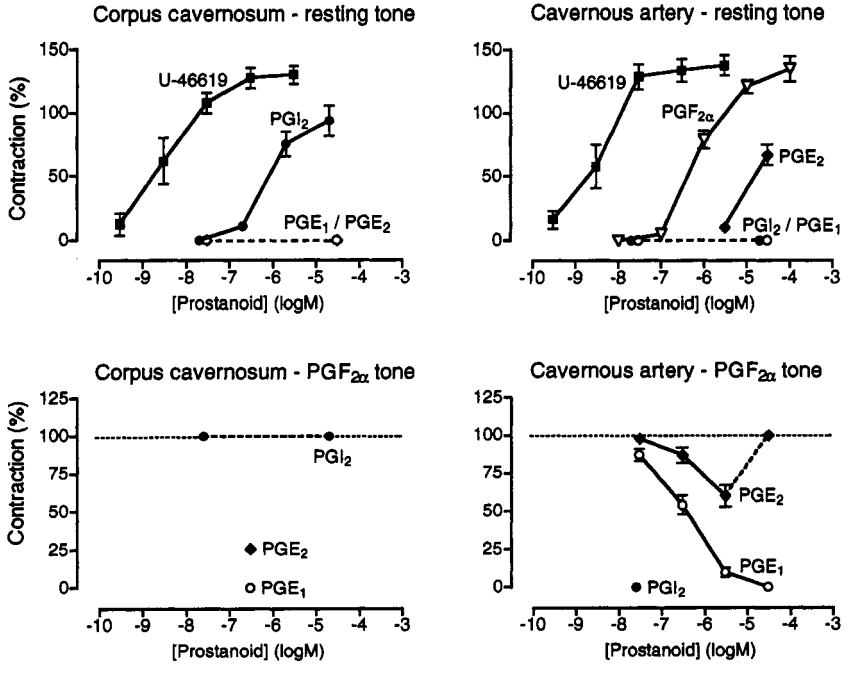

Figure 39. Log concentration response curves for prostanoid agonists on isolated vascular preparations from the human penis. Tone was induced by 1 μM $PGF_{2\alpha}$ in the lower panels. Broken lines joining data points indicate that absolute responses were not quoted in the original report, simply a description "no response over the concentration range ...". Data from Hedlund & Andersson,[18] with permission.

On the human cavernous artery preparation, the picture is quite different (Fig. 39): this vessel is also highly sensitive to the contractile action of U-46619, but prostacyclin now has no effect on resting tone and abolishes $PGF_{2\alpha}$-induced tone at a concentration of 25 nM; PGE_1 is a less potent relaxant and PGE_2 shows a bell-shaped curve. It is possible that the cavernous artery contains only a relaxant IP-receptor, for which the expected potency ranking would be prostacyclin > PGE_1 >> PGE_2. However, there may also be a relaxant EP-receptor present since the contractile action of PGE_2 could offset its relaxant activity. The profile of PGE_1 tells us little since we know that it activates EP_2, EP_4 and IP-receptors; its K_i values for these mouse cloned receptors in CHO cells are 10, 2.1 and 33 nM respectively,[21] see Table 2.

Returning to suitable animal preparations, rings of bovine coronary artery,[5] and dog and rabbit mesenteric arteries,[15,22] are all sensitive to IP agonists, but again they contain contractile TP-receptors and contractile and/or relaxant EP-receptors.

2.3 Pharmacological characterisation

Iloprost and cicaprost are better standard agonists than prostacyclin since the latter hydrolyses in the bathing fluid leading to a sharper response profile and modest underestimation of its agonist potency (see Chapter 2 for structures and stability data).[9,13]

Vascular IP-receptors appear to be of the IP_1 subtype, based on the high absolute potency of cicaprost, and its marginally greater potency over iloprost (1.5 - 3-fold) and somewhat greater potency over prostacyclin (3 - 20-fold), as typified by the data for human pulmonary artery shown in Figure 38.[9] This is substantiated by the weaker activity of another commonly used prostacyclin analogue, carbacyclin, which was 9.8, 2.1 and 3.6-fold less potent than cicaprost on human, rabbit and dog mesenteric arteries respectively,[15] and 4.3-fold less potent than prostacyclin on rabbit mesenteric artery.[23] Unfortunately, comprehensive comparison of IP agonist potency rankings for different vascular beds and for different species is virtually impossible, since in many studies only one or two prostacyclin analogues have been examined.

There has also been little work on comparing relaxant potencies of a range of IP agonists with their binding affinities in the same vascular preparation, mainly because of problems in obtaining good quality data on membrane preparations from vascular tissue. For example, Parfenova et al.[24] quoted an EC_{50} of 27 nM for saturable [^{3}H]-iloprost binding to membranes from pig cerebral microvessel cells grown in culture, but declined to carry out a Scatchard analysis due to the high level of nonspecific binding. A

secondary option is to compare relaxant potencies with more easily obtainable binding affinities for the IP_1-receptor on platelet membranes from the same species. Figure 38 shows that these two parameters correlate well for human pulmonary artery rings and human platelet membranes.[9] For the prostacyclin analogues (TEI-33 56 excluded), the predicted occupancy of vascular IP_1-receptors at the corresponding IC_{50} value ranges from 2.5 to 10% indicating moderately high efficacies. By the same procedure the nonprostanoid prostacyclin mimetics would appear to have lower efficacies and this aspect is discussed more fully in Chapters 3 and 5.

3. CELLULAR MECHANISMS INVOLVED IN RELAXATION

3.1 Adenylate cyclase/protein kinase A and dependence on the tone-inducing agent

Several studies on isolated arterial vessels and cultured vascular cells have implicated the adenylate cyclase/protein kinase A (PKA) system in the relaxant action of IP agonists (Fig. 40).[24-29] MacDermot & Barnes[30] found K_{act} values for adenylate cyclase in guinea-pig pulmonary (artery) tissue homogenates to be 26 nM for prostacyclin, 350 nM for PGE_1, and about 100 μM for PGE_2; these values correlated well with competition for [^{3}H]-PGI_2 binding in the same tissue.[31] Dong et al.[32] showed that the maximal relaxant effect of endothelium-derived prostacyclin on rabbit middle cerebral artery was inhibited by 56% by the adenylate cyclase inhibitor SQ 22536. The PKA blockers H-89 and Rp-8-CPT-cAMPS inhibited iloprost-induced dilatation in rat tail small arteries by 77 and 92% respectively.[33] Elevation of cyclic AMP has also been demonstrated in vivo in the open-chest dog by rapid freezing of coronary vessels during prostacyclin infusion.[34]

IP agonists and other agents that raise cyclic AMP levels inhibit vascular tone induced by a variety of spasmogens. However, inhibitory profiles against different spasmogens are not always identical and this is relevant to the processes whereby cyclic AMP causes relaxation. There are a number of reports where even quite high levels of tone induced by U-46619 were totally or almost totally inhibited by an IP agonist: in contrast, although the IP agonist completely inhibited low K^+ (25 mM) tone, it could only partially inhibit high K^+ (45 - 75 mM) tone (Fig. 40, inset).9,35,36 How can these observations be explained? High K^+ in the external fluid depolarises the plasma membrane and consequently opens voltage-gated (L-type) Ca^{2+}-

channels; the depolarisation also sensitises calmodulin / contractile proteins to Ca^{2+} (Fig. 40, lower section).[37]

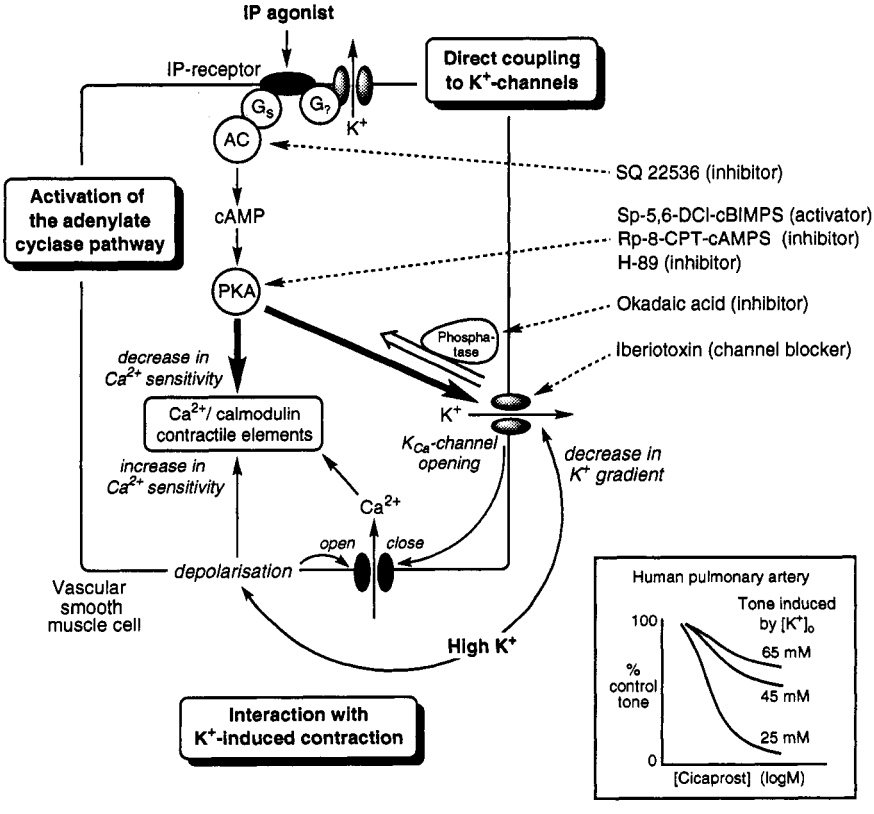

Figure 40. Potential mechanisms for the vasorelaxant actions of IP agonists. The bold and open arrows indicate phosphorylation and dephosphorylation respectively. The lower portion shows the mechanisms whereby IP agonists inhibit tone induced by high external K^+. The inset shows that cicaprost can only partially inhibit tone induced by high K^+ in human pulmonary artery rings.[9]

In opposition, a cyclic AMP elevator appears to have two major modes of action:[38] opening of K^+-channels to cause hyperpolarisation (and hence closure of L-type Ca^{2+}-channels) and reduction in the sensitivity of calmodulin / contractile proteins to Ca^{2+}. High $[K^+]_o$ will abrogate the first relaxation mechanism by moving the membrane potential closer to the K^+ equilibrium potential (E_K). Little K^+ will flow through the open channels, and this explains why the K^+-channel opener cromakalim is virtually ineffective against high K^+ tone. The second relaxation mechanism will still

operate however, and this was demonstrated for the IP system in guinea-pig aorta where 1 μM iloprost relaxed high K^+ tone by 25 - 30% with little effect on $[Ca^{2+}]_i$.[36] Yamagishi et al.[38] first proposed a similar explanation to account for incomplete relaxation of pig coronary artery by β_1-adrenoceptor agonists.

In contrast to this large body of evidence, Turcato & Clapp[39] have recently demonstrated that IP agonist-induced relaxation of guinea-pig aorta may be independent of cyclic AMP. In the absence of phosphodiesterase inhibition, iloprost (60 nM) induced about 65% relaxation of phenylephrine tone accompanied by a 3-fold rise in cyclic AMP. SQ 22536 abolished the rise in cyclic AMP, but had absolutely no effect on relaxation. Could there be a more direct link to K^+-channels in the plasma membrane (Fig. 40) and if so, what function does cyclic AMP serve in this situation?

Other mechanisms besides opening of K^+-channels and reduction of Ca^{2+} sensitivity could contribute to relaxation induced by IP agonists. It is known that forskolin and dibutyryl cyclic AMP can inhibit phosholipase C activation in vascular[40] and tracheal smooth muscle.[41] In addition, cyclic AMP elevation in tracheal smooth muscle promotes sequestration of Ca^{2+} into internal stores.[42] Another possibility is activation of the plasma membrane Na^+-K^+ ATPase to cause hyperpolarisation and lowering of $[Ca^{2+}]_i$. Fukuda et al.[43] have shown that in pig coronary artery the Na^+-pump inhibitor ouabain shifts the log concentration-response curve for PGE_1 to the right by about 0.8 log unit. However it is not clear whether PGE_1 was activating IP and/or EP-receptors in this preparation.

3.2 Opening of K^+-channels

Let us now examine information gained from electrophysiological studies about the nature of the K^+-channels involved in IP agonist-induced vascular relaxation. In a comprehensive series of experiments, Siegel et al.[35] showed that the hyperpolarisation of dog carotid artery by 1 μM iloprost was accompanied by a 3 - 4-fold increase in K^+ efflux as detected by ^{42}K washout. Using the whole-cell patch-clamp method on single vascular smooth muscle cells from rat portal vein (Fig. 41, Table 10), these workers also showed that the inward Ca^{2+} current in response to a depolarisation jump was not affected by iloprost (Ba^{2+} was the charge carrier and Cs^+ was present in the pipette solution to inhibit K^+ fluxes). When measured in either Ba^{2+} or Ca^{2+} solutions and with K^+ in the pipette, iloprost's action to increase outward K^+ current was much more dramatic and it was concluded that activation of IP-receptors opens both voltage-dependent and Ca^{2+}-activated K^+-channels (K_v and K_{Ca}). Table 10 shows that delayed rectifier and ATP-sensitive K^+-channels (K_{drf} and K_{ATP}) are also involved in IP-agonist-induced

relaxation of vascular smooth muscle cells. The K_{ATP}-channel blocker glibenclamide must be used with caution however, since it is also a weak TP-receptor antagonist (pA_2 6.2) and could affect U-466 19-induced tone.[44]

Table 10. Role of K^+-channels in vascular relaxation induced by IP agonists

In vitro preparation	*IP agonist*	*Method/inhibitors*	*Ion Channels*		*Ref. No.*
			identified	*excluded*	
Rat coronary circulation	Iloprost	Perfusion pressure/Glib	K_{ATP}		45
Rat aorta ring	Iloprost	Muscle tension/Glib		K_{ATP}	4s
Rat lung circulation	Iloprost	Perfusion pressure/Glib, CTX	K_{ATP} K_{Ca}		46
Rat tail small artery segment	Iloprost	Video-imaging/Glib, IBTX, TEA	K_{ATP} K_{Ca}		33
Rabbit coronary circulation	Iloprost PGI_2	Perfusion pressure/Glib	K_{ATP}		47
Rabbit middle cerebral artery ring	PGI_2*	Muscle tension/Glib, CTX, IBTX, DTX, 4-AP	K_{Ca} K_v		32
Cow coronary artery strip	Iloprost	Muscle tension/Glib	K_{ATP}		47
Guinea-pig aorta ring	Iloprost Cicaprost	Muscle tension/Glib, TEA, IBTX	K^{Ca}	K_{ATP}	48
Rat portal vein isolated cell	Iloprost	Whole cell patch-clamp	K_{Ca} K_v		35
Rat tail artery isolated cell	Iloprost	Whole cell and cell-attached patch-clamp/Glib, IBTX, 4-AP	K_{Ca}	K_{ATP}	33
Cow small coronary artery isolated cell	Iloprost	Cell-attached and inside-out patch-clamp	K_{drf}	K_{ca}	49

*Released as an EDRF. Glib = glibenclamide, CTX = charybdotoxin, IBTX = iberiotoxin, DTX = dendrotoxin, TEA = tetraethyl ammonium (halide), 4-AP = 4-aminopyridine. K_{ATP} K_{Ca}, K_v and K_{drf} are ATP-dependent, Ca_{2+}-dependent, voltage-dependent and delayed rectifier K^+-channels, respectively.

The patch-clamp experiments of Schubert et al.[33,50] on freshly isolated tail artery cells of the rat provide clear evidence of an interaction between the adenylate cyclase/PKA system and K_{Ca}-channel function in the relaxant action of IP agonists (Fig. 40) Iloprost-induced outward current was markedly inhibited by the K_{Ca}-channel blocker iberiotoxin and the PKA inhibitor Rp-8-CPT-cAMPS; this current was also enhanced by the phosphatase inhibitor okadaic acid. Iloprost did not open the K_{Ca}-channels by elevating $[Ca^{2+}]_i$. It may however promote phosphorylation of K_{Ca}-

channels to enhance their sensitivity to $[Ca^{2+}]_i$ as proposed by Sadoshima et al.[51] for isoprenaline action on cultured smooth muscle cells from rat aorta.

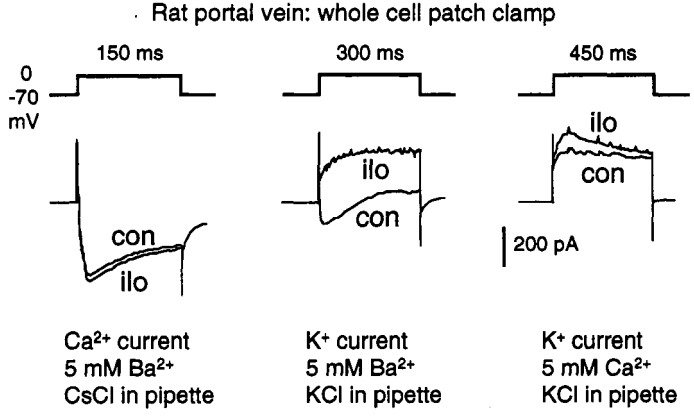

Figure 41. Effects of iloprost on Ca^{2+} and K^+ currents recorded in single vascular smooth muscle cells from the rat portal vein. Data from Seigel et al.,[35] with permission.

4. IN VIVO EFFECTS OF PROSTACYCLIN AND ITS STABLE ANALOGUES

4.1 Actions on peripheral vasculature

Prostacyclin given intravenously lowers the systemic blood pressure of all animal species examined so far. Early studies in the dog showed that arteriolar dilatation was the primary mechanism, with cardiac output and stroke volume increasing passively.[52] Stable analogues of prostacyclin such as iloprost and beraprost were also hypotensive in the dog, cat, rat and rabbit.[5,53-55] Prostacyclin induces dilatation in many arterial beds when given by either close-intraarterial infusion (e.g. coronary,[56] femoral,[56] and renal[57-59] arteries of the dog) or topical application (e.g. hamster cheek pouch,[60] rat cremaster muscle,[61] cat cerebrum[62]). Iloprost dilated rabbit ear artery segments to the same extent when tone was induced by either transmural nerve stimulation or phenylephrine,[63] indicating a direct (postsynaptic) action on the arterioles.

The cardiovascular effects of intravenous prostacyclin (Fig. 42) and several of its analogues in man are summarised in Table 11. Peripheral vasodilation was evident with each of the agents and facial flushing,

headache and nasal congestion often limited the dose that could be given. Vomiting occurred at the higher doses of prostacyclin and beraprost. Carbacyclin infusions produced modest inhibition of platelet aggregation (ex vivo), but no cardiovascular changes.[64] In one subject, 25 mg of carbacyclin by the oral route produced signs of peripheral vasodilatation, but also systemic hypertension.

Attempts to improve the therapeutic profile of prostacyclin analogues still continue. Etaloprost is an oral pro-drug in which an elongated a-chain is shortened by β-oxidation in vivo to give the highly potent cicaprost (see Chapter 2 for structure).[65] However, the activation process tends to be too fast to achieve a sustained plasma level of cicaprost. Another approach has been to incorporate isocarbacyclin methyl ester, which is highly lipophilic, into a lipid microsphere formulation (TTC-909) for intravenous use.[66] The isocarbacyclin methyl ester is then slowly released at the supposed site of action and de-esterified to give the active agent.[67] Transient orthostatic hypotension was observed with TTC-909 in one subject and postural hypotension in another, raising the usual dilemma of what to do when adverse effects appear with a sustained-release formulation.

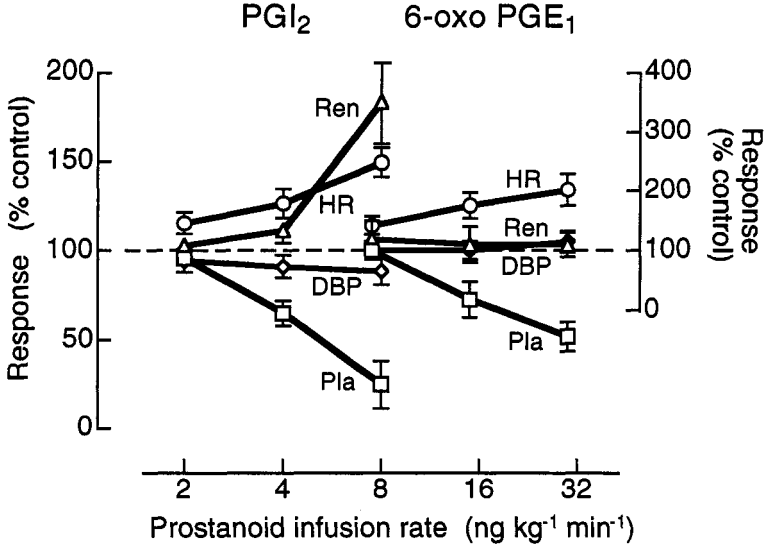

Figure 42. Effects of intravenous infusions of prostacyclin and 6-oxo PGE_1 on diastolic blood pressure (DBP), heart rate (HR), ADP-induced platelet aggregation (Pla) (all left y-axis) and plasma renin activity (Ren, right y-axis) in man. Data from Miyamori et al.,[68] with permission.

Table 11. Effects of prostacyclin and its analogues on cardiovascular function in man

Prostanoid / Subject group	*IV infusion rate (ng kg^{-1} min^{-1})*	*Effects*	*Ref. No.*
Prostacyclin / healthy subjects	0.5 - 2.5	Facial flushing.	69-72
	8	Hypotension, tachycardia, facial flushing, mild abdominal cramping, increase in plasma renin activity.	
	2.5 - 10	Hypotension, tachycardia, decrease of sinus cycle length and AV node refratoriness.	
	4–10	Headache, tachycardia, nausea and vomiting, increase in bleeding time, erythema at infusion site and "vagal reflex" after about 1 h.	
	10–15	Sweating, restlessness, sighing and yawning.	
	50	Bradycardia, pallor, nausea, marked hypotension.	
Iloprost / patients with occlusive disease of lower limbs	0.5 - 4 (also oral t.i.d.)	Hypotension, tachycardia, increase in cardiac index, fall in pulmonary vascular resistance, no change in mean pulmonary arterial pressure.	73
Carbacyclin / heathy subjects	20 - 80	No cardiovascular changes, inhibition of platelet aggregation.	64
	25 mg orally	Hypertension, facial flushing, headache.	
Ciprostene*/ healthy subjects	120 - 480	Headache, restlessness, perspiration, facial flushing, nausea and vomiting, jaw pain.	74
ONO-41483*/ healthy subjects	10	Hypotension, inhibition of platelet aggregation.	75
Beraprost / healthy subjects	75 - 150 µg day^{-1} t.i.d. orally	Facial flushing, heavy-headedness, nasal congestion, inhibition of platelet aggregation.	76,77
	200 µg single oral dose	Mild breathlessness, loss of appetite, vomiting, diarrhoea.	
TTC-909†/ healthy subjects	1 - 4 µg single IV dose	Temporary orthostatic hypotension, postural hypotension.	78

*9β-methyl carbacyclin; **15 -cyclopentyl-ω-pentanor carbacyclin; †Lipid microsphere formulation of isocarbacyclin methyl ester.

4.2 Renal actions

The renal actions of prostacyclin have been investigated in depth, particularly by infusion into a renal artery of the anaesthetised dog, which

largely avoids the systemic hypotension (and consequent antidiuresis) associated with the intravenous route.[57,58] Infusion of prostacyclin at 3 - 6 ng kg^{-1} min^{-1} induced equivalent increases in renal blood flow to PGE_2 at 0.5 - 1.5 ng kg^{-1} min^{-1}. However, PGE_2 was much more effective than prostacyclin in increasing urinary water and sodium output.[59,79] Jones et al.[59] suggested that the natriuretic actions of prostacyclin might be largely due to its renal haemodynamic activity, whereas PGE_2 is likely to have additional direct actions on sodium reabsorption in the ascending limb of Henlé and the distal tubule / collecting ducts and on antidiuretic hormone function (see Jones et al.[59] for references). However, Gullner et al.[80] have shown that in hypophysectomised/cortisol-treated dogs under maximal water diuresis, prostacyclin infusion enhances sodium excretion, probably by a direct action on the distal nephron.

Another facet of prostacyclin's action on the kidney is its ability to release renin, with consequent generation of angiotensin II and aldosterone. Early observations showed that (a) infusion of arachidonic acid into the renal artery of the rabbit,[81] rat[82] and dog[83-85] induces renin release, which is blocked by indomethacin, (b) renal medullary prostaglandins contribute to the elevated plasma renin levels in patients with Bartter's syndrome (see Levenson et al.[86] and Guay-Woodford[87]), and (c) prostacyclin is the dominant product synthesised by cyclo-oxygenase (COX) in renal arterioles close to juxtaglomerular cells.[88,89] In the presence of indomethacin, prostacyclin stimulates renin release from rat renal cortical slices with an IC_{50} of about 50 nM,[90] and this and other in vitro studies[91-94] support the idea that activation of IP-receptors on juxtaglomerular cells releases renin. Only a few prostacyclin analogues have been investigated for their renin-releasing activity. In the rat, intravenous administration of taprostene (CG 4203, see Chapter 2)[95] and the 15-cyclohexyl-ω-pentanor and 15-(3-thienyloxy-methyl)-ω-pentanor analogues of prostacyclin methyl ester[96] markedly stimulated renin release. However, direct release of renin is unlikely to be the sole mechanism involved, since blood pressure was reduced concomitantly by the prostacyclin analogues. Indeed, the angiotensin receptor antagonist saralasin enhanced the hypotensive action of taprostene and abolished the increase in blood pressure seen on stopping of the taprostene infusion.[95]

In vivo measurements in dog and man, support the view that endogenous prostacyclin can directly release renin.[97-100] However, the three main mechanisms effecting renin release are often difficult to study in isolation and consequently it is not easy to determine the involvement of intrarenally-generated prostacyclin in each mechanism.[101] In the first mechanism, a fall in blood pressure activates the systemic baroreceptors leading to increased sympathetic nervous tone to the kidney and activation of β_1-adrenoceptors

on juxtaglomerular cells. The consensus is against an involvement of prostacyclin in this mechanism,[101] since renin release from the rabbit isolated perfused kidney[102] and from rat renal cortical slices[90] induced by the β-adrenoceptor agonist isoprenaline was unaffected by COX inhibition. In addition, indomethacin failed to affect renin release resulting from renal nerve stimulation in the anaesthetised dog.[99,103]

In the second or renal baroreceptor mechanism, the renal afferent arteriole /juxtaglomerular cells sense changes in stretch or wall tension and release renin when renal perfusion pressure falls; the experimental evidence relating to a role for prostacyclin in this mechanism is mixed. In dogs with an intact filtering kidney, reduction in renal perfusion pressure to within the autoregulatory range (83 - 90 mmHg) caused release of renin that was inhibited by indomethacin; renin release elicited by lower perfusion pressures (53 - 60 mmHg) was not affected by indomethacin.[104,105] Moreover, in adrenalectomised dogs[106] and rats[107] with a single denervated nonfiltering kidney (in which the macula densa mechanism does not operate; see below), indomethacin and meclofenamic acid respectively failed to affect renin release in response to aortic constriction.

For the third renin release mechanism, in which the macula densa detects fluid flow and electrolyte composition within the distal tubule, the evidence is also contradictory. Thus, the elevated plasma renin activity in response to chronic sodium deprivation in the rat was abolished,[108] partially inhibited,[109] or unaffected[110] by indomethacin. In the dog, there is also evidence for [111] and against[112] prostacyclin involvement, and in man there is some evidence against.[113] In conclusion, prostacyclin biosynthesis is not a final common pathway in renin release, but may function to accentuate intrarenally-driven renin release mechanisms. Further studies would benefit from the use of a specific IP-receptor antagonist, which would not affect the activity of endogenous PGE_2 as COX inhibitors do.

It is possible that a metabolite of prostacyclin, 6-oxo PGE_1, may be responsible for some of the renin-releasing activity of prostacyclin.[114] The enzyme responsible, 9-hydroxy-prostaglandin dehydrogenase, is present in higher amount in the cortex of the rabbit kidney compared to the medulla and papilla, and this corresponds to the renin content.[114,115] When infused into the renal artery of the dog, 6-oxo PGE_1 was about four times more potent than prostacyclin in increasing renal blood flow and inducing renin release.[116] However, in human subjects its renin-releasing activity was minimal at doses that caused significant inhibition of platelet aggregation (Fig. 42).[68] 6-Oxo PGE_1, like PGE_1, is an agonist at both IP- and EP-receptors.[117]

4.3 Involvement of autonomic reflexes

In man, the intravenous infusion of prostacyclin can result in modest hypotension associated with marked tachycardia (Fig. 42). However, at higher infusion rates or longer infusion times severe hypotension, bradycardia and pallor develop (Table 11). The mechanisms may be similar to those found in conscious dogs, where prostacyclin[118] (and 6-oxo PGE_1[119]) stimulates sensory nerve endings in the heart to cause a reduction in sympathetic outflow from the brain. An instructive comparison was made with nitroglycerin (NTG), which reduced the internal pressure and end-diastolic diameter of the left ventricle by directly dilating veins; the consequent fall in arterial blood pressure actuated the baroreceptor reflex to increase heart rate. Neither section of the cervical vagi nor α_1-adrenoceptor blockade by prazosin affected the reduction in end-diastolic diameter. Prostacyclin on the other hand caused a more prolonged fall in blood pressure and a small reduction in heart rate. The accompanying fall in end-diastolic diameter was abolished by vagotomy and inhibited by 75% by prazosin. Thus, it may be concluded that prostacyclin has two main actions: the first is to directly dilate systemic arterioles (which will activate the normal baroreceptor reflex); the second is to activate vagal sensory receptors in the cardiopulmonary region, which results in reduction in sympathetic tone to the arterioles, veins and heart, thus reducing afterload, preload and heart rate. The nature of the sensory nerve endings stimulated by prostacyclin is discussed in Chapter 9.

4.4 Knockout of the IP-receptor gene

The removal of an organism's ability to manufacture a particular receptor by knockout of the corresponding gene is an important addition to receptor characterisation studies. As shown in Figure 43, the inhibitory action of cicaprost in platelet-rich plasma (PRP) from wild-type mice was absent in PRP from mice with homozygous knockout of the IP_1-receptor gene (IP_1-receptor-KO).[120] Similarly, high intravenous doses of cicaprost had no hypotensive action in anaesthetised mice with homozygous IP-receptor-KO, again demonstrating the identity of platelet and vascular IP_1-receptors within a species. PGE_2 had similar (transient) depressor activity in both wild-type and IP_1-receptor-KO mice confirming the specificity of the gene knockout procedure.

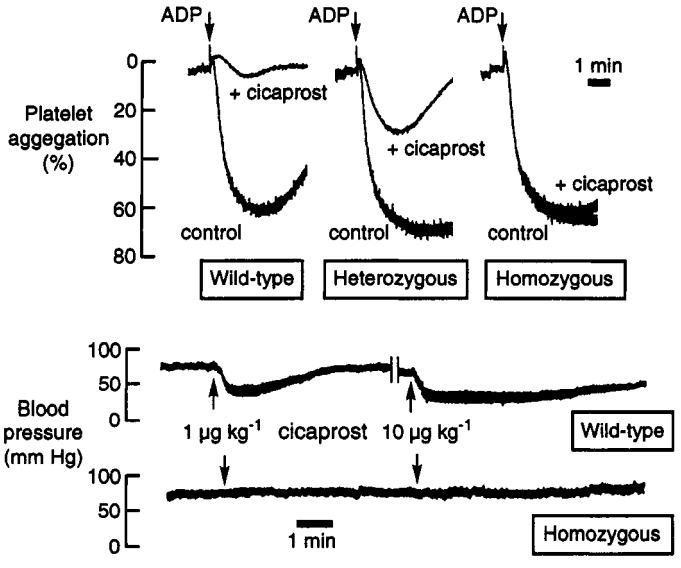

Figure 43. Effects of cicaprost on in vitro platelet aggregation and blood pressure of wild-type and IP_1-receptor-KO mice. Cicaprost concentration in platelet experiments was 10 nM. Data from Murata et al.,[120] with permission.

5. PROSTACYCLIN AS AN ENDOTHELIUM-DERIVED RELAXING FACTOR

5.1 The several EDRF systems in blood vessels

As discussed in Chapter 5, the finding that the endothelium of an artery can convert arachidonic acid or prostaglandin endoperoxides into prostacyclin,[121-123] raised the possibility that prostacyclin suppresses the reactivity of platelets in contact with the intimal surface, thus generating "antithrombotic cover" for the vessel.[124] However, if the prostacyclin were to diffuse into the circular smooth muscle layer, then vasodilatation might ensue. This type of endothelium-dependent vasodilatation has been investigated intensely following the report by Furchgott & Zawadski[125] in 1980 that the relaxant action of acetylcholine on rabbit aorta was abolished following removal of the endothelium. It was proposed that activation of muscarinic receptors on endothelial cells leads to production of an endothelium-derived relaxing factor or EDRF. In practice, at least three

EDRF systems exist in blood vessels (Fig. 44) and all are switched on by a rise in endothelial $[Ca^{2+}]_i$.

(i) Endothelial nitric oxide synthase (eNOS) converts L-arginine into nitric oxide (NO), which rapidly diffuses into smooth muscle cells to activate guanylate cyclase. This is often the dominant EDRF system.

(ii) Phospholipase A_2 (PLA_2) releases arachidonic acid from membrane phospholipids and COX and prostacyclin synthase then produce prostacyclin, which interacts with IP-receptors on the surface of the smooth muscle cell to activate adenylate cyclase.

(iii) A third enzyme system generates an endothelium-derived hyperpolarising factor or EDHF, which may be a cytochrome P450 mono-oxygenase metabolite of arachidonic acid,[126,127] although there is also evidence for and against it being the cannabinoid receptor agonist anandamide.[128,129] EDHF opens K^+-channels in the smooth muscle cell to cause membrane hyperpolarisation and closure of voltage-sensitive Ca^{2+}-channels, and its effect can usually be inhibited by appropriate K^+-channel blocker(s) (see later). It has also been suggested that in some vessels EDHF action is not chemical in nature, but involves a hyperpolarising current generated in the endothelial cell coupling electronically to the smooth muscle cell via gap junctions.[130-132]

The basic protocol for determining the contribution of each EDRF system to blood vessel relaxation involves constructing dose-response curves for the dilator stimulus in the presence of a NOS inhibitor (e.g. L-NAME, L-NNA or L-NOARG), a COX inhibitor (e.g. indomethacin), and a combination of the two. For example, on rabbit middle cerebral artery (Table 12, Fig. 44, inset), relaxant responses to acetylcholine (1 nM - 30 μM) were partially inhibited by both L-NOARG and indomethacin and almost abolished by a combination of the inhibitors,[32] indicating roles for both NO and prostacyclin as EDRFs. The remaining small relaxation may be due to EDHF, although it is difficult to establish that NOS and COX have been completely inhibited. Different EDRF profiles are found depending on the species and vascular bed examined. In substance P-induced relaxation of human umbilical artery, prostacyclin was dominant over NO,[133] whereas in human omental artery and vein prostacyclin's role was minimal.[134] The complexities of interpreting EDRF profiles are discussed in Section 5.4.

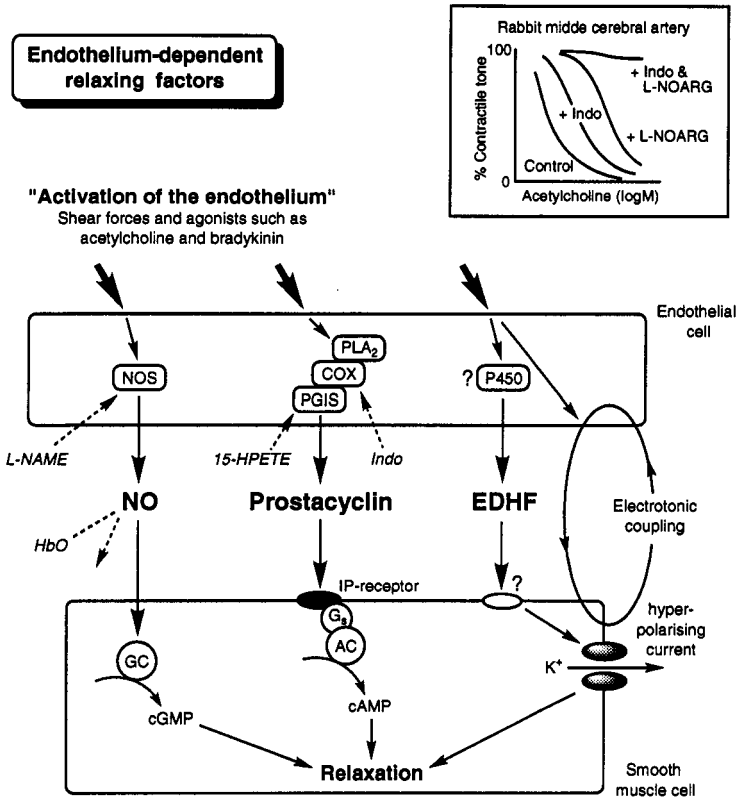

Figure 44. Endothelium-dependent relaxation systems present in blood vessels. Nitric oxide synthase (NOS) can be inhibited by L-NAME, cyclo-oxygenase (COX) by nonsteroidal anti-inflammatory agents (NSAIDS) such as indomethacin (Indo), and prostacyclin synthase (PGIS) by 15-hydroperoxy-eicosatetraenoic acid (15-HPETE). Nitric oxide (NO) levels can be reduced by binding to oxyhaemoglobin (HbO). EDHF = endothelium-dependent hyperpolarising factor; GC = guanylate cyclase; AC = adenylate cyclase. The inset shows the effect of L-NOARG and indomethacin alone and in combination on acetylcholine-induced relaxation of middle cerebral artery rings; from Dong et al.,[32] with permission.

Throughout this chapter, we have conveniently assumed that prostacyclin is the COX-generated EDRF in arterial preparations. However, unequivocal identification is lacking in most studies. Often the evidence merely consists of demonstrating that a functional IP system is present on the arterial smooth muscle as judged by its high responsiveness to prostacyclin or iloprost. In some cases, radio-immunoassay has been used to show a corresponding increase in the concentration of 6-oxo $PGF_{1\alpha}$, the hydrolysis product of prostacyclin, in the bathing fluid.[135] PGE_2 is the likely alternative to

prostacyclin, and it is pertinent that the endothelium of rabbit saphenous vein can convert exogenous arachidonic acid into PGE_2, which is then released to act on the sensitive EP_4 dilator system of the smooth muscle cells.[6] Moreover, the calcium ionophore A23187 also caused synthesis and release of PGE_2 from the venous endothelium. To discriminate prostacyclin from PGE_2, the bathing fluid can be assayed on platelet-rich plasma for anti-aggregatory activity,[136] since PGE_2 is a very weak platelet inhibitor. Exposing the artery to tranylcypromine, a low affinity inhibitor of prostacyclin synthase,[124] can also provide evidence for prostacyclin generation, provided that its contractile action is first blocked by an α-adrenoceptor antagonist such as phentolamine.[136]

5.2 Physiological role

Arteries constrict in response to raised intravascular pressure (the myogenic response) and dilate in response to increased intraluminal flow (shear stress). If correctly balanced at the organ level, these two mechanisms should provide appropriate distribution of blood flow without precipitous stealing of flow from one area to another. The flow-dependent dilatation is mediated by the endothelium[137] and involves a negative feedback role for EDRFs to maintain shear stress within normal limits.[138] The shear stress on the endothelium elevates $[Ca^{2+}]_i$ by opening divalent cation channels in the plasma membrane: a mechanotransducer system.[139,140] In addition, fluid flow induces membrane hyperpolarisation due to K^+ efflux[141-143] with different types of K^+-channels opening under pulsatile as opposed to steady pressure conditions.[144] The hyperpolarisation increases the electrochemical gradient for Ca^{2+} influx, a necessary event since endothelial cells do not have voltage-dependent Ca^{2+}-channels.[145]

Both prostacyclin and NO have been proposed as EDRFs involved in flow-dependent dilatation. In vitro studies on endothelium-intact gracilis muscle arterioles of the rat have shown that NO and prostacyclin make approximately equal contributions to the dilatation.[146] Also in rabbit femoral arteries, shear stress elicited a 11- to 12-fold increase in prostacyclin release (detected as 6-oxo $PGF_{1\alpha}$) and a 5- to 7-fold increase in NO.[147] However, in the perfused coronary circulation of the isolated rabbit heart, L-NNA completely inhibited flow-dependent dilatation, indicating no role for prostacyclin.[148] In vivo, there is also no consensus; Koller & Kaley[146] have shown that prostacyclin has a dominant role in the cremaster muscle microcirculation of the dog, whereas Holtz et al.[137] found no role for endogenous prostanoids in epicardial coronary arteries of the conscious dog.

Recent experiments conducted by Kaley and colleagues (G. Kaley, personal communication) on first-order gracilis arteries from mice with

knockout of the eNOS gene (eNOS-KO) are quite intriguing. Flow-dependent dilatation was similar in perfused vessels from wild-type and eNOS-KO, male and female mice. In wild-type, male and female vessels, NO and prostacyclin appeared to function equally as EDRFs, judging from the inhibitory effects of L-NNA and indomethacin. However, in eNOS-KO male vessels, indomethacin abolished the dilatation, showing that prostacyclin had accommodated for the loss of NO function. More surprisingly in eNOS-KO female vessels, indomethacin had minimal effect, and miconazole, a P450 mono-oxygenase inhibitor, now abolished the dilatation. Thus EDHF, and not prostacyclin, appeared to accommodate for the NO deficit in the eNOS-KO female vessel. Dilatation induced by carbacyclin was similar in all four situations, indicating that changes in sensitivity of the smooth muscle IP-receptor system were not responsible for the different EDRF profiles.

The blood pressure and heart rate of the IP-receptor-KO mouse were very similar to those of the wild-type mouse and this might indicate that prostacyclin has no role in circulatory control (at least in the mouse).[120] However, blood pressure is perhaps too crude a measure for the assessment of such an intrinsically complex mechanism as flow-dependent dilatation, which can operate at different levels of the vascular tree, and may involve compensation by one EDRF system for a deficit in another. The solution may be to directly measure flow-dependent dilatation in a IP-receptor-KO animal, preferably a species larger than the mouse.

5.3 Mechanisms of prostacyclin release

Cultured endothelial cells offer considerable advantages in the study of release mechanisms for EDRFs, including prostacyclin, and human umbilical vein and pig and cow aortae are common tissue sources. Using agonists for cell surface receptors, two main mechanisms may be distinguished. The first mechanism, which is typically elicited by ATP, bradykinin, histamine and thrombin, results in rapid EDRF generation and involves the classical G_q/phospholipase C pathway with subsequent activation of cytosolic PLA_2 (Fig. 45). In thrombin-stimulated human umbilical vein endothelial cells (HUTVEC), $[Ca^{2+}]_i$ rose to about 3 μM mainly due to internal release and then dropped to a sustained level of 1 μM after about 4 min as Ca^{2+} influx from the extracellular fluid became dominant; prostacyclin release (measured as 6-oxo $PGF_{1\alpha}$) was delayed by 10 - 20 s and reached a plateau after 4 min (Fig. 45, inset).[149] Diacylglycerol (DAG) also appears to increase prostacyclin release, this time through PKC activation, based on experiments with exogenous PKC activators such as 4β-phorbol 12-myristate 13-acetate (PMA);[150-152] the mechanism may involve

sensitisation of PLA_2 to Ca^{2+}.[41] However, PMA has also been shown to inhibit receptor- and ionophore-mediated EDRF release from intact vessels and isolated endothelial cells,[153,154] and to decrease both the peak and steady-state elevations in Ca^{2+} induced by ATP.[41] Buchan & Martin[155] also observed a reduction in $[Ca^{2+}]_i$ with PMA during the plateau phase of bradykinin, thrombin and histamine action on bovine aorta endothelial cells, and this was prevented by the PKC inhibitor staurosporine. The role of PKC is clearly complex.

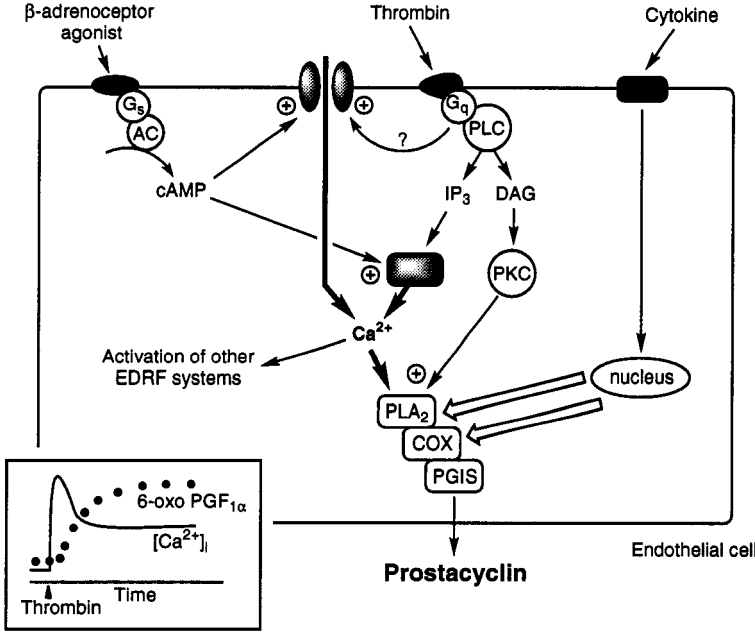

Figure 45. Mechanisms involved in prostacyclin release from endothelial cells. The major release mechanism (centre) involves activation of a cell surface receptor (e.g. thrombin receptor) to give a rapid release of Ca^{2+} from internal stores; the elevated $[Ca^{2+}]_i$ is maintained by influx from the extracellular fluid. The inset shows the correlation with prostacyclin release measured as its hydrolysis product 6-oxo $PGF_{1\alpha}$. PGIS = prostacyclin synthase. Modulating influences include: (a) diacylglycerol (DAG) activation of protein kinase C (PKC), (b) elevation of cyclic AMP levels by agonists (e.g. isoprenaline) acting on cell surface receptors, and (c) increased expression of PLA_2 and COX enzymes (unfilled arrows) by cytokine action on the cell.

Prostacyclin release through the second mechanism occurs with inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα), and is delayed by several hours due to the need for gene transcription and de novo synthesis of PLA_2 and COX-2.[156] In HUVEC, the

action of TNF, is associated with increased expression of the secretory type II PLA_2.[157]

Finally, a less common EDRF release mechanism involves the generation of cyclic AMP. For example, in bovine aorta endothelial cells isoprenaline (and forskolin) had no effect on the resting $[Ca^{2+}]_i$ or the transient Ca^{2+} release induced by thrombin, bradykinin or ATP. However internal release and influx of Ca^{2+} during the plateau phase were increased." Similar results were obtained by Graier et al.[158] in pig aorta endothelial cells, but Lückhoff et al.[159] reported that cyclic AMP inhibited endothelial Ca^{2+} mobilisation. In a few situations, prostacyclin itself may act as an endothelium-dependent vasodilator. Thus in pig coronary artery, perfusion of prostacyclin through the intact vessel caused EDRF release, which was detected as a relaxation of an endothelium-denuded ring of the same vessel.[160] The EDRF was mainly NO, since relaxations were markedly reduced by oxyhaemoglobin (an inactivator of NO, Fig. 44) and enhanced by superoxide dismutase *plus* catalase (inhibitors of NO breakdown). Schröder et al.[63] also observed greater relaxation to iloprost in rabbit isolated mesenteric artery with intact endothelium compared to a denuded preparation.

5.4 Interactions between EDRF systems

In studying the interactions between EDRF systems, it is important to appreciate that complications can arise when the vasodilator acts on both the endothelium and the circular smooth muscle (Table 12). In the case of monkey and dog mesenteric arteries[135] and dog temporal artery,[161] an agonist-induced relaxant component was still present after endothelium removal. This was abolished by indomethacin treatment, and in both dog arteries evidence was presented for the generation of prostacyclin within the smooth muscle layer. In contrast, in human and pig pulmonary,[162,163] monkey coronary[135] and rat hepatic arteries,[164] a vasoconstrictor response emerged after endothelium removal, and in the pig pulmonary and monkey coronary arteries this was due to generation of prostanoid(s), possibly TXA_2.[165]

The first situation that suggests an interaction between EDRF systems is where endothelium-dependent relaxation is independently abolished by two types of EDRF inhibitor. For example, in the dog isolated pulmonary artery, submaximal endothelium-dependent relaxation induced by bradykinin was virtually abolished by either L-NAME or indomethacin.[166] In addition, glibenclamide, a blocker of K_{ATP} channels (the target of EDHF in this preparation), partially inhibited bradykinin responses, but did not affect either the solitary NO component (indomethacin present) or the solitary

prostacyclin component (L-NAME present). It was proposed that a synergistic interaction between NO and prostacyclin occurs within the smooth muscle cell to enhance basal EDHF function (Fig. 46). NO-generated cyclic GMP inhibits cyclic AMP phosphodiesterase (PDE III); the consequent rise in cyclic AMP concentration then activates a protein kinase associated with the K_{ATP}-channel; channel opening then leads to hyperpolarisation of the plasma membrane and glibenclamide-sensitive relaxation.[167] The glibenclamide-insensitive relaxation also involves NO and prostacyclin, but they are non-interactive and opening of K_{ATP}-channels is not involved.

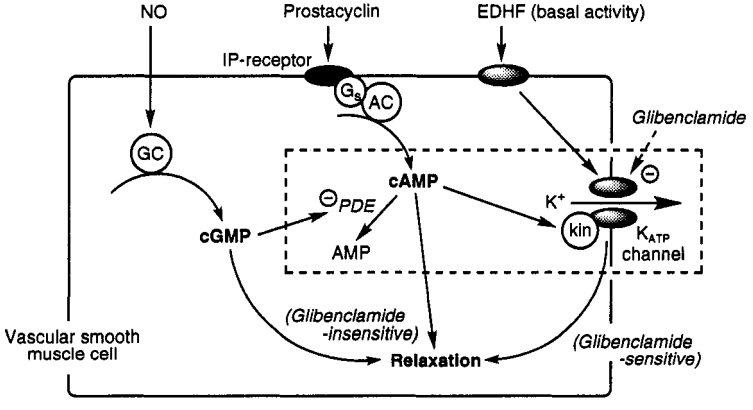

Figure 46. Potential interaction (within broken-line box) of endothelium-derived relaxing factors (EDRFs) in a vascular smooth muscle cell. The K_{ATP}-channel blocker glibenclamide inhibits relaxation induced by this interaction mechanism, but does not affect the relaxations induced by NO alone or prostacyclin alone. PDE = phosphodiesterase, kin = kinase.

In the second general situation, one type of EDRF inhibitor has no effect on the endothelium-dependent relaxation, but enhances the suppressive effect of a second type of inhibitor. For example, in the human isolated pulmonary artery, L-NOARG had no effect on the relaxant response to low concentrations of acetylcholine (10 - 300 nM), but enhanced the inhibition due to indomethacin.[162] NO has been shown to inhibit prostacyclin release from endothelial cells[168,169] and it may operate in this manner in the pulmonary artery. Inhibition of NOS by L-NOARG would remove the brake from prostacyclin synthesis and the enhanced prostacyclin release would compensate for the reduction in NO, resulting in little change in relaxation. In the recent studies of Zygmunt et al.[164] on the rat isolated hepatic artery, singly-applied L-NOARG and charybdotoxin/apamin (blockers of the target K^+-channels for EDHF) both failed to effect the relaxant response to

acetylcholine, whereas L-NOARG plus charybdotoxin/apamin shifted the dilator response curve to the right by 0.8 log units. This profile raises the possibility of reciprocal inhibition of EDRF synthesis.[169] However, exogenous NO failed to inhibit the EDHF component of acetylcholine-induced relaxation.

Table 12. Dilator mechanisms on isolated arteries

Artery	*Agonist*	*Action on endothelium*		*Action on smooth muscle*		*Ref: No.*
		Effect	*Mechanism*	*Effect*	*Mechanism*	
Human omental	SP	↓	NO and EDHF	-	-	134
Rabbit cerebral	ACh H*	↓ ↓	NO and PGI_2	-	-	32,170
Guinea-pig coronary	ACh	↓	PGI_2	-	-	171
Dog mesenteric	ACh	↓	NO and PGI_2	↓	PGI_2	135
Monkey mesenteric	ACh	↓	NO and PGI_2	↓	Prostanoid	135
Dog temporal	SP	↓	NO	↓	PGI_2	161
Human coronary	SP BK H	↓ ↓ ↓	weak NO and other EDRF	- - ↑	- - ?	172,173 174
Human pulmonary	ACh*	↓	NO and PGI_2	↑	?	162,163, 175-177
Monkey coronary	ACh	↓	NO, (PGI_2 and other EDRF?)	↓	Prostanoid	135
Pig pulmonary	ACh	↓	NO	↑	Prostanoid	163
Rat hepatic	ACh	↓	NO, PGI_2 and EDHF	↑	?	164
Human umbilical	SP	↓	PGI_2	-	-	133

Agonists: ACh = acetylcholine; BK = bradykinin; H = histamine; SP = substance P.
*Acting at H_3-receptor. **Acting at M_1 and $M_{1/3}$-receptors, respectively.
↓ = relaxation; ↑ = contraction.

In the Zygmunt et al. experiments[164] the role of prostacyclin was also investigated: indomethacin alone, indomethacin *plus* NOARG, and indomethacin *plus* charybdotoxin/apamin all had no effect on acetylcholine-induced relaxation. However, the combination of all three inhibitors abolished relaxation (and revealed a contractile response). Thus a prostacyclin component was present, which the authors emphasised could

only be reliably detected when each type of EDRF inhibitor and all their combinations were examined.

There is a gold mine of potential publications arising from the knowledge that EDRF profiles differ according to animal species, particular vessel, and even vasodilator agent examined. Whilst some of these studies will lead to important information about cellular mechanisms, they do not necessarily provide insight into the in vivo operation of EDRF systems.

6. CLINICAL ASPECTS

6.1 Role of prostacyclin in hypertension

The potential role of prostacyclin in hypertension cannot be considered in isolation since other prostanoids, particularly PGE_2 and PGH_2/TXA_2, also have potent actions on vascular smooth muscle and renal mechanisms. In addition, hypertension is a risk factor for acute myocardial infarction linked to coronary heart disease, and for progression to chronic heart failure, conditions where prostanoids may also have vital roles.

Early studies in the 1980's implicated prostanoids in human hypertension. Firstly, indomethacin had a mild hypertensive action in man associated with a rise in peripheral vascular resistance and a fall in cardiac index.[178,179] Secondly, several research groups reported decreased levels of 6-oxo $PGF_{1\alpha}$ in plasma and urine of essential hypertensive patients and strong negative correlations between 6-oxo $PGF_{1\alpha}$ levels and blood pressure.[180-138] There were also complementary increases in TXB_2 levels in plasma and urine. The conclusion was that reduction in prostacyclin levels may participate, at least in part, to the maintenance of blood pressure in patients with essential hypertension.

Around this period, several different rat models of hypertension were used to probe the involvement of prostanoids in specific hypertensive mechanisms, and we will compare these data with studies in man where possible. In dexamethasone-induced hypertension in the rat, the plasma concentration, urinary excretion, and release from blood vessel and kidney tissue of 6-oxo $PGF_{1\alpha}$ were all unchanged.[184] In contrast, the plasma concentration and urinary excretion of PGE_2 increased and were associated with reduced 15-hydroxy prostaglandin dehydrogenase (15-PGDH) levels in the kidney but not in the lung. PGE_2 production by renal medulla in vitro was also reduced. The authors concluded that deficits in circulating and renal prostacyclin and PGE_2 were not responsible for the hypertension. In contrast, Miyamori et al.[68] reported that mesenteric arteries from

dexamethasone-induced hypertensive rats produced less prostacyclin than control vessels under basal conditions and in response to angiotensin 11, and suggested that decreased prostacyclin synthesis contributed to the hypertension by allowing vasoconstrictor stimuli to exert greater effects.

On salt loading, the Dahl rat either becomes hypertensive (salt-sensitive) or remains normotensive (salt-resistant), Prostacyclin synthesis was increased in aortic rings of salt-sensitive rats on high salt intake compared to low salt intake; no change was observed in salt-resistant rats.[185] The authors concluded that enhancement of prostacyclin synthesis may be secondary to the hypertension. In contrast, PGE_2 synthesis was increased in the kidney medulla of both salt-sensitive and salt-resistant groups on salt loading. However, in the salt-sensitive group renal PGE_2 synthesis was lower and 15-PGDH activity higher. Thus, deficient renal PGE_2 levels leading to reduced sodium excretion may contribute to the development of hypertension in the salt-resistant rat. In related studies in patients with essential hypertension,186 salt loading following a low sodium diet resulted in variable increases in mean blood pressure. The percentage increases in mean blood pressure were negatively correlated with plasma PGE_2 levels and positively correlated with urinary 6-oxo $PGF_{1\alpha}$ levels. Peripheral renin activities also rose on salt loading, but these were positively correlated with plasma PGE_2 levels and not with urinary 6-oxo $PGF_{1\alpha}$ levels. The conclusions were similar to those for the Dahl rat, namely an adaptive role for prostacyclin to regulate blood pressure in salt loading and a natriuretic role for PGE_2, which may be defective in salt-sensitive hypertensives.

Most studies have been performed with the spontaneously hypertensive (SH) rat, using the Wistar-Kyoto (WKY) strain as a control. In a very recent study the role of prostacyclin in the SH rat was addressed using molecular biological techniques.[187] COX-1 mRNA and protein, PGI synthase mRNA and protein, and prostacyclin biosynthesis were higher in the thoracic aorta of the SH rat compared to the WKY rat, and showed delayed increases relative to the rises in systolic blood pressure (Fig. 47). However, at 5 weeks of age when hypertension had not yet set in, IP-receptor mRNA was already lower in the SH aorta compared to the WKY aorta and remained lower for up to 40 weeks. The authors suggested that decreased IP-receptor expression in the prehypertensive period could be one of the causes of the hypertension; increased endothelial production of TXA_2 may also be important.[188] In the chronic hypertensive state, the loss of vasodilator sensitivity may be caused by the persistently high intravascular pressure, and this has been studied in rats with a metal ring around the lower abdominal aorta (coarctation) such that the thoracic but not the abdominal aorta is subjected to high pressure. Relaxations of thoracic aorta induced by histamine (endothelium-dependent) and iloprost (endothelium-independent)

were attenuated by this procedure. In contrast, histamine-induced relaxation of the abdominal aorta was normal, as was the weak effect of iloprost. So in established hypertension in the SH rat, the increased contractility of the aorta may be the result of decreased sensitivity of the vascular smooth muscle to prostacyclin and increased endothelial and platelet production of the vasoconstrictors PGH_2 and TXA_2.[189,190] In opposition would be increased prostacyclin generation, which may be partly induced by the PGH_2/TXA_2 acting on TP-receptors on endothelial cells.[191,192] It would be of considerable interest to extend molecular biology and functional studies of this type to peripheral vessels in the SH rat, including the renal circulation.

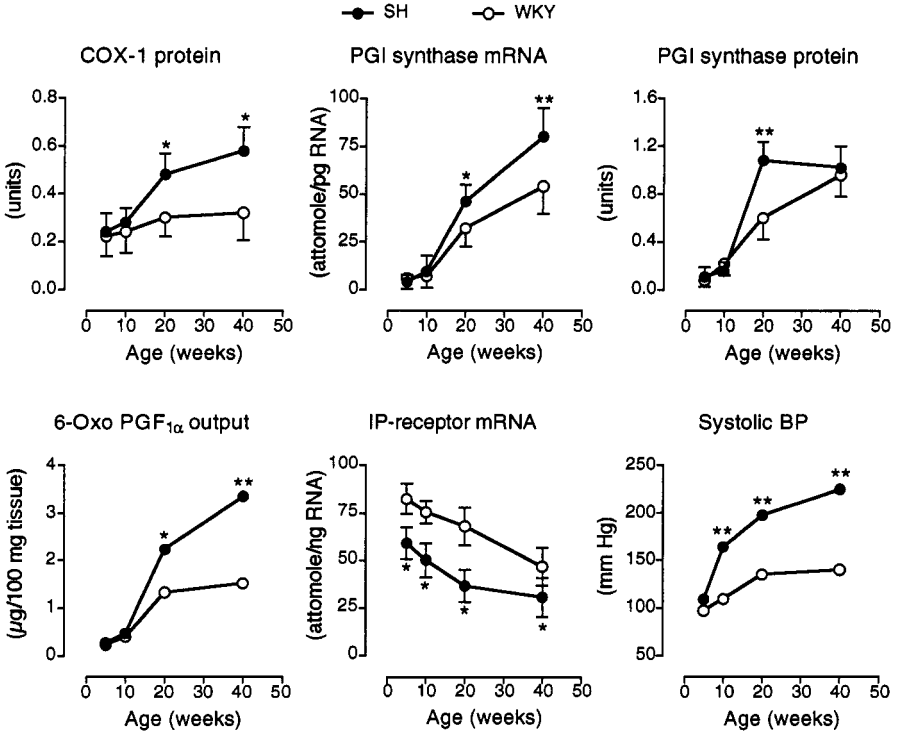

Figure 47. Comparison of the prostacyclin systems in thoracic aorta of spontaneously hypertensive (SH) and control (WKY) rats. Data from Numaguchi et al.,[187] with permission.

Indomethacin had no effect on the blood pressure of the SH rat.[193] However, the TX synthase inhibitor, 4-(imidazol-1-yl)-acetophenone suppressed the development of hypertension in the SH rat consistent with removal of the deleterious action of TXA_2 and maintenance of the beneficial action of prostacyclin.[194] In a second study, another TX synthase inhibitor

UK 38,485 lowered blood pressure in the established phase of hypertension;[195] urinary levels of 2,3-dinor-6-oxo $PGF_{1\alpha}$ were not changed by UK 38,485, indicating that shunting of PGH_2 to prostacyclin did not contribute to the antihypertensive effect. Turning to the most important element, essential hypertension in man, the combined TX synthase inhibitor and TP antagonist ridogrel reduced renal and extrarenal TXA_2 synthesis, increased renal and extrarenal prostacyclin synthesis, and suppressed platelet responsiveness to TP agonists, but failed to lower blood pressure.[196] Also, a more recent study of 133 untreated essential hypertensives by Ritter et al.,[197] has shown no significant correlation between systemic blood pressure and the urinary excretion rate of either 6-oxo $PGF_{1\alpha}$ (representing mainly renal prostacyclin production) or 2,3-dinor-6-oxo $PGF_{1\alpha}$, (extra-renal prostacyclin production). It may be that we need to return to an earlier hypothesis that PGE_2 is the more important prostanoid in essential hypertension.

6.2 Contribution of vasodilator prostanoids to antihypertensive drug action

In most studies, treatment with a COX inhibitor reduces the efficacy of clinically-used antihypertensive agents, including diuretics, angiotensin converting enzyme (ACE) inhibitors and α- and β-adrenoceptor antagonists (see Smith & Dunn[179]). These findings implicate endogenous prostanoids in the depressor actions of the drugs, but in a fair proportion of studies it is not clear whether prostacyclin or PGE_2 is the important mediator. Looking at the loop diuretic frusemide first, studies in salt-depleted dogs showed that frusemide increased renal blood flow and urinary sodium excretion; the former was abolished and the latter reduced by 50% in the presence of indomethacin.[198] In salt-loaded animals, frusemide did not increase renal flow and indomethacin did not affect frusmide-induced natriuresis. Thus the renal prostanoids released by frusemide act to decrease reabsorption of salt and water in the proximal tubule and may also inhibit sodium transport out of the thick ascending loop of Henlé. Subsequent experiments in man have shown that prostanoid-dependent increases in renal plasma flow, glomerular filtration rate and plasma renin activity due to frusemide were similar in normal subjects and patients with mild essential hypertension.[199]

Most information in this area derives from work on ACE inhibitors, particularly captopril where early studies showed that indomethacin reduced its acute hypotensive action in rabbit[200] and man.[201] ACE inhibitors prevent angiotensin II formation and also the deactivation of bradykinin by kininase. Using the superfused cat terminal ileum as a (brady)kinin detector, Pontieri et al.[202] showed that 0.15 and 10 mg kg^{-1} captopril raised arterial bradykinin levels by about 4 nM in the anaesthetised dog. Since indomethacin reversed

the blood pressure fall to captopril without affecting the response of the kinin-detecting tissue to exogenous bradykinin, it was concluded that the bradykinin induced the biosynthesis of dilator prostanoids. The three main sites at which elevated levels of bradykinin/prostanoid(s) may act are the peripheral blood vessels, the kidney, and the sensory/central nerves controlling sympathetic outflow, and each will be discussed in turn.

ACE inhibitors have endothelium-dependent relaxant actions on isolated blood vessels. For example, on rat isolated aorta, captopril, lisinopril and ramipril all stimulated prostacyclin biosynthesis, effects that were abolished by competitive block of bradykinin receptors.[203,204] However, Guivernau et al.[205] have linked the stimulation of prostacyclin synthesis to captopril's SH group, since 2-mercaptoethanol had the same effect, and both effects were inhibited by the SH-blockers N-ethylmaleimide and ethacrynic acid. They stated (but gave no data) that bradykinin was not involved, since bradykinin could not be detected by radio-immunoassay in supernatants of aortic rings. Goldschmidt & Tallarida[206] have also implicated the SH group, but this time as a superoxide (O_2^-)-scavenger protecting EDRF (NO). They used rabbit aortic rings and showed that captopril-induced endothelium-dependent relaxation was not affected by indomethacin, and the non-SH ACE inhibitor enalaprilat had no relaxant effect. However, very high concentrations of captopril (100 - 600 μM) were used in this study. On dog femoral artery rings, the relaxant actions of captopril and M-1 (the active metabolite of the ACE inhibitor delapril and a non-SH agent) were partially blocked by aspirin and abolished by the NOS inhibitor L-NMMA.[207] Relaxation was only partially inhibited by a bradykinin receptor antagonist. In a more physiological experiment, anaesthetised rats were dosed with either bradykinin antagonist or vehicle followed by enalaprilat; aortae were then removed and incubated in drug-free buffer.[208] Aortic prostacyclin synthesis was elevated by enalaprit, but not when the bradykinin antagonist was also administered. Enalaprilat added directly to aortic rings did not affect prostacyclin production. Clearly, there is some disagreement as to the role of prostacyclin in the vasodilator action of ACE inhibitors, some of which may be due to genuine differences between species.

In the conscious dog, captopril produces a shortlasting renal vasodilatation (< 2 h), which corresponds in time to small increases in urinary 6-oxo $PGF_{1\alpha}$ levels.[209] However, sodium excretion is still rising at this time and it appears unlikely that prostacyclin is responsible for this effect. The rate urniary excretion of 6-oxo $PGF_{1\alpha}$ after captopril was much less than the rates obtained during intravenous prostacyclin infusion, implying that an increase in systemic prostacyclin synthesis is unlikely to contribute significantly to the hypotensive action of captopril.

In other studies in conscious dogs, captopril decreased renal sympathetic nerve activity, but increased this parameter after indomethacin administration.[210] As expected, sodium nitroprusside, at doses causing a similar fall in blood pressure to captopril, increased renal nerve activity. The authors suggest that captopril, either directly or through bradykinin generation, induces prostacyclin biosynthesis close to cardiac or lung sensory afferents (see Section 4.3) and this results in reduction of sympathetic outflow to the kidney; this would be beneficial in terms of lowering blood pressure.

Turning to recent studies involving longer term antihypertensive treatment in man, the evidence is against an important role for prostacyclin. Gerber et al.[211] showed that neither captopril nor enalapril (twice daily for two weeks) stimulated prostacyclin production as judged by urinary 2,3-dinor-6-oxo $PGF_{1\alpha}$ levels, and their hypotensive actions were not inhibited by indomethacin. In a more comprehensive study, Ritter et al.[197] treated newly-diagnosed patients for one year with one of four antihypertensive drugs, the ACE inhibitor quinapril, the diuretic bendrofluazide, the β-blocker metoprolol, and the Ca^{2+}-channel blocker amlodipine. Renal and extrarenal prostacylin production were no different before and after treatment with any of the drugs.

6.3 Atherosclerosis

Atheromatous changes in blood vessels are the starting point for a range of pathological cardiovascular conditions and in view of the ability of the blood vessel endothelium to synthesise prostacyclin, it has been natural to investigate its role in these conditions. The initial events in atherosclerosis involve (a) migration of monocytes into the intima due to changes in endothelial function, but not necessarily loss of the endothelial layer,[212] (b) concentration of cholesterol ester (CE) in macrophages with formation of fatty streaks, and (c) proliferation of smooth muscle cells.

Prostacyclin generation by vessels or cultured smooth muscle cells from cholesterol-fed rabbits and from patients with atherosclerosis is reduced compared to controls.[213-216] In addition, studies on cultured human and bovine aorta endothelial cells show that low-density lipoprotein (LDL) and its oxidised product, which are risk factors for ischaemic vascular disease, inhibit prostacyclin biosynthesis.[217,218] The natural anti-oxidants vitamin E and selenium, which are thought to afford protection against oxidative attack on the blood vessel wall, enhance prostacyclin synthesis by endothelial cells,[218-221] and this has been linked to activation of PLA_2.[222] The crucial link between these observations may be the ability of atherosclerotic vessels to synthesise increased amounts of lipoxygenase products, such as 15-

hydroperoxy-eicosatetraenoic acid (15-HPETE), which are inhibitory to PGI synthase.[223] Mathur et al.[224] have also suggested that depressed phospholipase activity accounts for low prostaglandin output from cholesterol-rich macrophages. (See Chapter 8, Section 3.6 for further information on prostacyclin and macrophages in atherosclerosis.)

Apart from its vasodilator and platelet inhibitory actions, what benefit could prostacyclin bring to the atherosclerotic situation? Firstly, prostacyclin could inhibit CE accumulation through its ability to enhance CE hydrolase activity. In rabbit aorta smooth muscle cells in culture, the EC_{50} for this effect was about 50 nM and PGE_2 was inactive.[225] CE hydrolase activity was increased by membrane-permeant dibutyryl cyclic AMP and inhibited by the adenylate cyclase inhibitor dideoxy-adenosine, Cyclic AMP levels were correspondingly raised by prostacyclin, strongly suggesting that the IP-receptor couples to adenylate cyclase. In cells cultured from atherosclerotic lesions of human aorta, carbacyclin at 2.8 μM decreased CE levels by about 40% over 6 days (IC_{50} ~ 200 nM); 6β-PGI_1, a weak IP agonist, was a less potent inhibitor of CE levels.[226]

Secondly, prostacyclin may inhibit proliferation of smooth muscle cells in the intima, a process thought to be initiated by mitogenic substances, such as platelet-derived growth factor, thrombin and oxidised arachidonate products, released from damaged endothelial cells, leukocytes and platelets. Prostacyclin and its stable analogue RS-93427 (see Chapter 2) inhibit this mitogen release,[227,228] and this correlates with inhibition of DNA synthesis in rabbit aorta smooth muscle cells,[229] and inhibition of [^{3}H]-thymidine incorporation into cultured human atheroma cells.[226]

The picture painted above may not fit all clinical circumstances and FitzGerald et al.[230] have demonstrated higher urinary output of the prostacyclin metabolite 2,3-dinor-6-oxo $PGF_{1\alpha}$ in severe atherosclerotic patients who had evidence of platelet activation. They have proposed that the rise in prostacyclin production may be the result of platelet interactions with the endothelium.

There are several treatment modes for atherosclerosis that may involve a prostacyclin component. For example, the well-known protective effect of oestrogen replacement therapy against cardiovascular disease in postmenopausal women may be partly due to stimulation of prostacyclin synthesis in endothelial cells, and this area has been recently reviewed by Mikkola et al.[231] Low-dose aspirin therapy has also been tried in man with the intention of inhibiting TXA_2 biosynthesis without affecting prostacyclin biosynthesis.[232] However, it appeared that the selectivity obtainable in young healthy men was not achievable in older atherosclerotic men and their age-matched controls, with significantly greater inhibition of prostacyclin production occurring in the latter two groups. A more direct approach would

be to give a prostacyclin analogue chronically, and studies in animal models of atherosclerosis, particularly in the rabbit, support the strategy. Iloprost showed a beneficial effect in atherosclerosis induced with soyabean oil extract.[233] Beraprost improved serum lipid profiles, reduced serum concentrations of TXB_2 and 6-oxo $PGF_{1\alpha}$ and the TXB_2/6-oxo $PGF_{1\alpha}$ ratio, and reduced stenosis of an aortic anastomosis and intimal thickening along the suture line.[234] Also, oral administration of the selective IP agonist cicaprost at a subhypotensive dose (5 $\mu g\ kg^{-1}\ day^{-1}$) reduced the extent of atheromatous lesions, improved endothelial-dependent relaxations to acetylcholine and substance P, and reduced platelet and leukocyte hyper-reactivity.[235] Of particular interest is octimibate, a nonprostanoid developed in an anti-atherosclerosis programme directed at inhibition of acyl-CoA:cholesterol acyltransferase (ACAT),[236] which was subsequently shown to be an IP agonist (see Chapter 3). Octimibate is a weak ACAT inhibitor[237] and a weak agonist at rabbit IP-receptors[238] (see Chapter 3), but the relative contributions of these two mechanisms to the reduction in serum cholesterol levels and aortic plaque formation seen in the rabbit is not clear.[239] When tested in patients with primary hypercholesterolaemia, octimibate (~4 mg $kg^{-1}\ day^{-1}$ for 10 days) improved the serum lipid profile, but adverse symptoms of indigestion, dizziness and muscular ache were common.[240]

An exciting new approach to atherosclerosis treatment involves localised transfer of the PGI synthase gene to at-risk blood vessels in vivo with the intention of enhancing prostacyclin biosynthesis. In one study, human PGI synthase gene was transferred to rat carotid arteries which had been damaged by balloon inflation.[241] This procedure resulted in the increased 6-oxo $PGF_{1\alpha}$ levels in the carotid arteries (3 days), a strong human PGI synthase immunoreactivity signal in neointimal cells (7 days), and reduced neointima formation (14 days). A beneficial effect was also seen in a similar model involving transfer of rat PGI synthase gene;[242] neointima formation was reduced and endothelium regeneration was enhanced at 14 days post-injury.

6.4 Peripheral vascular disease

Longterm treatment of patients with an IP agonist for the purpose of generally arresting the development of atherosclerotic lesions has not been attempted and clearly there are major problems of formulation and side-effects to be considered. However, there are a number of studies where IP agonists have been used to treat specific types of peripheral vascular disease.

Intermittent claudication, in which exercise brings on pain in the legs forcing the patient to rest, has been treated with either iloprost or beraprost. Following a typical iloprost intravenous infusion protocol (2 $ng\ kg^{-1}\ ml^{-1}$ for 6 - 8 h per day for 3 - 14 days), treadmill walking time was improved for one

to two months post-therapy, whereas the inhibition of platelet aggregability did not outlast the infusion period. Iloprost was also beneficial in patients with more severe forms of leg ischaemia, where complete vessel occlusion occurs, ulcers develop and amputation is often necessary (see Grant & Goa[243] for a review). Beraprost was of benefit in both intermittant claudication[244,245] and diabetic angiopathy.[246]

PGE_1 has been used for some time in intermittent claudication with variable sucess. [247-249] Improved therepy has been reported with an aclated-esterified prodrug of PGE_1 (AS-013) incorporated into lipid microspheres, which give a more selective delivery of PGE_1 to the vessel wall.[248] From a mechanistic standpoint, the question that continues to arise with PGE_1 is whether its major effects are exerted via EP- or IP-recepotrs (see Section 2.2). In this context, the PGE_1 analogue misoprostol, which has potent EP_2 and EP_3 agonist activity but little IP agonist potency, has recently been shown to be beneficial in intermittent claudication.[250]

6.5 Pulmonary hypertension

Primary pulmonary hypertension (primary PH) is a serious condition characterised by increased pulmonary artery pressure and pulmonary vascular resistance and consequent excessive load on the right heart. Vasodilator therapy appears warranted, but reactivity to dilators declines as medial hypertrophy of vessels progresses to fibrosis of the intima.

The stimulus for using prostacyclin in primary PH in man derives from early observations of its potent pulmonary dilator action in laboratory animals. In the cat, prostacyclin decreased perfusion pressure when injected into a lobar pulmonary artery and was about 15 times more potent than PGE_1, consistent with activation of IP-receptors.[251] In addition, prostacyclin inhibited the vasoconstriction and platelet aggregation induced by intralobar infusion of ADP. Prostacyclin also reduced pulmonary vascular resistance in the neonatal sheep.[252]

One of the first uses of prostacyclin in human primary PH was the treatment of an 8-year-old girl with severe idiopathic primary PH.[253] Her plasma levels of 6-oxo $PGF_{1\alpha}$ and TXB_2 before treatment were 400 (normal <50) and 850 (normal 65) pg ml^{-1} respectively. During treatment with prostacyclin at 44 ng kg^{-1} min^{-1}, the circulating 6-oxo $PGF_{1\alpha}$ level rose to 2200 pg ml^{-1} while the TXB_2 level fell to 170 pg ml^{-1}, probably indicating a reduction in platelet activation. Prostacyclin produced greater pulmonary vasodilatation than PGE_1, isoprenaline or tolazoline (α-adrenoceptor antagonist), without reducing systemic vascular resistance. Subsequently, continuous intravenous infusion of prostacyclin was used as a stop-gap until lung/heart-lung transplantation became feasible.[254] However, prostacyclin

has progressively become more important in longterm therapy of primary PH,[255-257] and survival rates are similar to those for single lung transplantation; see reviews by Kneussl et al.[258] and Barst.[259]

The acute response to a pulmonary vasodilator is usually determined as a guide to longterm therapy. However, prostacyclin differs from other dilators (e.g. calcium channel blockers) in that longterm administration can lead to considerable haemodynamic improvement despite a poor acute response. In PH associated with acute respiratory failure, there was evidence of increased stroke volume due to decreased afterload during prostacyclin therapy.[260] An important aspect of prostacyclin therapy is its ability to improve the structure and function of the right ventricle.[261]

One issue that has arisen is the relative merits of NO versus prostacyclin in the therapy of primary PH. Test inhalation of NO appears to have the edge on prostacyclin infusion in that it is less expensive, easier to administer and has a shorter time to peak effect.[262] NO poses difficulties in terms of its mode of administration over long periods and its safety is not yet known. Prostacyclin on the other hand is acceptable to patients in the longterm when delivered by a lightweight portable infusion pump into a central catheter inserted into a subclavian or jugular vein.[254,263,264] The moderate side effects listed in Table 11 are common and are easily dealt with by altering the dosage rate.

Whether the chemical instability of prostacyclin contributes to a selective action on the pulmonary circulation is not clear. After all, the loss of activity during transit from pulmonary to systemic arterioles must be quite small. In relation to this, the stable prostacyclin analogue beraprost given by mouth showed a good therapeutic profile in patients with either primary or secondary PH in the shortterm.[265] Clearly, there is scope for experimentation with other prostacyclin analogues in man to see if any are improvements on prostacyclin.

6.6 Congestive heart failure

The 1980's saw the successful shortterm use of prostacyclin in patients with severe heart failure (New York Heart Association class III or IV); the commonest use is in pulmonary hypertensive crisis after cardiac transplantation and after cardiothoracic surgery on infants (see Haywood et al.[266]). The rationale for therapy involves reductions in the afterload of both the right and left ventricles, possibly accompanied by suppression of platelet reactivity.[266-268] Prostacyclin administered by an indwelling central venous catheter reduced right and left atrial filling pressures, increased cardiac index, and reduced pulmonary and systemic vascular resistances with little change in their ratio.[269-211]

The 1990's saw prostacyclin being used for longterm treatment of severe heart failure, a much more difficult proposition since the death rate often exceeds 50% even with the best available therapy with diuretics, vasodilators and positive inotropic agents. A number of drugs, such as dobutamine, amrinone and milrinone, have all showed promising profiles in small-scale studies, but increased mortality and poor side-effect profiles have emerged in larger randomised clinical trials, leading to their abandonment (see Haywood et al.[266]). Sad to say, prostacyclin has followed a similar path. Early studies indicated significant benefits,[272] but the Flolan International Randomized Survival Trial (FIRST) was cut short because prostacyclin therapy did not improve walking distance or quality of life and was associated with an increased risk of death.[273] Montalescot et al.[274] have suggested that the mortality is due to a positive inotropic effect of prostacyclin.

6.7 Lupus nephritis

Proliferative glomerulonephritis (lupus nephritis) is an often fatal progression of systemic lupus erythematosus (SLE). Glomerular thrombi occur frequently in active lupus nephritis and their presence is associated with a reduced ability of plasma to generate prostacyclin.[275] Patrono et al.[276] showed that urinary excretion of TXB_2 as increased, while 6-oxo $PGF_{1\alpha}$ excretion was decreased. This group went on to show that renal function was improved following treatment with the TP antagonist BM 13177.[277] Subsequently it was shown that the TX synthase inhibitor DP-1904 reduced urinary TXB_2 levels without affecting 6-oxo $PGF_{1\alpha}$ levels and this was associated with an improved creatinine clearance.[278] Neither low-dose aspirin nor indomethacin improved kidney function. The overall conclusion was that TXA_2 generated within renal tissue (but not necessarily from platelets) may be responsible for some of the deterioration in renal haemodynamics and also that it is important to maintain prostacyclin production. We await further developments in this area.

7. CONCLUDING REMARKS

The 1980's saw an explosion of interest in the vascular actions of prostacyclin. Now that the pace has slowed, we can see that it does have important roles in normal and aberrant cardiovascular function. In addition, prostacyclin and its analogues have specific uses in the treatment of vascular disease. In some cases, their limited efficacy may be because we are attempting to salvage the unsalvageable. For the future, genetic manipulation of the PGI synthase/IP-receptor system offers the hope of both

more precise knowledge of pathophysiological function and improved therapy.

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Chapter 7

IP-receptors on neutrophils

1. INTRODUCTION

Neutrophils (polymorphonuclear leukocytes) are defence cells that are recruited from the blood into inflamed tissues or sites of inflammation by chemotactic agents. Once recruited, neutrophils phagocytose and usually kill bacteria through O_2-derived radicals and proteolytic enzymes; unfortunately these latter factors are also toxic to the host and therefore neutrophils need to be under tight control to prevent damage to the host itself. However patients who have a complete deficiency or extremely low levels of the neutrophil adhesion molecule CD18 also have severe defects in their inflammatory responses due to the lack of appropriate neutrophil responses.[1] The clinical features include soft tissue lesions, impaired pus formation, defective wound healing, and increased morbidity and mortality from bacterial infections. Thus while neutrophils are an essential component of our defense systems, we shall see later (Section 3.2) that they are also a dangerous component of ischaemia-reperfision injury.

As prostaglandins can inhibit a wide range of neutrophil functions when activated by a range of chemotactic stimuli, it is possible that they represent an endogenous mechanism designed to modulate neutrophil activity under physiological and pathological conditions.[2-5] There are several types of chemotactic molecules with different origins: *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) is a product of bacteria; the complement fragment C5a is formed in blood plasma and inflammatory exudates upon complement activation; opsonised zymosan (OZ) is a form of immune complex; platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) derive from membrane

phospholipids and are released by activated neutrophils; and interleukin-8 (IL-8) is produced by monocytes. A more detailed explanation of neutrophil biology and their role in inflammation and disease can be found in Hellewell & Williams.[6]

2. EFFECT OF IP AGONISTS ON NEUTROPHIL FUNCTION

Unlike most other cell types involved in inflammation, neutrophils are reported to be poor producers of prostaglandins,[7,8] having rather low cyclo-oxygenase (COX) activity relative to lipoxygenase (LOX),[8,9] and synthesising relatively low amounts of prostacyclin and PGE_2.[10,11] Therefore although prostacyclin and its mimetics are clearly able to regulate a variety of neutrophil functions, prostacyclin itself does not appear to play an autocrine role in these cells. Instead, it seems that endothelial cells may provide this source of prostacyclin, since cultured endothelial cells can synthesise prostacyclin in the presence of FMLP-activated human and rat' peritoneal neutrophils.[12] Although there is plenty of evidence for an involvement of IP-receptors in regulating rat peritoneal neutrophil function, there have been no studies characterising IP-receptors by radioligand binding experiments. Indeed there is only one report of [^{3}H]-PGD_2 and [^{3}H]-PGE_2 binding with high affinity to human neutrophils.[13]

In an attempt to simplify experiments needing large numbers of neutrophils, many researchers have turned to using the human promyelocytic leukaemic cell line HL-60, which when differentiated with dimethylsulphoxide, displays many aspects of neutrophil activity.[14,15] However, we have shown that PGE_1, PGE_2, and to a lesser extent cicaprost and iloprost, can cause an unexpected aggregation response in neutrophil-like HL-60 cells.[16] Furthermore, this PGE_2-induced aggregation response is not mediated by elevations in cyclic AMP or changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and may involve a novel intracellular signalling pathway for EP_2 (and IP)-receptors.[17] Now in contrast to our data, others have shown that PGE_2 can increase $[Ca^{2+}]_i$ in neutrophil-like HL-60 cells, but again this effect is not mimicked by forskolin or cell-permeable forms of cyclic AMP.[18] Because of this lack of consistency in data derived from HL-60 cells, we have tended to concentrate on discussing data from neutrophils wherever possible. The reader should note that studies of IP-receptors in human neutrophils utilise cells taken directly from venous blood, whereas neutrophils from rats, rabbits and guinea-pigs come from the peritoneal cavity following injection of an inflammatory agent. Therefore, what might

at first appear to be a species-dependent property of IP-receptors, may actually be due to the differences between resting and elicited cells.

2.1 Adenylate cyclase

An early, but inconsistent,[19] event in human neutrophil activation is a transient (5 - 15 s) elevation in cyclic AMP which is thought to act as a negative regulator of neutrophil function.[20-23] In the presence of a phosphodiesterase (PDE) inhibitor, nonactivated human neutrophils consistently respond to 10 μM PGE_1 and PGE_2 with a 3 - 4-fold increase in cyclic AMP production,[24,25] whereas variable results have been obtained with IP agonists. Thus, prostacyclin and iloprost were inactive at concentrations <10 μ M, but iloprost at 10 μM produced a 2-fold increase in cyclic AMP.[24] Yet in another study, prostacyclin appeared much more potent producing a 3-fold increase in cyclic AMP at approximately 300 nM, even in the absence of a PDE inhibitor.[26] In a comparative study of IP agonists related to taprostene, it was clear that while these compounds produced only minor increases in cyclic AMP alone, they dramatically potentiated the cyclic AMP response to FMLP with maximal effects observed at 10 μM.[27] In yet another study, iloprost and carbacyclin were also inactive at 10 μM, but cicaprost (10 μM) produced a significant 4-fold increase in cyclic AMP, being equipotent with PGE_2 and the DP agonist ZK 110841.[25] Because of this discrepancy between the adenylate cyclase response of human neutrophils to three IP agonists, Armstrong & Talpain[25] questioned the selectivity of cicaprost, especially since there was no comparative data on the effect of cicaprost in other assays of human neutrophil function. They also noted that in neutrophil-like HL-60 cells, cicaprost was much weaker than PGE_2, achieving a maximum increase in cyclic AMP which was merely 15% of the PGE_2 response.[25] The greater response of human neutrophils to PGE1 and PGE_2 compared with IP agonists has also been seen in the presence of FMLP[27] and PAF,[28] yet in the presence of PAF, much smaller increases in cyclic AMP production were found.[28] These results contrast with those of Sedgwick et al.[29] who found that the adenylate cyclase response of human neutrophils to stimulation by PGE_1 was similar in the presence of FMLP, OZ or phorbol myristate acetate (PMA). No cross-desensitisation was seen between PGE_1/PGE_2 and iloprost-mediated increases in cyclic AMP, suggesting that PGE_1 and PGE_2 act here on EP-receptors, whereas iloprost and prostacyclin act on IP-receptors.[28] All in all, one can conclude that measurements of cyclic AMP production by human neutrophils in response to IP agonists, in the absence or presence of neutrophil activators, leads to highly inconsistent results, not least because of the inate variablity of neutrophils isolated from human subjects.[27]

In the presence of FMLP, rat peritoneal neutrophils produced a significant increase in cyclic AMP in response to beraprost, prostacyclin and PGE_2 (all at 1 μM) with increases over basal levels of 70, 84 and 59%, respectively.[30] Although Ham et al.[31] noted a 20% increase in cyclic AMP levels in response to FMLP, our own studies have shown that activation of rat peritoneal neutrophils with FMLP produces no consistent effect on cyclic AMP production, and that FMLP does not affect the adenylate cyclase response of neutrophils to prostaglandins.[32] In rat peritoneal neutrophils we found that 1 μM cicaprost, iloprost and PGE_2 stimulated adenylate cyclase activity with an increase over basal levels of 262, 270 and 254%, respectively.[32,33] Cicaprost and iloprost have relatively high potency, comparable with PGE_2, with EC_{50} values of 20 nM and 44 nM, respectively, and appear to act as full agonists for stimulating adenylate cyclase in rat peritoneal neutrophils. The related nonprostanoid prostacyclin mimetics BMY 42393 and BMY 45778 gave responses typical of partial agonists, failing to produce the same maximal level of adenylate cyclase activation as seen with cicaprost and iloprost; the related nonprostanoid octimibate produced no statistically significant effect even at 10 μM;[32] see Figure 48.

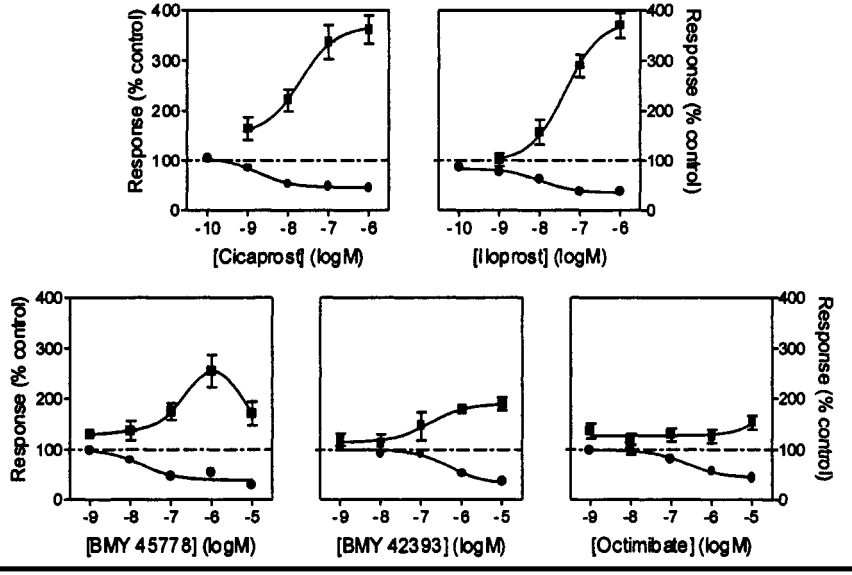

Figure 48. The effects of IP agonists on rat peritoneal neutrophil activity. Stimulation of [^{3}H]-cyclic AMP production (■). Inhibition of FMLP-stimulated aggregation (●). Reprinted from Wise,[34] with permission.

It is conceivable that the different responsiveness of human and rat peritoneal neutrophils to IP agonists may be because the human neutrophils

are nonelicited cells, taken directly from the blood of the donor. Elicited mouse, rat and human macrophages all gain in their adenylate cyclase response to prostacyclin (Chapter 8, Table 16), therefore it is possible that human blood neutrophils are more similar to resident macrophages.

2.2 Calcium fluxes and phospholipid turnover

FMLP, LTB_4 and PAF all produce different patterns of time-dependent changes in IP_3 production, and release different amounts of Ca^{2+}, which may be responsible for the different repertoire of responses to these chemotactic factors.[35,36] Changes in $[Ca^{2+}]_i$ were thought to play a central role in the activation process since $[Ca^{2+}]_i$ was consistently elevated in stimulated cells and some biological responses could be mimicked by treating the cells with calcium ionophores such as A23187. However some responses persist under conditions where $[Ca^{2+}]_i$ remains at low or below resting levels.[37] Therefore attempts to relate IP agonist-mediated changes in $[Ca^{2+}]_i$ to changes in functional responses are complicated by the particular Ca^{2+}-dependency of the process being studied.

Although both PGE_1 and PGE_2 can inhibit FMLP and PAF-stimulated Ca^{2+} influx in human neutrophils, iloprost was inactive even at 10 μM.[28] We have also shown that PGE_2 (1 μM) significantly inhibited FMLP-stimulated increases in $[Ca^{2+}]_i$ in human neutrophils[16] but cicaprost, octimibate, BMY 42393 and BMY 45778 were inactive (unpublished observations). Beraprost (100 μM) produced about 50% inhibition of FMLP-stimulated $[Ca^{2+}]_i$ in human neutrophils, but this is still rather a weak effect?[38] Neutrophil-like HL-60 cells behaved here in a similar fashion to human neutrophils, with PGE_1 and PGE_2 inhibiting FMLP-stimulated increases in $[Ca^{2+}]_i$ with IC_{50} values of 140 nM and 30 nM, respectively, and cicaprost (1 μM) being inactive.[16] In contrast, IP agonists were active in rat peritoneal neutrophils[32] and inhibited FMLP-stimulated increases in $[Ca^{2+}]_i$ by decreasing the influx of extracellular Ca^{2+}.[30] PGE_2 also inhibits the influx of extracellular Ca^{2+} in response to FMLP in rat and guinea-pig peritoneal neutrophils.[39,40]

Cicaprost and iloprost showed similar potency for inhibiting FMLP-stimulated increases in $[Ca^{2+}]_i$ and for stimulating adenylate cyclase in rat peritoneal neutrophils (Table 13). Unfortunately, high concentrations of both octimibate and BMY 45778 fluoresce at the wavelength used to detect Ca^{2+} binding to Fura-2, and thus their potency in inhibiting FMLP-stimulated increases in $[Ca^{2+}]_i$ cannot be readily measured.[32] It is clear however that cicaprost and iloprost have no effect alone on rat peritoneal neutrophil $[Ca^{2+}]_i$, which suggests that unlike recombinant IP_1-receptors expressed in CHO cells (Chapter 4), IP-receptors in rat peritoneal neutrophils are unable to couple to G_q-proteins.

Table 13. Summary data for IP agonists in rat peritoneal neutrophils

Drug	*Cyclic AMP production* (EC_{50} nM)	*Inhibition of FMLP-stimulated aggregation* (IC_{50} nM)	*Inhibition of FMLP-stimulated increase in* $[Ca^{2+}]_i$ (IC_{50} nM)	
Cicaprost	20	2	24	
Iloprost	44	5	75	
BMY 45778	245	20	>1,000	*
BMY 42393	160	462	18,000	*
Octimibate	>10,000	286	>1,000	

Data from Wise.[32] *Estimate only as data limited by autofluorescence of nonprostanoid.

In rat peritoneal neutrophils, beraprost, prostacyclin and PGE_2 also inhibited FMLP-stimulated inositol phospholipid turnover, with IC_{50} values of approximately 1 μM.[30] However, it is unlikely that this reflects a change in initial IP_3 production in response to FMLP, because as noted above, IP agonists affected the influx of extracellular Ca^{2+} rather than the release of Ca^{2+} from intracellular stores. Kainoh et al.[30] therefore concluded that IP agonists modify inositol phospholipid turnover by an indirect route.

2.3 Chemotaxis and adhesion

Neutrophil adhesion in response to chemotactic factors is characterised by the expression of the β_2 integrins CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1) on the surface of the neutrophil; the latter adhesion molecule being most important for FMLP-stimulated human neutrophils.[41] Monoclonal antibodies directed against CD11b or CD18 decrease rat peritoneal neutrophil adherence to and emigration from rat mesenteric venules.[42] Cyclic AMP modulators are thought to affect neutrophil rather than endothelial cell function; thus, they inhibit FMLP-mediated adhesion of human neutrophils to endothelial cells by decreasing the expression of Mac-1 on neutrophils.[41] In contrast, iloprost mediated inhibition of human neutrophil adhesion to endothelial cells was apparently independent of Mac-1.[43]

Human neutrophils extrude about 50% of their cyclic AMP, but there are conflicting reports concerning the chemotactic nature of cyclic AMP.[44,45] Prostanoids themselves are similarly not chemotactic for human neutrophils but can inhibit FMLP-stimulated chemotaxis.[46,47] This statement requires slight clarification though since it has been reported that EP_3 agonists can stimulate human neutrophil chemotaxis at low concentrations.[48] Presumably the typical concentrations of PGE_2 used in chemotaxis experiments is high enough for the more dominant EP_2/EP_4-receptor-mediated inhibitory effect to be measurable.

In rat peritoneal neutrophils, both beraprost and prostacyclin inhibited FMLP-stimulated chemotaxis with IC_{50} values of 30 nM; similar to the effect of PGE_2.[30] Although it is rare to find a functional response of human neutrophils modified by IP agonists, prostacyclin has been reported to suppress the adherence of nonactivated human neutrophils to endothelial cells.[26] Surprisingly, prostacyclin inhibited human neutrophil adhesion to endothelial cells at concentrations (approximately 300 nM) which had no effect on OZ-stimulated respiratory burst or phagocytic activity, suggesting that prostacyclin might play a role in regulating neutrophil adhesiveness to endothelial cells without compromising host defence.[26]

In a model of inflammatory skin lesions, prostacyclin (50 ng kg^{-1} min^{-1}, i.v.) significantly inhibited neutrophil migration in response to intradermal injection of zymosan-activated plasma in dogs.[5] In contrast, intradermal iloprost significantly enhanced accumulation of neutrophils induced by zymosan-activated plasma (ZAP), but not by the passive cutaneous anaphylactic (PCA) reaction, in guinea-pig skin.[49] In similar models of inflammation in rabbit skin, prostaglandins are both pro-inflammatory and anti-inflammatory depending on the route of administration; thus, when given locally, prostaglandins are pro-inflammatory, and when given systemically, prostaglandins are anti-inflammatory.[49]

2.4 Aggregation

Neutrophils aggregate in response to activators such as FMLP, LTB_4 and PAF, and this homotypic aggregation is thought to be a prerequisite for the successfull emigration of fast-moving neutrophils from the blood stream into the tissue space.[50,51] Neutrophil aggregation provides a mechanism for increasing the density of neutrophils near the site of inflammation/infection by sequestration of neutrophil aggregates in the microvasculature.[52] Rat peritoneal neutrophil aggregation can be inhibited by anti-Mac-1, but not by anti-LFA-1 monoclonal antibodies.[53] Recent evidence indicates though that while neutrophils isolated from the bone marrow of mice deficient in Mac-1 are defective in homotypic aggregation, these mice have a normal inflammatory response when challenged by thioglycollate intraperitoneally; indeed neutrophil emigration into the peritoneal cavity is inhibited far more when the mice are treated with monoclonal antibodies against LFA-1.[1]

FMLP causes human neutrophil aggregation and this is a direct effect, not the consequence of LTB_4 biosynthesis.[54] The FMLP-stimulated aggregation of human neutrophils can be inhibited by PGE_1 and PGE_2, but not by $PGF_{2\alpha}$.[55] A similar profile is seen with rat peritoneal neutrophils, but in addition, IP agonists are potent inhibitors of aggregation (Table 14) while PGD_2 and U-46619 are inactive.[32,33] With the as yet unexplained exception

of BMY 42393, the potency of cicaprost, iloprost, octimibate and BMY 45778 for inhibition of rat peritoneal neutrophil aggregation is independent of the neutrophil activator used (Table 14). Although the octimibate-related prostacyclin mimetics behave as partial agonists for stimulation of adenylate cyclase activity (Section 2.1), they all produced the same maximal inhibition of FMLP-stimulated rat peritoneal neutrophil aggregation;[32] see Figure 48. The question of whether or not cyclic AMP mediates the functional effects of IP agonists on neutrophils will be discussed later in Section 2.8.

Table 14. Comparison of the inhibitory effect of IP agonists against different neutrophil activators

Drug	*Inhibition of FMLP-stimulated aggregation (IC_{50}, nM)*	*Inhibition of PAF-stimulated aggregation (IC_{50} nM)*
Cicaprost	2.1	1.3
Iloprost	4.5	nd
BMY 45778	20	18
BMY 42393	462	88
Octimibate	286	184

Data from Wise[32] and Wise & Chow.[56] nd = not determined.

2.5 Respiratory burst

Neutrophils exhibit a respiratory burst, characterised by an increase in O_2 consumption, activation of the hexose monophosphate shunt and production of reactive O_2-derived free radicals. When a variety of reactive O_2 species (ROS) were measured, it was shown that PGE_1 could significantly decrease superoxide anion, H_2O_2 and $OH^{\cdot}$ production by OZ-stimulated human neutrophils, but PGE_1 did not affect the levels of ROS generated by a cell-free ROS generating system.[57] Similarly, PGE_2, prostacyclin and iloprost were reported to have no scavenging properties in a cell-free system.[28] Therefore the inhibitory effects of PGE_2 and IP agonists result from direct cellular actions.

PGE_1, PGE_2 and PGD_2 inhibited FMLP- and OZ-stimulated respiratory burst by human neutrophils with IC_{50} values ranging from 80 - 800 nM, but beraprost, cicaprost, iloprost, prostacyclin and 6-oxo $PGF_{1\alpha}$ were much less potent (iloprost and prostacyclin had IC_{50} values of 2 - 10 μM and approximately 30 μM, respectively).[2,28,29,38,58-60] $PGF_{2\alpha}$ and U-466 19 were almost inactive in inhibiting FMLP-stimulated superoxide anion generation by human neutrophils,[58] yet PGE_1 and taprostene inhibited FMLP-stimulated respiratory burst with IC_{50} values of 100 - 300 nM.[27] Furthermore, the potency of inhibitory prostanoids was dependent on the neutrophil activator used; thus, PGD_2 and PGE_1 were equipotent against FMLP and OZ, but

PGE_2 was more potent against the FMLP-stimulated respiratory burst.[2,28] Prostaglandins fail to modulate superoxide anion production when added 10 s or later after FMLP stimulation of human neutrophils, suggesting that they act at an early step in the neutrophil response pathway.[60]

The lack of cross-desensitisation between PGE_1 and iloprost for inhibition of neutrophil respiratory burst (and for stimulation of cyclic AMP),[28] clearly indicates that PGE_1 and iloprost are acting at different receptors, and suggests that PGE_1 activation of EP_2/EP_4-receptors, and not IP-receptors, is the dominant effect of PGE_1 in human neutrophils. The compound OP-1206 was made in an attempt to design a PGE_1 analogue with antiplatelet activity equivalent to that of prostacyclin.[24] However, although PGE_1, PGE_2 and OP- 1206 inhibited FMLP-stimulated superoxide anion production by human neutrophils, both iloprost and prostacyclin were inactive, even at 10 μM. As we have seen above, IP agonists are much less potent than PGE_2 (and EP_2 agonists) at inhibiting the respiratory burst of human neutrophils, which might suggest that OP-1206 is both an EP_2 agonist (responsible for antineutrophil activity) and an IP agonist (responsible for antiplatelet activity).

As seen above for human neutrophils, rabbit peritoneal neutrophils also failed to respond to prostacyclin.[61] Unfortunately PMA was used as the stimulus here and there are important differences to note between modulation of receptor- and nonreceptor-stimulation of the respiratory burst in neutrophils (see Section 2.8). Therefore this data alone does not allow us to make any conclusion yet as to the role of IP-receptors in modulating rabbit neutrophil functions. Canine neutrophils are also similar to human neutrophils, in that high concentrations of IP agonists (about 100 μM of prostacyclin and iloprost) are needed to produced 50% inhibition of OZ-stimulated superoxide anion production.[4,5] In 1983, Ham et al.[31] noted that superoxide anion production by rat peritoneal neutrophils stimulated with FMLP was significantly inhibited by PGE_1 and PGE_2, with IC_{50} values of 10 - 100 nM, and that this inhibition might be associated with cyclic AMP productoin. Unlike human neutrophils, FMLP-stimulated superoxide anion production by rat peritoneal neutrophils was potently inhibited by both prostacyclin and beraprost.[30]

2.6 Enzyme release

The bacteria-destroying proteolytic enzymes of neutrophils are contained within granules of which the neutrophil has two types: azurophilic and specific. The azurophilic granules contain a variety of enzymes, including β-glucuronidase and lysozyme (which is also found in the specific granules),

and the release of enzymes from azurophilic granules is under tighter control than the specific granule release.[62]

While both PGE_1 and PGE_2 significantly inhibited FMLP- and PAF-stimulated β-glucuronidase release from human neutrophils, neither iloprost or prostacyclin had any significant effect at 10 μM.[28] However, both PGE_2 and prostacyclin were effective in inhibiting OZ-stimulated β-glucuronidase and lysozyme release from rabbit peritoneal neutrophils, whereas TXB_2 and $PGF_{2\alpha}$ were inactive,[61] suggesting that rabbit peritoneal neutrophils are more similar to rat peritoneal neutrophils than to human neutrophils; thus supporting the idea that the elicitation process used to harvest peritoneal neutrophils accounts for the apparent difference between species.

2.7 Leukotriene B_4 production

Leukotriene B_4, the major arachidonic acid metabolite of neutrophils, is itself a potent chemoattractant and activator of neutrophils and thus may serve as an amplification factor in inflammatory processes.[28] Despite this, it is difficult to detect LTB_4 formation and release by FMLP, PAF or arachidonic acid-stimulated human neutrophils, therefore exogenous arachidonic acid needs to be added in these assays.[63] Although both PGE_1 and PGE_2 could inhibit PAF- and FMLP-stimulated LTB_4 release from human neutrophils, an equivalent concentration of iloprost (1 0 μM) had no significant effect;[63] this was one of the first studies to note the relative lack of effect of IP agonists on human neutrophil function. In OZ-stimulated human neutrophils, iloprost, U-46619 and $PGF_{2\alpha}$ were at least one order of magnitude weaker than PGE_2 or PGD_2 in inhibiting LTB_4 release; thus, iloprost had an IC_{50} value of approximately 2 μM compared with 720 nM and 9 10 nM for PGE^2 and PGD_2, respectively.[58]

In rat neutrophils, PGE_1 and PGE_2 were slightly more potent inhibitors of FMLP-stimulated LTB_4 release (IC_{50} ~ 10 nM) than in human neutrophils (IC_{50} ~30 - 100 nM).[31] In rat peritoneal neutrophils, the order of potency of prostanoids was $PGE_1 \approx PGE_2$ > MMM-I-135 (prostacyclin analogue) > $PGA_1 \approx PGA_2 > PGI_2 > PGF_{2\alpha} > PGF_{1\alpha} > PGD_2$. This order of potency and the low potency of prostacyclin (IC_{50} 16 μM) suggests that these inhibitory effects could be attributed to activation of EP_2/EP_4-receptors rather than IP-receptors.

2.8 Mechanisms of action

All the prostanoid receptors which are inhibitory for neutrophils (DP, EP_2/EP_4, IP) couple to activation of adenylate cyclase[64] and it has been clear for some time that the inhibitory effect of prostaglandins on neutrophil

function is often mimicked by cyclic AMP,[29] and that the neutrophil-suppressant potencies of prostaglandins generally correlates with their potencies for activation of adenylate cyclase.[9] However, this in itself is not sufficient proof of a second messenger role for cyclic AMP. For example, the effect of prostaglandins on superoxide anion generation is dependent on the specific stimulus and increased cyclic AMP in activated neutrophils by itself is not sufficient to limit superoxide anion induced by all agonists.[2,28] Thus, while 10 μM iloprost produced an equivalent increase in cyclic AMP as 1 μM PGE_1 in human neutrophils, only PGE_1 produced a significant inhibition of superoxide anion release.[28] Total cellular cyclic AMP is also not well correlated with inhibition of human neutrophil chemotaxis to all stimuli. In one study, PGE_1 needed a PDE inhibitor to inhibit FMLP-stimulated chemotaxis, but was active alone against LTB_4-stimulated chemotaxis; 8-bromo cyclic AMP was inactive against both stimuli.[45] If we attempt to relate forskolin-induced increases in cyclic AMP and functional responses, we see that it has no significant effect on the early responses of a neutrophil to FMLP (shape change, transmigration, priming) yet forskolin inhibits the late events (respiratory burst, PLA_2 activation).[65] The differences in neutrophil stimulus and response sensitivities to cellular cyclic AMP suggests that cyclic AMP alone does not regulate neutrophil function, unless we implicate a role for highly compartmentalised changes in cyclic AMP.

The general consensus of opinion is that prostaglandins only inhibit receptor-activated neutrophils, and do not inhibit activation by receptor-independent processes such as the use of PMA, arachidonic acid or A23187. Thus PGE_1 and PGE_2 had no effect on A23187-stimulated LTB_4 release,[63] or against arachidonic acid-stimulated respiratory burst,[28,60] or against PMA-stimulated respiratory burst in human neutrophils.[29,60,66] Interestingly, neither dibutyryl cyclic AMP nor theophylline could inhibit PMA-stimulated respiratory burst in human neutrophils, whereas they were both effective inhibitors of FMLP- or OZ-stimulated responses.[29] Prostacyclin (30 μM) similarly failed to inhibit PMA-stimulated respiratory burst in human neutrophils,[60] and although beraprost inhibited FMLP-stimulated respiratory burst, it was also inactive against PMA and A23187 in human neutrophils.[38] In contrast, prostacyclin appeared able to inhibit PMA-stimulated enzyme release in rabbit peritoneal neutrophils, but this effect could be overcome by increasing the concentration of PMA,[61] and iloprost still inhibited PMA-stimulated human neutrophil adhesion to endothelial cells.[43] Another surprise is that the PDE-IV inhibitor nimesulide could inhibit both FMLP- and PMA-stimulated respiratory burst in human neutrophils, but no completely satisfactory explanation was offered.[67] If the release of eicosanoids by neutrophils is relevant to their overall response profile, then it

may be worth noting that while PMA is a potent stimulant of neutrophil oxidative metabolism and degranulation (i.e. enzyme release), unlike OZ and calcium ionophores, PMA does not release arachidonic acid.[8]

It has been suggested that the general failure of PGE_1 and dibutyryl cyclic AMP to inhibit PMA-stimulated respiratory burst and enzyme secretion is because PMA acts directly on protein kinase C by a Ca^{2+}-dependent mechanism.[66] This implies that cyclic AMP modulators need to influence Ca^{2+} to produce an inhibitory effect. Despite this conclusion, and the observations that cyclic AMP modulators can inhibit the elevation of $[Ca^{2+}]_i$ in activated neutrophils, there are sufficient anomalies to suggest that Ca^{2+} fluxes are not the major target for the inhibitory action of prostaglandins.[22,66]

Using results from studies of PGE_1 and PGE_2, we can see that the maximal inhibition seen with these prostaglandins shows considerable variation which cannot be entirely accounted for by differences in concentration of neutrophil activator used. Thus the efficacy of PGEs in inhibiting neutrophil functions is as follows: respiratory burst (90 - 100%), chemotaxis and LTB4 release (80%), aggregation (60%), enzyme release (30 -50%), increases in $[Ca^{2+}]_i$ (20 - 40%). In addition, Kainoh et al.[30] noted that beraprost was more effective in inhibiting FMLP-stimulated rat peritoneal neutrophil chemotaxis than in inhibiting the respiratory burst, and suggested that the inhibitory mechanisms used by beraprost to inhibit these neutrophil functions are unlikely to be identical. Of course, such conclusions can be confused by the use of different neutrophil activators; thus, C5a and FMLP show similar potency for stimulating aggregation, C5a has greater potency than FMLP for releasing lysozyme, and FMLP is more potent than C5a in stimulating the respiratory burst in human neutrophils.[68] Therefore it is difficult to tell if these results may account for the observation that C5a-stimulated human neutrophil aggregation and lysozyme release is more sensitive to inhibition by PGE_1 and PGE_2 than is the respiratory burst.[68] However, when a larger group of prostanoids and a larger range of responses are studied, we see that the relative potencies are similar, irrespective of the stimulus being used or the function being examined, and the most likely explanation for these results is that prostanoids inhibit a common pathway involving a fundamental mechanism of neutrophil activation.[28,58]

Much of the mechanistic work on the inhibition of neutrophil function by prostaglandins has naturally concentrated on PGE_2 since IP agonists have relatively little effect on human blood-derived neutrophils. Since all the inhibitory prostanoid receptors (i.e. DP, EP_2/EP_4 and IP) couple to activation of adenylate cyclase,[64] we shall include the mechanistic studies on PGE_2 here for reference. At the simplest level, studies have looked for a correlation between concentrations of prostanoids needed to stimulate cyclic

AMP and to inhibit neutrophil functions. Thus, a correlation exists for inhibition of the respiratory burst,[28-31,61,68,69] and for inhibition of LTB_4 release.[31] A more detailed study in human neutrophils using cyclic AMP mimetics and antagonists indicates that inhibition of the respiratory burst by PGE_2 is clearly dependent on cyclic AMP,[69] whereas the correlation between the extent of cyclic AMP production and inhibition of respiratory burst for IP agonists such as PGE_1, taprostene, naxaprostene and CG 4303 is far from ideal.[27] Although a correlation was noted for inhibition of chemotaxis by PGE_2 and prostacyclin in rat peritoneal neutrophils,[30] and inhibition of adhesion by iloprost in human neutrophils,[43] no such quantitative relationship was found in rabbit peritoneal neutrophils,[44] and a more thorough study of human neutrophils indicated that the inhibitory effect of PGE_2 and EP_2 agonists was independent of cyclic AMP.[47] Agents which increase cyclic AMP, including PGE_1, tend to inhibit chemotaxis and spontaneous motility of rabbit peritoneal neutrophils, but there is a poor concentration-response relationship.[44] Explanations ventured were that these quantitative discrepancies may indicate that the rise in cyclic AMP is coincidental with but bears no relationship to the inhibition of neutrophil chemotaxis and locomotion, or that there is compartmentalisation of cyclic AMP in the neutrophil.

Contradictory results exist for inhibition of lysozyme release; for example, there is a poor correlation in human neutrophils,[68] and a better correlation for PGE_2 and prostacyclin in rabbit peritoneal neutrophils.[61] There is also a poor correlation for PGE_2-mediated inhibition of C5a-stimulated human neutrophil aggregation,[68] and for FMLP-stimulated rat peritoneal neutrophil aggregation by PGE_2 and IP agonists,[32,70] but we cannot yet rule out a role for highly localised changes in cyclic AMP which might inhibit phosphatidylinositol 3-kinase activity, resulting in the antiaggregation effect of PGE_2 in rat peritoneal neutrophils.[70]

The involvement of cyclic AMP in mediating the antiaggregatory effect of IP agonists is far from clearcut. Figure 48 clearly shows that if IP agonists do inhibit aggregation by increasing cyclic AMP, then very small (undetectable) changes are all that are required in this highly-coupled system. For example, the nonprostanoid prostacyclin mimetics octimibate, BMY 42393 and BMY 45778 are all relatively weak activators of adenylate cyclase, yet all produce the same maximal inhibition of aggregation as seen with cicaprost and iloprost. In addition, although the inhibitory effect of cicaprost in rat peritoneal neutrophils can be potentiated by PDE inhibition, it cannot be blocked by protein kinase A (PKA) inhibitors.[32] We also studied the effect of PDE and PKA inhibitors on iloprost, octimibate, BMY 42393 and BMY 45778 and concluded that these enzyme inhibitors can only

modulate the effect of IP agonists when very small changes in cyclic AMP are involved.[32]

Prostaglandin-mediated inhibition of many neutrophil functions occurs at concentrations well below the half-maximal concentration for activation of adenylate cyclase, therefore submaximal activation of adenylate cyclase can provide substantial regulation.[71] But as pointed out,[71] activation of adenylate cyclase alone may be insufficient in the control of neutrophils. If increasing neutrophil cyclic AMP leads to inhibition of neutrophil activity, then we might expect the converse effect on inhibiting cyclic AMP production. Indeed, it has been reported that EP_3 agonists can stimulate chemotaxis at low concentrations:[48] and that EP_3 agonists increase the release of LTB_4 in OZ-activated human neutrophils.[58]

Chromatographic evidence suggests that the PDE isozyme present in the cytosol of human neutrophils is predominantly PDE-IV (cyclic AMP specific), and PDE-IV inhibitors such as IBMX, nimesulide, Ro-20-1724 and zardaverine all increase basal and FMLP-stimulated cyclic AMP and inhibit the respiratory burst.[67,72] Furthermore, rolipram and Ro-20-1724 have little or no effect on FMLP-stimulated chemotaxis in human neutrophils, and unlike the respiratory burst, the antichemotactic effect of PGE_2 cannot be inhibited by PKA inhibitors.[47] We find very little effect of rolipram on basal cyclic AMP levels in rat peritoneal neutrophils, but it produces a substantial enhancement of PGE_2 and IP agonist-stimulated cyclic AMP.[32,70] Rolipram is a fairly potent inhibitor of rat peritoneal neutrophil aggregation (IC_{50} 194 nM),[32] but has slightly different effects when used with PGE_2 or cicaprost. Thus, the inhibitory concentration-response curve for cicaprost is potentiated by rolipram, while there is only an additive effect with PGE_2.[32,70]

It is unlikely that inhibition of FMLP-stimulated increases in $[Ca^{2+}]_i$ is responsible for the anti-aggregatory effect of IP agonists in rat peritoneal neutrophils. IP agonists are at least 10-fold less potent in inhibiting FMLP-stimulated increases in $[Ca^{2+}]_i$ compared with inhibiting the aggregation response, despite the observation that the EC_{50} for FMLP in stimulating increases in $[Ca^{2+}]_i$ was18-fold less than the EC_{50} for stimulating rat peritoneal neutrophil aggregation.[32] In addition, IP agonists can activate ATP-sensitive K_+ channels (K_{ATP}) in coronary artery smooth muscle,[73,74] in a cyclic AMP-independent fashion, but the K_{ATP} antagonist glibenclamide had no significant effect on IP agonist activity in rat peritoneal neutrophils.[32]

An alternative mechanism could involve a direct effect of prostaglandins on the FMLP receptor. PGE_2 does not inhibit [^{3}H]-FMLP binding to human neutrophils,[19] but neutrophils isolated from rats treated with 15-methyl PGE_1 showed a decrease in binding affinity for FMLP to its receptor and a decrease in the ability to secrete lysosomal enzymes, which might account

for the anti-inflammatory nature of PGEs.[75] One must bear in mind that prostaglandins inhibit neutrophil responses to a wide range of stimuli, and there are no other reports of prostaglandins decreasing the affinity of other chemotactic agents to their receptors.

2.9 Characterisation of neutrophil IP-receptors

Because cicaprost and iloprost were nearly as potent in inhibiting rat peritoneal neutrophil aggregation as in inhibiting human platelet aggregation, we attempted to compare the potency of a range of IP agonists in both assays (Table 15). Characterising IP-receptors using agonist potency ratios has many hazards, one of which is clearly shown in Table 15. Rather than comparing IC_{xx} values, it is usual to compare the order of potency of agonists relative to the standard compound when looking for evidence of receptor subtypes. A comparison of data in columns 4 and 7 of Table 15 would suggest that IP-receptors in human platelets differ from those in rat peritoneal neutrophils; the nonprostanoid prostacyclin mimetics being relatively more potent at rat IP-receptors. However, the nonprostanoid prostacyclin mimetics have a high degree of protein binding[76,77] and this factor becomes highly relevant when one assay, i.e. platelet aggregation, is performed in the presence of plasma proteins. Thus, reanalysis of the results with reference to BMY 45778 as the standard compound gives a more reliable interpretation; i.e. although these IP agonists are 4-fold less potent in rat peritoneal neutrophils compared with human platelets, the order of potency is similar and it is probable that there is no difference between these IP-receptors. Therefore, the IP-receptor of rat neutrophils is likely to be similar to the IP_1-receptor of human platelets. However, in Chapter 5 we suggested that the human platelet IP1-receptor differed from the rat platelet IP_1-receptor. Therefore the question arises over whether the rat platelet and rat neutrophil IP_1-receptors are different, and with the pharmacological tools available to us at the moment, we are unable to satisfactorily answer this question.

Stahlberg et al.[27] had earlier shown a similar order of potency for IP agonists to inhibit platelet aggregation and to inhibit the neutrophil respiratory burst, i.e. taprostene > naxaprostene > CG 4303. However, because considerably higher (~ 20-fold) concentrations of prostacyclin analogues were required to affect neutrophil function, the authors suggested that IP-receptors in neutrophils are distinct from those of platelets. Since in general, human neutrophils do not respond well to IP agonists, and given that few researchers have access to a complete range of IP agonists for testing, then we have no same-tissue comparisons available for the IP-receptors of rat and human neutrophils. We have though attempted a same-

species comparison and discovered that the IP-receptor of rat peritoneal neutrophils differs from that which inhibits the spontaneous contractile activity of rat colon (see Chapter 1 0).[78]

Table 15. Comparison of IP agonist potency in studies of human platelet and rat neutrophil aggregation

Drug	*Inhibition of human platelet aggregation (assayed in platelet-rich plasma)*			*Inhibition of rat neutrophil aggregation (assayed in protein-free buffer)*		
	IC_{50} (nM)	*EMR VS. cicaprost*	*EMR VS. BMY 45778*	*IC_{30} (nM)*	*EMR VS. cicaprost*	*EMR vs BMY 45778*
Cicaprost	0.8	1	0.03	2.6	1	0.12
Iloprost	1.6	2	0.06	2.5	0.96	0.96
	2.0	5.9	0.17			
BMY 45778	27	34	21	21	8	1
Octimibate	200	250	278	278	107	13
	1020	1275				
EP 185	100-300	125-375	500	500	192	24
BMY 42393	1200	1500	368	368	139	18

Data on cicaprost, BMY 45778, EP 185 and BMY 42393 are from Ref. No. 78. Data on iloprost and octimibate are from Ref. No. 79-81. IC_{30} values were used for the neutrophil aggregation data in this table because all compounds except EP 185 failed to produce more than 60% maximal inhibition. EMR = equi-effective molar concentration.

3. ROLE OF NEUTROPHILS IN DISEASE

3.1 Adult Respiratory Distress Syndrome

The etiology of Adult Respiratory Distress Syndrome (ARDS) remains unknown, but there is clear evidence that neutrophils are involved in the component of lung injury. Endothelial cells in the lung are thought to be damaged by ROS released from phagocytes and neighbouring endothelial cells. It is thought that PGE_1 may prove effective in the treatment of ARDS by lessening of autoinjury to endothelial and alveolar cells partly through its action to reduce superoxide anion production by neutrophils.[57] Furthermore, when human neutrophils were infused into isolated buffer-perfused rat lungs and subsequently stimulated with PMA, iloprost pretreatment reduced the extent of lung injury.[43] Although this protective effect of iloprost occurred despite the continued production of superoxide anion and upregulation of Mac-1 , the authors could not entirely discount the neutrophil rather than the endothelial cells as the target site for iloprost since iloprost (30 μM) was

able to inhibit PMA-stimulated human neutrophil adhesion to endothelial cells despite having no effect on Mac-1 expression. Following soft tissue trauma in anaesthetised pigs, neutrophils accumulate in the lungs, and there is some evidence that iloprost plays a protective role in this trauma model by affecting neutrophil pulmonary sequestration.[82]

3.2 Ischaemia-reperfusion injury

A period of ischaemia causes irreversible cellular injury which may be exacerbated by inadequate restoration of blood flow if the arteries are occluded with platelet thrombi. An alternative view is that reperfusion itself can result in a drastic acceleration of tissue necrosis, resulting in ischaemia-reperfusion injury. An excellent discussion of the roles played by neutrophils in this process can be found in Williams;[83] we will concentrate here on the influence of IP agonists on ischaemia-reperfusion injury.

The ability of prostacyclin to alter responses to acute myocardial ischaemia was studied in open-chest, anaesthetised cats, where prostacyclin infusion (0.5 nmoles kg^{-1} min^{-1}, i.v.) protected the ischaemic myocardium by reducing oxygen demand, primarily through reduction in cardiac work, and perhaps by inhibiting platelet aggregation and preserving myocardial cell integrity.[84] The possible involvement of neutrophils in this cytoprotective effect of IP agonists was noted in 1985 when examining the effects of iloprost (0.6 μmoles kg^{-1} min^{-1}, i.v.) in the reperfused ischaemic myocardium of cats.[85] The interstitial spaces of the ischaemic myocardium in control animals frequently contained neutrophils, whereas the extravasation of neutrophils was rarely seen in the iloprost-treated group. This was later confirmed in dogs where the cytoprotective nature of prostacyclin (50 ng kg^{-1} min^{-1}, i.v.),[5] iloprost (100 ng kg^{-1} min^{-1}, i.v.)[4] and beraprost (300 ng kg^{-1} min^{-1}, i.v.)[86] during myocardial ischaemia and reperfusion was related to an inhibition of neutrophil migration and the decreased production of cytotoxic ROS. By inhibiting neutrophil infiltration, monoclonal antibodies against CD11b or CD18 decreased ischaemia-reperfusion damage in rat heart[87] and rat brain,[88] further implicating the neutrophil as a major contributor to tissue damage following ischaemia-reperfusion injury. Previously it had been assumed that prostacyclin, by reducing arterial blood pressure, would decrease myocardial ischaemia by reducing afterload and thus myocardial oxygen demand. However, this does not appear to be the case because in a model of myocardial ischaemia in the dog, the prostacyclin analogue SC39902 produced the same changes in cardiovascular responses as prostacyclin, yet afforded no cytoprotection.[5] Furthermore, prostacyclin, but not SC39902, inhibited canine neutrophil respiratory burst and only prostacyclin inhibited neutrophil migration into inflammatory skin lesions.

Thus, the cytoprotective nature of prostacyclin is independent of changes in blood flow to the previously ischaemic myocardium and is more likely due to inhibition of neutrophil activation. Furthermore, the coronary release of prostacyclin is greater in lipopolysaccharide-treated rats, and it may be this factor which attenuates the deterioration of myocardial function in response to ischaemia-reperfusion in these animals.[3] Certainly, 6-oxo $PGF_{1\alpha}$ is the principal prostaglandin released from cardiomyocytes in vitro in response to oxidative stress and 30 nM iloprost significantly limits cell damage.[89] Factors causing oxidative stress (e.g. H_2O_2 or doxorubicin) in cardiomyocytes led to induction of COX-2, mediated by activation of extracellular signal-regulated kinases (ERK) 1/2, with the consequent elevation in 6-oxo $PGF_{1\alpha}$.The COX-2 specific inhibitor NS398 prevented the elevation in 6-oxo $PGF_{1\alpha}$ and potentiated doxorubicin-induced cell damage.[89] It is therefore possible that the heart is capable of limiting the damage caused by ischaemia-reperfusion injury by the generation of prostacyclin and the subsequent inhibition of neutrophil activity; the use of aspirin immediatedly following an ischaemic event may therefore need to be re-evaluated.

The processes involved as a result of spinal cord injury may also relate to those of ischaemia-reperfusion injury. For example, compressive spinal cord trauma in rats can be significantly attenuated by infusion of iloprost (100 ng kg^{-1} min^{-1}), to a similar extent as seen in neutropenic rats.[90] Consistent with the neutrophil as the target site of action of iloprost, neutrophil infiltration of the spinal cord was significantly attenuated by iloprost.

4. DISCUSSION

Regardless of the functional measure under consideration, human neutrophils are little affected by IP agonists,[2,24,28,59,63] and inhibitory prostanoid receptors can be ranked in order of inhibitory potency as:$EP_2 \approx$ DP >>> IP > FP, TP. The situation differs for rat peritoneal neutrophils where the order of inhibitory potency is: $EP_2 \approx$ IP >>DP > FP, TP.[30-33] Because many of the in vivo inflammatory models used in research involve rodents rather than humans, then this species difference between the relative roles of PGE_2 and prostacyclin needs to be taken into consideration, along with the primary cell type of interest. If we look briefly at another similar inflammatory cell such as the eosinophil, we see that the respiratory burst of the human eosinophil responds in the same way as the human neutrophil in that it can be inhibited by PGE_2 and PGD_2, but not by cicaprost.[59] Similarly, PGE_1 and PGE_2 suppress eosinophil accumulation in guinea-pig skin, but iloprost has no effect.[49] In addition, the homotypic aggregation of elicited

guinea-pig eosinophils is also unaffected by IP agonists and is inhibited by PGE_2 (via the EP_2-receptor), but this time PGD_2 is without effect.[91]

Schrör & Hecker[24] suggested that prostacyclin and its analogues, while being effective antiplatelet agents, might not cause any direct antineutrophil action in humans because of the absence of an antineutrophil potential. Clearly this concept needs re-evaluating because it may be the elicited neutrophils which are responsible for the tissue damage in chronic inflammatory conditions, As described in Chapter 8, elicited monocytes from mouse, rat and humans increase their responsiveness to prostacyclin compared with nonelicited cells. It is possible therefore that human neutrophils might be relatively unresponsive to IP agonists because they are not elicited cells. It is conceivable therefore that human neutrophils could increase their responsiveness to IP agonists during the priming process which will occur during transendothelial cell migration. It is clear that the activation status of a cell is highly influential on the expression of IP-receptors, thus, human basophils express high affinity binding sites for [^{3}H]-iloprost and these are downregulated following cell incubation with the activating cytokine interleukin-3, with a matching concentration-dependent decrease in cyclic AMP production in response to IP agonists.[92] It remains to be seen whether the cytokines which will influence neutrophils during transendothelial cell migration have any effect on IP-receptor expression.

These conclusions are however as yet unproven. In a study comparing blood and tissue (elicited) neutrophils, we begin to see some consistency between species.[93] Using human saliva neutrophils as representative of tissue neutrophils, Kanamori et al.[93] showed that tissue neutrophils are less (rather than more) responsive than blood neutrophils to the inhibitory activity of EP_2 and DP agonists, when measuring FMLP-stimulated respiratory burst. Identical results were found when comparing rabbit blood and peritoneal neutrophils. Unfortunately IP agonists were not included in this study, so for the moment, our hypothesis that tissue neutrophils gain in responsiveness to IP agonists remains unchallenged.

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Chapter 8

IP-receptors on monocytes/macrophages and lymphocytes

1. Introduction

As we have seen in Chapter 7, circulating neutrophils are the first of the body's white cell defence force to limit microbiological invasion or respond to tissue damage. Next in line are the blood-borne monocytes, which when transformed into macrophages, are capable of phagocytosis and chemotaxis, and many other responses also seen with neutrophils. If the local inflammatory reaction cannot contain the infection, then invading microorganisms are carried to the regional lymph nodes via the lymphatics. Here the macrophage-like cells of the reticuloendothelial system phagocytose the microorganisms and act as antigen-presenting cells to facilitate both cell-mediated and antibody-mediated immune responses involving lymphocytes. Antigen presenting cells additionally release cytokines such as interleukin-1 (IL-1) to facilitate the induction phase of these immune responses. In turn, lymphocytes can trigger macrophage activation by releasing chemokines. Thus the activity of monocytes/macrophages and lymphocytes are closely interrelated.[1]

Although we are primarily interested here to discuss the role of IP-receptors on monocytes and lymphocytes, we again find that the predominant inflammation-related prostanoid studied to date is prostaglandin E_2 (PGE_2) rather than prostacyclin. Given that the most popular anti-inflammatory drugs in current use are the nonsteroidal anti-inflammatory drugs (NSAIDs), and since these drugs will inhibit all prostanoid production (i.e. both prostacyclin and PGE_2), then it is important for us to try and pick

out the differences in activity of prostacyclin and PGE_2 with a view to highlighting the potential therapeutic use of specific prostanoid receptor agonists and antagonists should they become available.

Studies using macrophages in particular are often hampered by the lack of readily accessible, and location-appropriate human cells. For example, usable numbers of monocytes can be harvested from human peripheral blood, and allowed to settle on tissue culture plates for several days; the adherence process being a stimulus for differentiation into macrophages.[2] However, these cells are not identical to those involved in an inflammatory reaction. Thus, during inflammation and in response to a chemotactic signal, monocytes would normally migrate from the blood vessel into the tissue space, and in the process they would be influenced by a variety of chemical factors including prostanoids and chemokines. Therefore, despite the fact that blood mononuclear phagocytes are probably precursors of practically all macrophages, once they enter diverse anatomical sites the blood-borne cells ultimately develop tissue-determined differences.[3] The influence of these changes in monocytes/macrophages has been studied in the mouse where it is clear that the production of prostacyclin is highly dependent on the stage of cell development and the factors present in its local environment.[4] With the clear role played by macrophages in inflammatory diseases, and the usual need to treat these disorders only when they have become already well established in the body, then it is these tissue macrophages which really need to be studied. While macrophages can be harvested from the synovial fluid of patients with arthritis,[5] or from the peritoneal cavity of patients undergoing CAPD (continuous ambulatory peritoneal dialysis as treatment for kidney failure),[6-8] it is always difficult to obtain sufficient cells, especially those which can serve as the correct control cells. Obviously a more complete understanding of the human macrophage would allow one to make better comparative data with the more accessible rodent macrophage. Microscopic examination of rat peritoneal macrophages shows gross differences between resident and elicited cells.[8] Thus, the elicited cells are larger and contain ingested particles and many vacuoles, i.e. all the features of activated macrophages. The human peritoneal macrophages of CAPD patients do not represent the same type of resident macrophage as seen in rodents. According to Bonta et al.[8] these cells are more similar to the elicited rather than the resident macrophages of rats, probably due to the stimulating action of the dialysis treatment. Much of the literature therefore deals with macrophages from nonhuman species (or cell lines maintained in culture), which may lead us into problems if there are species-specific properties of IP-receptors (see Chapter 4). Therefore when considering the role played by IP-receptors in macrophages, we must bear in mind the source

of the cells (i.e. species and tissue) before attempting to infer properties onto human macrophages.

In contrast to macrophages, circulating human lymphocytes are readily accessible and are suitable for studying many of the functions of lymphocytes. However, when it comes to assessing the function of IP-receptors on lymphocytes, we find that relatively little work has been done in this particular area, with work on PGE_2 taking greater priority.

2. MONOCYTES/MACROPHAGES

2.1 Prostacyclin production

Current evidence indicates that among the family of leukocytes, cells of the monocyte/macrophage lineage are the most potent producers of prostaglandins, and the range of prostanoids produced is species-dependent. Thus mouse, but not human, monocytes are a significant source of prostacyclin.' For human macrophages, prostanoid production follows the order: thromboxane A_2 (TXA_2) >> PGE_2 > prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); in mouse macrophages we see that PGI_2 = PGE_2 >> TXA_2; and in rabbit macrophages, TXA_2 and prostaglandin D_2 (PGD_2) are the cell's major cyclo-oxygenase (COX) metabolites. Furthermore, the relative amounts of prostacyclin, PGE_2 and TXA_2 produced by macrophages are markedly influenced by the cell activator used and the time period studied.[10] In addition, it is important here also to consider the range of COX products produced by other inflammatory cells because of the potential for transcellular metabolism. For example, platelet derived prostaglandin H_2 (PGH_2) can be used by lymphocytes to produce prostacyclin,[11] and the anti-inflammatory agent 15-epi-lipoxin A_4 (15-epi-LXA_4) is produced by human leukocytes using 15R-15-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15R-HETE) derived from aspirin-treated macrophages or vascular endothelial cells.[12] The use of nonmacrophage COX intermediates by macrophages still remains to be studied in detail.

The complement of prostaglandins produced by macrophages is also highly dependent on the tissue source of the cells, therefore it is clear that when we are considering the function of IP-receptors on monocytes/macrophages, we must also be aware not only of the species, but also the source/state of these cells. We also need to be aware of the normal production of COX products by these cells. In 1977, Humes et al.[13] showed that thioglycollate-elicited mouse peritoneal macrophages responded to zymosan-challenge by producing only relatively small amounts of [^{3}H]-

prostaglandins from [^{3}H]-arachidonic acid-labelled phospholipids. In contrast, resident cells produced a 24-fold increase in PGE_2 and a 15-fold increase in 6-oxo $PGF_{1\alpha}$ (the stable metabolite of prostacyclin), despite both cell populations incorporating similar amounts of [^{3}H]-arachidonic acid. The process of phagocytosis itself is insufficient to increase macrophage prostaglandin synthesis, e.g. ingestion of immunologically inert latex particles has no effect on PGE_2 production, yet phagocytosis of inflammatory stimuli such as zymosan, antigen-antibody complexes and opsonised bacteria (IgG plus complement) releases prostaglandins from macrophages.[13]

When peritoneal murine macrophages were elicited and harvested following injection with *Listeria monocytogenes,* it was shown that immune activation of macrophages in vivo also suppressed the arachidonic acid and prostaglandin endoperoxide-dependent enzymes with the notable exception of thromboxane synthase.[14] Thus, immunising mice activates macrophages and leads to a 80% reduction in COX and lipoxygenase (LOX) metabolites relative to resident cells. The most striking change was seen with prostacyclin production; for example, if we consider the ratio of TXA_2:PGI_2:PGE_2 in resident macrophages as 1:25:5, then in immune activated macrophages, the ratio changed to 1:1:1. Resident murine alveolar and peritoneal macrophages also produce significantly more prostacyclin than elicited peritoneal cells in response to the calcium ionophore A23187.[4]

In a comprehensive study of eicosanoid production by resident murine macrophages, Rouzer et al.[15] found that in addition to PGE_2 and TXA_2, pulmonary tissue macrophages, but not alveolar macrophages, produced prostacyclin in response to zymosan challenge. Furthermore, LOX products seemed to predominate in pulmonary tissue macrophages, especially leukotriene C_4, whereas COX products dominated in peritoneal macrophages, especially PGE_2. The local environment therefore seems to play an important determining factor in the production of prostacyclin by macrophages.

Resident murine peritoneal and plural macrophages produce prostacyclin and PGE_2 in equal amounts, but their metabolic response following elicitation by *Corynebacterium parvum* injection is highly location-specific and involves changes in the production of prostacyclin and TXA_2, but not PGE_2.[16] Macrophages were harvested, labelled with [^{3}H]-arachidonic acid, and stimulated with a maximum phagocytic stimulus (zymosan). Following an intraplural injection of *C. parvum,* intraplural, but not intraperitoneal, macrophages released less prostacyclin than resident macrophages in response to zymosan challenge. Similarly, following an intraperitoneal injection of *C. parvum,* intraperitoneal, but not intraplural, macrophages also released less prostacyclin. These results suggest that a downregulation of

specific arachidonic acid metabolites occurs as a localised process involving a contained macrophage population in the region of the inflammatory stimulus. Injection of several types of insoluble and soluble irritants into the pleural cavity of rats resulted in a three-fold increase in prostacyclin and TXA_2, with much smaller changes in PGE_2 and $PGF_{2\alpha}$ in peritoneal fluid after 24 h.[17] Even 3 to 5 days after intrapleural injection of dextran, peritoneal macrophages liberated higher amounts of prostanoids compared to untreated control cells. Consistently higher levels of prostacyclin production by peritoneal macrophages were found in response to intrapleural injection of dextran or calcium pyrophosphate. Therefore during the development of an acute inflammation reaction at one site (i.e. the pleural cavity), cells present at a distant site (i.e. the peritoneal cavity) display an increased capacity to release prostanoids, even several days after resolution of the local inflammatory process. The important difference between these two studies is that the latter[17] has measured prostanoid production in unstimulated macrophages, while the former[16] has measured prostanoid production by macrophages in response to activation by zymosan, but this in no way entirely explains the different results. In a comparative study it was noted that basal prostacyclin generation was much lower in guinea-pig elicited peritoneal macrophages (0.08 ± 0.06 pmol 106 $cells^{-1}$) than in rat elicited macrophages (4.5 ± 1.3 pmol 106 $cells^{-1}$)[18] It was also noted that guinea-pig resident peritoneal macrophages could generate prostacyclin in response to *N*-formyl-methionyl-phenyl-alanine (FMLP), platelet activating factor (PAF), zymosan and A23187, yet rat resident peritoneal macrophages produced prostacyclin only in response to zymosan and A23187. Again we see though that elicited peritoneal macrophages from rats and guinea-pigs show reduced prostacyclin generation, in response to activation, compared to resident cells.

Looking at the limited data on elicited macrophages from humans, we see that peritoneal macrophages from CAPD patients produce readily measurable levels of prostacyclin (approximately 0.2 ng ml^{-1} 10^6 $cells^{-1}$) and TXA_2 (approximately 0.6 ng ml^{-1} 10^6 $cells^{-1}$),[6] although the spontaneous production of 6-oxo $PGF_{1\alpha}$, PGE_2 and TXB_2 (the stable metabolite of TXA_2) falls during Peritonitis.[7] Furthermore, even in the presence of exogenous substrate (arachidonic acid), human rheumatoid synovial fluid macrophages fail to release prostacyclin.[5]

There is therefore overwhelming evidence that elicited mouse and human macrophages lose their capacity to produce prostacyclin, and it is possible that this decreased prostacyclin production by macrophages upregulates IP-receptors (or conversely, prevents prostacyclin-mediated IP-receptor downregulation), thus making the macrophages more responsive to prostacyclin. Evidence for this idea can be found when looking at the

adenylate cyclase response of resident and elicited macrophages to stimulation by prostacyclin and PGE_2 (Table 16). In addition, macrophages isolated from rats receiving a sublethal dose of lipopolysaccharide (LPS) show a significant increase in levels of IP1-receptor mRNA.[19] This upregulation of IP-receptors and associated increased cyclic AMP response to cicaprost are thought to underlie the "tolerance" of these rats to subsequent doses of LPS.

Table 16. The relative ability of prostacyclin and PGE_2 to stimulate cyclic AMP production by macrophages

	Mouse		*Rat*			***Human CAPD patients*	
	Res.[20]	*Elic.*[20]	*Res.*[8]	*Elic.*[3,8]	**Gran.*[21]	*Res*[7,8]	*Elic.*[7]
PGI_2	+	+++		+/++	+	++/++	+++
PGE_2	+	+++	+	++/++	++++++	+/+	+++
Basal			++	+		++++	

*Except for these granuloma (Gran.) cells, all other macrophages were obtained from the peritoneal cavity, as resident (Res.) or elicited (Elic.) cells. **See Section 1 for a description of resident and elicited cells in CAPD patients.

Of increasing interest in current inflammation research is the interaction between the activity of inducible nitric oxide synthase (iNOS) and COX-2. It now seems that agents which increase cyclic AMP production, for example, prostacyclin, iloprost and PGE_2 can all inhibit bacterial LPS-stimulated expression of iNOS and COX-2 mRNA, and reduce nitric oxide (NO) release and COX activity by the murine macrophage cell line J774.[22] Although not tested using prostacyclin, it is clear that the suppression of LPS-induced iNOS induction by PGE_2 was functionally significant, in that it protected against the mild cytotoxicity of the NO generated in response to endotoxin. Pang & Hoult[22] therefore suggest that macrophages possess a feedback regulatory suppression of COX-2 induction by a prostaglandin-driven cyclic AMP-mediated process. The authors also noted that the marked ability of phosphodiesterase (PDE) inhibitors to inhibit iNOS and COX-2 activity also suggests that such prostaglandin-mediated inhibition is normally held in check by active PDE enzymes in these cells.

2.2 IP-receptor binding studies

[^{3}H]-Prostacyclin binds to rat peritoneal macrophages in a saturable and reversible manner, with IC_{50} values of approximately 6 nM in whole cells, and 400 nM in a membrane preparation in which the B_{max} value increased four-fold to 1200 fmol mg protein^{-1}.[23] Unfortunately, satisfactory interpretation of these experiments is hampered by the low amount of

specific binding compared to total binding (45 - 50)% and the conversion of a biphasic to a monophasic binding curve on the use of a membrane preparation. Surprisingly, PGE_2 was more potent in inhibiting [^{3}H]-prostacyclin binding and in stimulating cyclic AMP production in these elicited peritoneal macrophages; results which might suggest that we are looking at [^{3}H]-prostacyclin binding to PGE_2 receptor sites rather than to IP-receptors, since by definition the prostanoid receptor is classified according to the activity of the most potent natural prostaniod.[24] The authors subsequently suggested that PGE_2 elevated cyclic AMP in rat cells through binding to a greater number of binding sites, i.e. receptors for both PGE_2 and prostacyclin, whereas prostacyclin can only act through the IP-receptor.[8] However, this conclusion still does not explain the higher affinity of PGE_2 compared with prostacyclin for competition with [^{3}H]-prostacyclin binding to rat peritoneal macrophages.

2.3 Adenylate cyclase

Using elicited rat peritoneal macrophages, it was shown that PGE_2 can inhibit the cyclic AMP-elevating effect of prostacyclin, but prostacyclin did not modify the responsiveness of macrophages to PGE_2.[25] Similar effects were seen using *rac*- 13,14-didehydrocarbacyclin (DDH-carbacyclin), which is a stable analogue of prostacyclin and therefore not liable to undergo rapid decomposition. With the benefit of hindsight, it is possible to conclude that the biphasic inhibitory effect of PGE_2 could be due to low concentrations of PGE_2 stimulating EP_3-receptors (which are inhibitory for adenylate cyclase), whereas the higher concentrations of PGE_2 could begin to activate the EP_2/EP_4-receptors which couple positively to adenylate cyclase; especially since the low inhibitory concentrations of PGE_2 failed to increase cyclic AMP levels alone. Bonta & Adolfs[25] speculated that endogenous PGE_2 rather than endogenous prostacyclin has the dominant role in regulating the levels of cyclic AMP in elicited macrophages. Furthermore, to the extent that the levels of cyclic AMP are of importance in governing the activation of macrophages, then these and other results implicate endogenous PGE_2 as a major feedback regulator of immuno-inflammatory processes in which macrophages are involved. We shall see now that this original assessment of the relative importance of prostacyclin and PGE_2 is dependent on both the species studied and the activation state of the macrophages, and may require some modification.

Elicited human peritoneal macrophages can be obtained from CAPD patients who have the complication of peritonitis. It is clear that cyclic AMP levels in macrophages from normal CAPD patients decline sharply during the period of peritonitis, but are restored after recovery from infection, and

are closely matched by a fall in the spontaneous production of 6-oxo $PGF_{2\alpha}$, PGE_2 and TXB_2 during peritonitis.[7] The lower cyclic AMP content of inflammatory macrophages compared to resident macrophages (Table 16) is more marked when the membrane adenylate cyclase assay is performed in the presence of GTP, but whether or not the difference lies at the receptor or effector level is unclear.[26] Macrophages from the uncomplicated (normal) stage of CAPD were more responsive to DDH-carbacyclin than to PGE_2, but we should note that these resident human peritoneal macrophages may be more similar to elicited rather than resident rodent macrophages.[8] However, during inflammation, the cells displayed a marked increase in sensitivity toward prostaglandins, especially toward PGE_2. Therefore for elicited human peritoneal macrophages, PGE_2 is again the dominant prostaglandin, for although macrophages show improved sensitivity to both PGE_2 and prostacyclin, it is the change in response to PGE_2 which is the most pronounced.

The evidence presented so far suggests a real difference in the response of macrophages dependent on the species studied. For example, a comparison of elicited rat peritoneal macrophages and human macrophages from CAPD patients indicates that rat and human cells are similar in their response to DDH-carbacyclin, but differ in their response to PGE_2 (Fig. 49). Thus DDH-carbacyclin stimulates cyclic AMP production with EC_{50} values of 384 nM and 3 10 nM, and maximum stimulation in the presence of the PDE inhibitor IBMX of 785% and 720% control in rat and human macrophages respectively (nonlinear regression analysis of data from Bonta et al.[8]) In contrast, rat macrophages are much more responsive to the cyclic AMP-elevating effects of PGE_2 than are human macrophages: EC_{50} values were 95 nM and 149 nM, and maximum stimulation was 1066% and 362% control in rat and human macrophages, respectively.

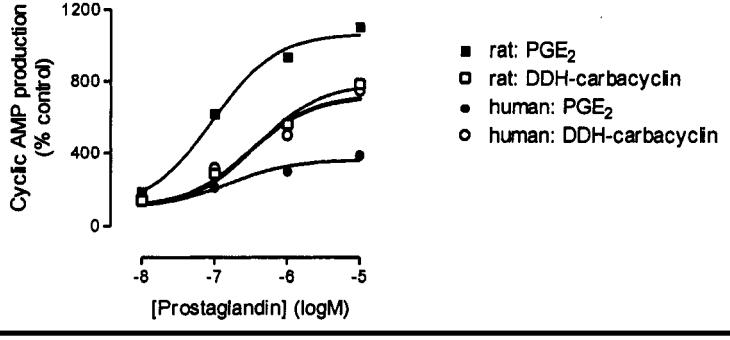

Figure 49. Effect of DDH-carbacyclin and PGE_2 on macrophages from the peritoneal cavity of humans and rats. Adapted from Bonta et al.,[8] with permission.

Results so far suggest that in the rat, PGE_2 appears to be the more important mediator of macrophage function, with prostacyclin being more important in humans. These observations are further supported by the work of Elliott et al.[5] who studied the adenylate cyclase response of human synovial fluid macrophages. When these cells were harvested from rheumatoid patients with an active synovitis, PGE_2 produced a doubling of cyclic AMP production, peaking at approximately 30 nM, whereas DDH-carbacyclin produced a four-fold increase in cyclic AMP production at approximately 300 nM. These experiments also indicated that a low basal level of cyclic AMP production was indicative of a good response to prostacyclin.

Prostacyclin or DDH-carbacyclin appear able to stimulate more cyclic AMP production in elicited macrophages when compared to resident cells (Table 16); presumably the infiltrating monocytes undergo a change following the inflammatory stimulus (e.g. thioglycollate or starch), resulting in macrophages which are more responsive to stimulation by prostaglandins than are resident cells. Morley[27] suggested that the hyposensitivity of macrophages towards PGE_2 could be part of the mechanism maintaining the inflammation response. Indeed, the loss of responsiveness to prostacyclin, and the marked gain in responsiveness to PGE_2 seen in granuloma macrophages is quite striking[21] and distinguishes the granuloma cell response from that of elicited peritoneal cells. PGE_2, but not DDH-carbacyclin, stimulated cyclic AMP production by guinea-pig alveolar macrophages, and the responses to both prostaglandins were significantly enhanced in antigen-challenged sensitised guinea-pigs.[28] In these latter cells, the response to DDH-carbacyclin was relatively minor compared to PGE_2, though comparable to the response to histamine. When these results are expressed relative to the altered basal level of cyclic AMP in the different macrophage samples, prostacyclin clearly stands out in its enhanced ability to stimulate adenylate cyclase and Beusenberg et al.[28] suggest that this may be due to improved receptor-effector coupling.

Furthermore, we have shown in Chapter 7 that for neutrophils there is often a poor correlation between the production of cyclic AMP and the final functional response to prostacyclin and PGE_2. Therefore, it may be premature to speculate on the relative importance of prostacyclin and PGE_2 in controlling macrophage function in any species until the efficiency of receptor-effector coupling has been considered. Bonta et al.[21] concluded that macrophages, together with lymphocytes, are pivotal targets for both the anti-inflammatory and immuno-modulatory effects of E-prostaglandins; but the question remains as to why, when compared to elicited rat macrophages, do macrophages from granulomatous tissue lose their response to

prostacyclin, and why do elicited mouse and rat peritoneal macrophages gain in their response to prostacyclin (Table 16).

If we examine responses of macrophage-like cell lines, we see that prostacyclin is much less potent than PGE_2 in stimulating cyclic AMP production in the human monocytic cell line THP-1, with EC_50 values of 40 μM and 22 μM, respectively.[29] Similarly, iloprost is less potent than PGE_2 in stimulating cyclic AMP production by the human monocytic cell line Mono Mac 6, with EC_{50} values of 1.7 μM and 54 nM, respectively.[30] In addition, the nonprostanoid prostacyclin mimetics octimibate and BMY 42393 stimulate cyclic AMP accumulation by elicited mouse peritoneal macrophages; at 10 μM they produced increases of 80% and 140% respectively.[31] It is noticeable that mouse macrophages are much less responsive to these nonprostanoid prostacyclin mimetics than are human monocytes, which is either due to species differences or the different effect of IP agonists on monocytes vs macrophages. [31]

3. FUNCTIONAL EFFECTS OF IP AGONISTS ON MONOCYTES/MACROPHAGES

3.1 Inflammation

Peritoneal macrophages are commonly used for experimental purposes, but one has to bear in mind that in order to obtain sufficient cells for experimental use, these cells are usually elicited by intraperitoneal injection of thioglycollate or starch. Therefore these cells have already undergone one cycle of chemotaxis and transendothelial cell migration in order to move into the peritoneal cavity; as such they are representative of macrophages present at the site of inflammation. An additional method for studying macrophages actively involved in an inflammatory reaction is to use the carrageenin-induced granuloma model. This is an immune-related inflammatory model in which activated macrophages, but not lymphocytes, participate,[3] and has proved crucial in recognising the different effects of PGE_2 and prostacyclin on macrophage function. Thus, the adenylate cyclase response of rat peritoneal macrophages to PGE_2 and prostacyclin is similar, while rat granuloma macrophages respond to PGE_2 but not to prostacyclin (Table 16). During the macrophage phase of granuloma tissue growth, local administration of prostacyclin did not result in any appreciable reduction in granuloma formation, but PGE_2 significantly inhibited the tissue-proliferative macrophage-phase of granulomatous inflammation.[21] It is not

clear why granuloma macrophages should become more sensitive to PGE_2 yet lose their sensitivity to prostacyclin, but it has been suggested that endogenous formation of PGE_2 modulates the ability of the cells to respond to exogenously added prostaglandin in terms of cyclic AMP levels.[20] Therefore, Bonta et al.[21] concluded that the distinction between the responsiveness of granuloma-derived macrophages to PGE_2 and prostacyclin in vitro, is directly related to the difference between these two substances as inhibitors of the macrophage-phase of immune-related inflammatory granuloma in vivo.[21]

3.2 Chemotaxis

Transendothelial migration of monocytes occurs in response to an inflammatory stimulus and this process depends on the initial adhesion of monocytes to endothelial cells. The chemotactic peptide FMLP can stimulate the adhesion and migration of human peripheral blood monocytes to porcine aortic endothelial cells in culture.[32] Prostacyclin had no effect on basal or FMLP-stimulated monocyte adhesion, and did not affect the monocyte cell surface expression of the adhesion molecules CD11/CD18, yet produced a concentration-dependent inhibition of FMLP-stimulated chemotaxis.[32] Presumably therefore prostacyclin inhibits chemotaxis by affecting monocyte motility. Elevated monocytic cyclic AMP has been reported to inhibit chemotaxis,[33] and 1 μM prostacyclin produced a small (2.3-fold) but significant increase in cyclic AMP in these human monocytes.[32] However, prostacyclin also produced a significant increase (1.7-fold) in cyclic GMP production which is more generally associated with an increase in chemotaxis.

The ability of various prostaglandins to inhibit monocyte chemotaxis induced by monocyte chemoattractant protein-1 (MCP-1) has been studied using THP-1 cells.[29] Although PGE_1 and PGE_2 showed high potency (IC_{50} values of 2.8 nM and 0.9 nM respectively), prostacyclin and isocarbacyclin were much less potent with IC_{50} values of 30 μM and 300 μM, matching their low potency in stimulating adenylate cyclase.

Using a model of atherosclerosis in hyperlipidaemic hamsters, Kowala et al.[31] found that both octimibate and BMY 42393 reduced the number of mononuclear cells on the luminal surface of the aortic arch. These nonprostanoid prostacyclin mimetics also inhibited FMLP-stimulated chemotaxis by human peripheral blood monocytes in vitro with IC_{50} values of approximately 10 nM. Together these results suggest that by inhibiting mononuclear cell adhesion to the artery wall, and/or by inhibiting chemotaxis, then monocyte diapedesis could be reduced resulting in fewer intimal macrophage-foam cells accumulating in vivo.

3.3 Phagocytosis

In addition to the complications of interpreting data across species and tissue sources, we also have to consider the nature of the functional test being studied. For example, following preincubation with indomethacin to inhibit endogenous prostanoid production, resident rat peritoneal cells (80% macrophages) lose their ability to perform CR1 (opsonin-receptor)-dependent phagocytosis, yet remain fully able to perform FcR (immunoglobulin-receptor)-dependent phagocytosis of bacteria.[34] It is likely therefore that the COX products which are formed by macrophages in response to CR1-opsonised bacteria, play an obligatory role in the phagocytosis of these bacteria.[34] Although the effect of prostacyclin was not directly measured in this study, we can see a marked difference between the effect of PGE_2 and PGE_1; both prostanoids potentiated CR1-mediated phagocytosis at concentrations from 100 μM to 10 nM, but only inhibited phagocytosis at concentrations above 100 nM. In contrast, PGE_2 had only a minor inhibitory effect on FcR-dependent phagocytosis at the highest concentration tested (10 μM), but PGE_1 inhibited this response at concentrations from 1 to 100 nM. It is possible therefore that both PGE_2 and PGE_1 activate EP_3-receptors (inhibitory for adenylate cyclase) at low concentrations, thus facilitating phagocytosis, and at higher concentrations activate EP_2/EP_4-receptors (stimulatory for adenylate cyclase), thus inhibiting CR1-mediated phagocytosis. In contrast, FcR-mediated phagocytosis is not influenced by stimulation of EP_2, EP_3 or EP_4-receptors, but activation of IP-receptors by PGE_1 (and high concentrations of PGE_2) leads to inhibition of FcR-mediated phagocytosis. The authors themselves concluded that endogenous PGE_2 and/or prostacyclin produced by rat peritoneal cells are involved in the complement activation-dependent bacterial phagocytosis by rat peritoneal cells but not in the antibody-dependent phagocytosis.[34] It would be interesting now to re-evaluate these results using the currently available selective EP and IP-receptor ligands.

3.4 Cytokine production

Early studies clearly indicated that LPS-stimulated resident murine macrophages produced similar amounts of both prostacyclin and PGE_2, and that exogenous prostacyclin and PGE_2 could suppress macrophage interleukin-1 (IL-1) production.[35] Furthermore, addition of NSAIDs facilitated LPS-stimulated IL-1 production by both peripheral blood monocytes and resident peritoneal macrophages, implicating endogenous prostacyclin and/or PGE_2 as normal regulators of macrophage cytokine production. Thus the data support the conclusion that the cytokine IL-1, as

with classical hormones, can regulate its own production through a self-induced inhibitor, PGE_2 (and/or prostacyclin?). One complicating factor comes from the observation that aspirin-pretreatment of mice differentially affects the release of cytokines by LPS-elicited and thioglycollate-elicited macrophages, and alters their morphology.[10] This study found that mouse thioglycollate-elicited macrophages release more prostacyclin than PGE_2 whereas the converse was true for LPS-elicited peritoneal macrophages. If, as proposed in Section 2.1, decreased macrophage prostacyclin production increases the responsiveness of macrophages to subsequent activation by prostacyclin, then care is needed to standardise these factors in functional assays in order to properly assess the relative significance of prostacyclin and PGE_2 in controlling macrophage functions. In addition, the ability of aspirin to influence transcellular biosynthesis[12] (see Section 2.1) and generate 15-epi-LXA_4 may need to be considered here.

Iloprost inhibited LPS-stimulated tumour necrosis factor-α (TNF_α) production by elicited mouse peritoneal macrophages with high potency (IC_{50} value approximately 300 μM) by preventing TNF_α mRNA transcription.[36] In a similar experiment, the nonprostanoid prostacyclin mimetics octimibate and BMY 42393 also inhibited LPS-stimulated TNF_α production by elicited mouse peritoneal macrophages, with IC50 values of approximately 1 μM.[31] It is difficult though to make any sound judgements on the relative potency of the IP agonists in these two studies because of the obvious variability between experiments performed by different groups. In vivo studies indicated that endotoxin-induced mortality in galactosamine-sensitised mice could be significantly reduced by iloprost administration, which suggests that prostacyclin modulates endotoxin-induced and TNF_α-mediated inflammation in septic shock.[36]

The tumouricidal activity of all three differentiation states of macrophages (resident, elicited and activated) can be enhanced by the antitumour drug 5,6-dimethyl-xanthenone-4-acetic acid ((5,6-MeXAA).[37] This enhanced macrophage tumouricidal activity can be completely inhibited by iloprost and PGE_2, with IC_{50} values of approximately 1 nM and 1 μM, respectively, by a mechanism thought to involve the inhibition of TNF_α production. The growth of tumour cells themselves (e.g. mouse myelomonocytic leukaemia cells, WEHI-3B) can be influenced by prostaglandins, with DDH-carbacyclin and PGE_2 showing a synergistic cytostatic effect with IL-1, and with DDH-carbacyclin itself being cytostatic at concentrations >30 nM.[38]

In human peripheral blood monocytes, cicaprost inhibited LPS-stimulated TNF_α generation with an IC_{50} value of 10 nM, being almost 10-fold more potent than PGE_1 and PGE_2.[39] Iloprost (50 nM) abolished TNF_α secretion and inhibited IL-1β secretion by 45% in LPS-stimulated THP-1

cells.[40] Iloprost also abolished the LPS-induced increase in TNF, mRNA and reduced that of IL-1β mRNA, suggesting that iloprost exerts its effect, at least in part, at the level of gene transcription. The U937 cell line is an alternative source of monocyte-like cells, and as seen with THP- 1 cells, both prostacyclin and PGE_2 inhibited IL-1 expression by endotoxin-stimulated U937 cells.[41] Furthermore, this study showed that IL-1 production by peripheral blood monocytes was also inhibited by PGE_2; unfortunately prostacyclin was not tested. In addition though, cyclic AMP mimetics also inhibited IL-1 production by U937 cells, and the inhibitory effect of prostacyclin and PGE_2 was potentiated by IBMX. More detailed analysis of the site of action of cyclic AMP suggests that it acts posttranscriptionally.

We have also done some work with another human monocytic cell line known as Mono Mac 6. When stimulated with LPS, these cells secrete TNF, and this response can be inhibited by iloprost, PGE_2 and the adenosine A_2-receptor agonist NECA with IC_{50} values of approximately 30 nM, 3 nM and 30 μM respectively,[30] (Fig. 50). Octimibate failed to inhibit LPS-stimulated TNF, production, but clearly antagonised the effects of iloprost and PGE_2, but not NECA. The effect of octimibate on LPS-stimulated TNF_α production was mirrored by its effect on cyclic AMP production by Mono Mac 6 cells. The ability of octimibate to antagonise iloprost in Mono Mac 6 cells presumably reflects the partial agonist nature of octimibate, and would suggest that IP-receptor-mediated inhibition of LPS-stimulated TNF_α production is a poorly coupled system. As anticipated, one would not expect octimibate to inhibit the activity of a nonprostanoid receptor agonist such as NECA. Similarly, there is no other evidence that octimibate interacts with the EP_2/EP_4-receptor system, therefore its ability to antagonise PGE_2 is somewhat surprising.

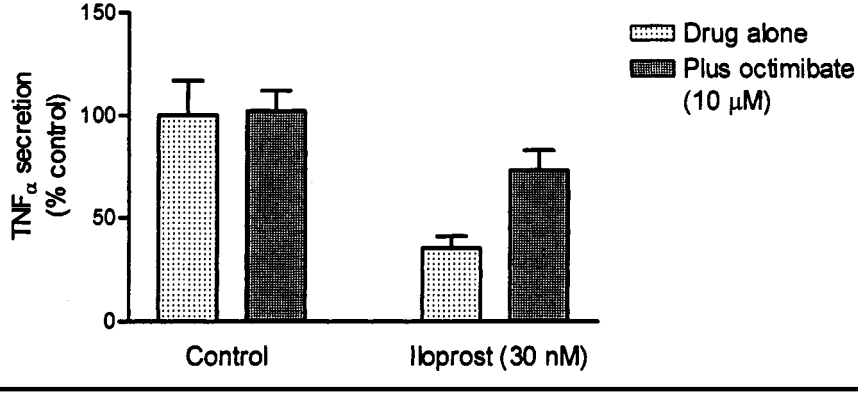

Figure 50. Effect of octimibate on LPS-stimulated secretion of TNF_α from the human monocytic cell line, Mono Mac 6. Reprinted from Wise et al.,[30] with permission.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a multifunctional cytokine which has been suggested to contribute to the pathogenesis of various inflammatory disorders by raising the functional responsiveness of effector cells, particularly neutrophils. Incubation of human peripheral blood monocytes with LPS produces more than a 10-fold increase in GM-CSF release which was significantly inhibited by cicaprost with an IC_{50} value of approximately 20 nM, and which appears to result from inhibition of GM-CSF mRNA expression.[42] Therefore, IP agonists may have therapeutic application in septic shock and other forms of acute lung injury in which neutrophils are believed to play a central role. All the data presented above points to a role of prostacyclin and/or PGE_2 in the autoregulation of monocyte expression of cytokine activity that may then modulate inflammatory and immunologic reactions.

3.5 Tissue factor procoagulant activity

Tissue factor (thromboplastin, factor III) is a membrane-bound glycoprotein that plays a critical role in initiating blood coagulation, and can be synthesised by monocytes and cells of the vascular endothelium in response to inflammatory stimuli and injury. Exposure to LPS leads to transcription of the tissue factor gene[43,44] and expression of procoagulant activity[45-47] by both cell types. In addition, endothelial tissue factor expression is also stimulated by exposure to the cytokines IL-1β and TNF_α,[48-51] both of which are secreted by activated monocytes.[52,53]

Crutchley et al.[40] have shown that the prostacyclin analogues iloprost, carbacyclin and ciprostene (10 nM to 1 μM), had no direct effect on human umbilical vein endothelial tissue factor expression in response to LPS, IL-1β or TNF_α, but indirectly decreased expression due to the inhibition of monocyte cytokine secretion. As shown in Section 3.4 above, IP agonists can inhibit monocyte cytokine production. Thus pretreatment of THP- 1 cells with 50 nM iloprost before the addition of LPS led to a marked inhibition of the ability of the THP-1 cell-conditioned medium to induce endothelial tissue factor expression.[40] Although TNF_α-stimulated endothelial cells showed a slight increase in prostacyclin production, incubation with indomethacin had no effect on tissue factor expression, indicating that endogenous prostacyclin does not modulate tissue factor expression by endothelial cells.[40]

Iloprost and other IP agonists can inhibit endotoxin-stimulated procoagulant activity in both human peripheral blood mononuclear cells (monocytes plus lymphocytes; iloprost IC_{50} value of 20 nM[54]), and in THP-1 cells (Table 17).[55] When experiments are performed in whole blood, monocyte procoagulant activity is amplified by T lymphocyte cooperation.

However, because iloprost can directly inhibit endotoxin-induced activity in THP-1 cells, these results suggest that iloprost acts primarily on the monocyte and not at the level of lymphocyte cooperation.[54] The order of potency of IP agonists to inhibit endotoxin-induced procoagulant activity in THP-1 cells (cicaprost = iloprost > PGE_1 > carbacyclin = ciprostene) strongly suggests that this effect is mediated by activation of the IP_1-receptor, and as such might be expected to involve the production of cyclic AMP. Iloprost produced a concentration-dependent increase in THP- 1 cell cyclic AMP production, with a 27-fold increase at 5 nM.[55] Iloprost therefore appears to increase cyclic AMP levels in THP-I cells over the same concentration range at which it inhibited tissue factor expression. The PDE inhibitor lBMX enhanced both iloprost-stimulated cyclic AMP production, and potentiated the inhibitory effects of iloprost on THP-1 cell tissue factor expression. The effects of iloprost could be mimiked by forskolin and dibutyryl cyclic AMP, but not by dibutyryl cyclic GMP or sodium butyrate; results which strongly suggest that IP agonists inhibit monocyte tissue factor expression by stimulating adenylate cyclase. Because the combination of iloprost and IBMX was more potent in inhibiting tissue factor expression than would be predicted from their effects on cyclic AMP, it was suggested that possible additional mechanisms should be considered.[55]

Table 17. Inhibition of induced monocyte (THP-1 cell) procoagulant activity by IP agonists in vitro

Procoagulant-inducer	*IC_{50} values (nM)*			
	Iloprost	*Cicaprost*	*Carbacyclin*	*Ciprostene*
IL-1β (2 U ml-1)	5	nd	500	500
TNFα (5 ng ml-1)	5	nd	500	500
Endotoxin (100 ng ml-1)	5	5	500	500

Data from Crutchley et al.[55] nd = not determined.

Increased tissue factor expression by monocytes may contribute to the disseminated intravascular coagulation associated with disorders such as allograft rejection, bacterial infection, carcinoma, and immune disease (for reviews see Edwards & Rockles,[56] and Lyberg[57]). It has also been suggested that increased monocyte tissue factor expression may contribute to the postoperative thrombotic state. Thus IP agonists may have clinical potential for the treatment of thrombotic disorders in which elevated monocyte procoagulant activity plays a role; especially since iloprost can significantly blunt further development of procoagulant activity in cells that are already expressing elevated levels of activity.[54]

3.6 Atherosclerosis

Atherosclerosis is a complex disorder characterised by thickening of the arterial intima, and there is evidence that prostacyclin may function as an important regulatory factor in aortic cholesteryl ester metabolism, with an inverse correlation between prostacyclin and sterol accumulation.[58] Vascular smooth muscle cells are an important element of an atherosclerotic lesion, and their role in atherosclerosis has been discussed in Chapter 6. Here we will concentrate on the relationship betweem macrophages and prostacyclin in atherosclerosis. For example, as a result of the accumulation of low density lipoproteins (LDLs) and liposomes in arteries, we see monocyte adhesion to the endothelium and migration into the subendothelial space.[59-62] These monocyte-derived macrophages collect modified arterial LDL via the scavenger receptor, are transformed into foam cells,[63] and appear to be modulated by prostaglandins.

When stimulated by acetyl-LDL, murine peritoneal macrophages increase their levels of prostacyclin and TXA_2 and decrease PGE_2, but these changes are minor compared to the increased eicosanoid production in response to zymosan or A23187.[64] Furthermore, the major [^{3}H]-arachidonic acid metabolite released in response to acetyl-LDL were LOX products, whereas COX products were dominant in zymosan or A23187-stimulated macrophages. In contrast, another study found that activated cholesterol-rich (i.e. acetyl-LDL treated) mouse peritoneal macrophages released less prostacyclin and PGE_2 than control cells, and that LOX products predominated in PMA, A23187 or zymosan-stimulated macrophages.[65] Mathur et al.[65] proposed that the decreased production of prostacyclin by cholesterol-rich macrophages would be unable to counterbalance the increased TXA_2 production by cholesterol-rich platelets, resulting in an increased propensity for vessel spasm and thrombosis. To confuse the picture further, we should note that another study found that oxidised LDL doubled the production of prostacyclin and trebled PGE_2 in mouse peritoneal macrophages, but acetyl-LDL had no effect on PGE_2 (prostacyclin was not measured in this part of the experiment).[66] Overall we see that with regard to the increase in prostaglandins, acetyl-LDL stimulated macrophages behave similarly to zymosan-activated macrophages although the former have a slower time course.

In mouse peritoneal macrophages, octimibate (5 μM) significantly decreased the uptake of acetyl-LDL by the scavenger receptor.[31] Therefore, when tested in vivo, the IP agonists octimibate and BMY 42393 may have reduced the size of foam cells by inhibiting the uptake of oxidised LDL.[31] Although octimibate was originally identified as an inhibitor of acyl coenzyme A cholesterol acyltransferase (ACAT), this property cannot

entirely account for the effectiveness of these nonprostanoid prostacyclin mimetics in models related to atherosclerosis. It has therefore been proposed that because the IP agonists octimibate and BMY 42393 suppressed monocyte-macrophage atherogenic activity and cytokine production, they would be useful in inhibiting the development of early atherosclerosis.[31]

4. LYMPHOCYTES

Although prostaglandins may be important in various disease states, their role in the immune system of normal individuals has been suggested as relatively minor because the immune responses of humans and animals given longterm and sometimes high-dose COX inhibitors appears to be quite normal.[67] By the early 1980's, it became clear that in addition to the well known role of prostaglandins as mediators of inflammation, they had a less well recognised role as negative regulators of humoral and cellular immunity (for reviews see Goodwin & Webb,[67] and Morley[27]) By the early 1990's, it was apparent that PGE_2 was the important prostanoid involved with cells of the immune system; showing both stimulatory and inhibitory properties. Therefore, PGE_2 is now considered as a regulatory modulator of immunity (for reviews see Phipps et al.,[68] Plescia & Rack,[69] and Ninnemann[70]).

As a consequence of the cloning of the IP1-receptor in 1994, it became apparent that IP_1-receptors may have some role to play in lymphocytes with the discovery of IP_1-receptor mRNA in the mouse[71] and rat spleen and thymus;[72] (see Chapter 4, Tables 6 and 7). Surprisingly, no IP_1-receptor mRNA was detected in human thymus[73] although IP1-receptor cDNA clones have been isolated from this source.[74] It has therefore been suggested that the differences between IP_1-receptor mRNA levels in human and mouse spleen and thymus reflect different stages of maturation of thymocytes in different lymphatic organs of rodent and human specimens.[75] Further experiments using in situ hybridisation techniques demonstrated that hybridisation signals were visible only in the medulla and not the cortex of rat thymus, and were predominantly in thymocytes and not stromal cells.[76] The IP_1-receptor mRNA is therefore exclusively present in the thymic medulla where $CD4^+8^-$ and $CD4^-8^+$ thymocytes are harboured. Since prostacyclin is produced by thymic stromal cells and IP_1-receptor mRNA is localised to thymocytes and is distributed in lymphocytes in the white pulp of the spleen,[76] then we should look further now for the functional significance of these unexpected observations.

4.1 Prostacyclin production

Early conflicting results suggested that normal lymphocytes alone do not produce prostaglandins, and any apparently positive results were due to contamination by monocytes or platelets.[77] It seems that lymphocytes can utilise PGH_2 from other cell sources and transform it into prostacyclin using their own prostacyclin synthase,[11] and this transcellular metabolism may complicate the picture. For example, human lymphocytes labelled with [^{14}C]-arachidonic acid failed to form 6-oxo $PGF_{1\alpha}$ when stimulated with phytohemagglutinin (PHA) or A23187. However, when incubated with activated platelets, or with PGH_2, lymphocytes produced 6-oxo $PGF_{1\alpha}$ which presumably indicates that prostacyclin was responsible for the anti-aggregatory activity of lymphocytes when co-incubated with platelets.[11]

The human leukaemic T cell line Jurkat produces about 10-times as much prostacyclin as PGE_2 under basal conditions, but cell activation by exposure to PHA or concanavalin A decreased the total release of [^{3}H]-prostaglandins from [^{3}H] -arachidonic acid -labelled cells.[77] Therefore, Aussel et al.[77] suggested that endogenous prostaglandin synthesis by lymphocytes might serve to keep them in a quiescent state, while a reduction in prostaglandin synthesis is required as part of the activation sequence. You will note that we could draw a similar conclusion with regard to macrophages, as cell activation was often accompanied by decreased prostacyclin and PGE_2 synthesis (see Section 2.1 above).

4.2 Adenylate cyclase activity

As seen for activated (elicited) macrophages (Table 16), cyclic AMP production in response to PGE_2 also increases in activated human T lymphocytes when compared to resting cells,[78] but there is no equivalent data for prostacyclin or other IP agonists. Certainly in the T-cell line Jurkat, resting cells are at least 10,000-fold more sensitive to PGE_2 than to carbacyclin,[79] thus in the absence of a PDE inhibitor, carbacyclin showed little activation of adenylate cyclase at concentrations < 100 μ M.

5. FUNCTIONAL EFFECTS OF IP AGONISTS ON LYMPHOCYTES

5.1 Activation and differentiation

PGE_2 can shape the immune response by stimulating the production of IgE antibody by B lymphocytes and the synthesis of T-helper type 2 cytokines (e.g., IL-4, IL-10), while inhibiting production of T-helper type 1 cytokines (e.g., interferon-γ (IFNγ), IL-12). In an extensive study to determine the EP-receptor subtypes involved in these processes, it was shown that 10 μM cicaprost and iloprost failed to mimic the ability of PGE_2 to inhibit class II MHC hyperexpression by mouse B lymphocytes.[80] Cicaprost and iloprost also failed to prevent B lymphocyte enlargement and proliferation when stimulated with LPS and IL-4, and did not enhance IgE production.[80] Because the effect of PGE_2 appeared to be mediated by EP_2 and/or EP_4-receptors, and since the effect seemed to be cyclic AMP-dependent, then we might conclude that mouse B lymphocytes lack functional IP-receptors. In contrast, there is one study supporting an inhibitory role for IP-receptors in mouse T lymphocytes. Thus, 100 nM iloprost inhibited the secretion of IL-2, IL-4 and IFNγ from mouse T lymphocytes primed with the contact-sensitising agent picryl chloride.[81]

Again in a study primarily aimed at defining the EP-receptor subtype mediating the activity of PGE_2, we are able to find further negative data for IP agonists and lymphocytes. Thus, 1 μM prostacyclin failed to increase the cytosolic level and secretion of matrix metalloproteinase (MMP)-9 by the human leukaemic T cell line HSB.2;[82] MMPs degrade diverse components of the extracellular matrix and therefore aid the migration of T-cells out of blood vessels.

In contrast to these negative studies, the antimetastatic effect of iloprost appears to rely on its immunomodulatory action in addition to its antiplatelet activity.[83] In their mouse model of experimental metastasis, Costantini et al.[83] showed that iloprost had a far more potent and persistent antimetastatic activity compared with prostacyclin, lasting up to 6 h after tumour cell challenge. Iloprost treatment increased natural killer (NK) lytic activity of spleen cells and T lymphocyte mediated cytotoxicity ex vivo, but had no major modulation of the humoral antibody (B lymphocyte) responses. It is important to note though that the immunomodulating effects of iloprost were highly dependent on the drug concentration and dosing routes used. In addition, there is further data which suggests a role for IP-receptors in T lymphocytes. For example, the benzindene prostacyclin analogue 15 AU8 1 inhibits human mixed lymphocyte culture responses in a concentration-

dependent manner; thus inhibiting both PHA- and anti-CD3-activated human lymphocytes with IC_{50} values of approximately 300 nM and 14 nM, respectively.[84]

5.2 Organ rejection

Prostacyclin, PGE_2 and PGD_2 are recognised as antirejection metabolites.[1,85] They share an ability to increase cyclic AMP with resulting stabilisation of inflammatory cells, vasodilation (thus maintaining blood flow), and for PGE_2 additional suppression of immunological recognition and rejection of the transplanted tissue. In contrast, TXA_2, which is vasoconstrictor and can aggregate platelets, will reduce graft survival.

The human foetus exists in an environment rich in prostaglandins.[86] The concentration of 6-oxo $PGF_{1\alpha}$ in foetal plasma at midterm is much higher than in the maternal peripheral plasma, suggesting an important role for this antirejection metabolite during early pregnancy.[87] MacKenzie et al.[87] suggested that prostacyclin maintains the ductus arteriosus and the tone of the placental and umbilical vasculature. However, Narumiya's IP_1-receptor knockout mice seem to have no problems in bringing their pups to full term,[88] suggesting that there is redundancy in the system and the role played by prostacyclin can be taken by another player.

Prostacyclin can prolong allograft survival, possibly via an antiplatelet effect and/or vascular effect. In acute kidney rejection, cortical prostacyclin formation is significantly enhanced, suggesting that this might be a self-protecting mechanism; unfortunately this process is subsequently overwhelmed by irreversible rejection.[89] The accumulation of platelets in transplanted organs undergoing acute rejection is therefore critical, and this problem may be countered by prostacyclin. In addition, prostacyclin may reduce the extent of organ rejection by an immunosuppressive effect; OP-41483 (15-cyclopentyl-ω-pentanor carbacyclin) alone provides some protection against canine renal allograft rejection,[90] and as shown in Section 5.1 above, the prostacyclin analogue 15 AU81 is a potent inhibitor of human T lymphocyte activation and can potentiate the immunosuppressive activity of cyclosporine and prolong the survival of rabbit renal allografts by an as yet unclear mechanism.[84] Iloprost also synergises with cyclosporine, prolonging the survival of rat cardiac allografts.[91] This synergistic effect of IP agonists on cyclosporine could be used to broaden its therapeutic window by potentiating immunosuppression and mitigating nephrotoxic effects.

6. DISCUSSION

Elicited macrophages clearly lose their capacity to produce prostacyclin, and this in turn may render them more susceptible to regulation by exogenous IP agonists. It is possible that the decreased production of prostacyclin by macrophages upregulates IP-receptors (or conversely, prevents prostacyclin-mediated IP-receptor downregulation), thus making the macrophages more responsive to subsequent prostacyclin challenge. It is time to recognise that PGE_2 is not the only important prostaglandin involved in inflammatory conditions. Although much of the early work in this field of research noted interesting similarities and dissimilarities between PGE_2 and prostacyclin, few of these observations on macrophage function have been pursued. Limited data on human THP-I cells suggests that the IP-receptors on macrophages are similar to the platelet IP_1-receptor; perhaps now that we have a better selection of IP agonists to work with, it is time to reevaluate to role of IP-receptors in regulating macrophage function in particular. For example, it has been suggested that the general cytoprotective action of IP agonists could probably be explained in part as a consequence of IP agonist-induced macrophage modulation.[36] and we have seen here that IP agonists can inhibit many macrophage functions such as chemotaxis, phagocytosis and cytokine production. As such we might expect IP agonists to be useful in the treatment of septic shock and coagulation disorders, and in the prevention of atherosclerosis. The studies reported here highlight the need to understand the distinction between resident and elicited macrophages, since these phenotypes clearly influence the ability of macrophages to respond to prostacyclin.

Of the limited data examining lymphocyte responses to IP agonists, we could tentatively conclude that IP-receptors are unimportant for mouse B lymphocytes. In contrast, mouse T lymphocytes and human T lymphocytes, but not human T lymphocyte cell lines, are responsive to IP agonists. Again, with the better selection of IP and EP agonists currently available, it is time to more thoroughly characterise the role of lymphocyte IP-receptors, and to monitor their responsiveness during different stages of lymphocyte activation. The significance of the discovery of IP_1-receptor cDNA in rat thymocytes is yet to be appreciated but may be relevant to the ability of IP agonists to potentiate the immunosuppressive effect of cyclosporine. Because of their multifunctional properties, IP agonists may ultimately find a place in facilitating organ transplants.

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Chapter 9

IP-receptors on sensory neurones

1. INTRODUCTION

In addition to demonstrating that vascular IP-receptors were not involved in homeostatic regulation of blood pressure, the IP_1-receptor knockout mice of Murata et al.[1] provided a much greater surprise in highlighting the role of IP_1-receptors in inflammatory pain. Thus, the inflammatory pain responses of IP_1-receptor knockout mice were reduced to the levels seen in indomethacin-treated animals and these results clearly identify prostacyclin as an important mediator of inflammation and pain, challenging the supremacist role of prostaglandin E_2 (PGE_2). These results came at a time when we were beginning to appreciate that IP-receptors had a function in neuronal systems, and were becoming increasingly aware that the role of prostacyclin in sensory systems had long been overlooked in favour of PGE_2.[2,3] It has been pointed out previously that if prostaglandins produce their sensitising effects by a common mechanism, then we would expect a common order of potency for the arachidonic acid metabolites.[4] Indeed this is the case; in many instances prostaglandin E_1 (PGE_1) and other IP agonists are more potent than PGE_2. In this chapter we aim to bring to the fore the data on prostacyclin, with reference to studies using PGE_2 where appropriate.

One characteristic of inflammation is hyperalgesia in which there is an enhanced response to a painful stimulus and a decrease in pain threshold. Inflammatory mediators can be released from a variety of sites such as the injured tissue, the microvasculature or the primary afferent terminals,[4] and can sensitise or excite peripheral nerve endings and contribute to the

phenomenon of hyperalgesia. It is possible that different cyclo-oxygenase (COX) products sensitise skin to nonmechanical, noxious stimuli (e.g. chemical or thermal). Nevertheless, while it is possible that different COX products selectively modulate different hyperalgesic stimuli, results suggest that prostacyclin and PGE_2 are the most significant hyperalgesic arachidonic acid metabolites for mechanical stimuli.[5]

Although prostaglandins alone rarely produce a pain response in man, they clearly potentiate the pain response to other stimuli.[6] Furthermore, Crunkhorn & Willis[7] showed that nanogram doses of PGE_1 and PGE_2 injected intradermally into the human forearm induced weal and flare responses, clearly implicating PGEs in inflammatory responses in humans. Even though prostacyclin alone may not produce overt pain, it does appear crucial in mediating some of the algogenic effects of bradykinin, which is a well-characterised inflammatory agent.[8-11]

The major metabolite of prostacyclin, 6-oxo $PGF_{1\alpha}$, is found in high concentrations in inflammatory exudates, indicating that prostacyclin is synthesised and released in areas of inflammation.[12-14] Prostaglandin levels are increased in the synovial effusions of patients with rheumatoid or psoriatic arthritis, and since both steroids (e.g. methylprednisolone) and COX inhibitors (e.g. indoprofen) can decrease synovial prostacyclin, but only indoprofen decreased PGE_2 in these circumstances, then prostacyclin may be seen as a major prostaglandin involved in synovial inflammation.[14] Presently the source of this prostacyclin is unclear as it could derive from vascular or synovial cells.

In most studies examining the effects of prostacyclin on sensory neurones, the primary action of prostacyclin is to sensitise the neurones to the excitatory effects of other agents.[4,15-17] This sensitising action of prostacyclin is seen at nanomolar concentrations, i.e. the same range as the concentrations of prostacyclin found in inflammatory exudates.[12-14] Geppetti et al.[18] also showed that prostacyclin increased the outflow of calcitonin gene-related peptide (CGRP) from isolated guinea-pig heart; this increase was blocked by capsaicin pretreatment, suggesting that the release was from capsaicin-sensitive sensory neurones. Thus we see that IP-receptors may be involved in both sensitising the terminals of primary sensory afferents and additionally may act on these same terminals to release inflammatory neuropeptides. Furthermore, it has been suggested that in spite of the functional specialisations of the peripheral and central presynaptic terminations, the primary sensory neurones associated with inflammation respond as an integral unit.[19] Thus, any biophysical or biochemical membrane event associated with a lowering of the peripheral threshold triggered at one neuronal terminal may be rapidly spread throughout the neurone to influence functioning at other terminals of the same neurone.

Whether or not IP agonists release neuropeptides/neurotransmitters from both the peripheral and central terminals of primary afferent nociceptive fibres remains to be clarified. In addition to causing hyperalgesia in the periphery, prostaglandins may therefore be involved in regulation of nociceptive sensory input at the level of the spinal cord.[20] Whether prostacyclin can be included here remains unknown, but since spinal cord levels of prostacyclin exceed those of PGE_2 after injection of Freund's adjuvant into the rat paw,[21,22] then the possibility is high.

2. BIOCHEMICAL EVIDENCE

2.1 [^{3}H]-iloprost binding

Autoradiographic studies of [^{3}H]-iloprost binding in the rat central nervous system (CNS) show that the regions with the highest density of specific binding sites (nucleus tractus solitarius (NTS), area postrema and spinal trigeminal nucleus caudalis) possess a common feature in that they receive the primary sensory afferents.[23] Furthermore, with respect to vagal afferent fibres, removal of the nodose ganglion (Fig. 51) significantly reduced [^{3}H]-iloprost binding in the NTS, suggesting that the binding sites are located presynaptically on the central terminals of the primary sensory neurones. Ligation studies of the vagus nerve also support the conclusion that [^{3}H]-iloprost binding sites are on the presynaptic terminals of the primary viscerosensory neurones, showing that [^{3}H]-iloprost binding sites in the NTS are transported there by axoplasmic flow from the nodose ganglion. Sectioning of the dorsal root of the spinal nerve (dorsal rhizotomy) resulted in significant loss of [^{3}H]-iloprost binding in the superficial layers of the dorsal horn on the operated side, again indicating a presynaptic location on the central terminals of the peripheral unmyelinated or finely myelinated primary sensory fibres (Fig. 52). Additionally, [^{3}H]-iloprost binding sites were also detectable in intact nodose ganglia (25 fmol mg tissue^{-1}) and intact dorsal root ganglia (DRG: 4 fmol mg tissue^{-1}), whereas [^{3}H]-PGE_2 binding was less than 1.5 fmol mg tissue^{-1} in these ganglia.

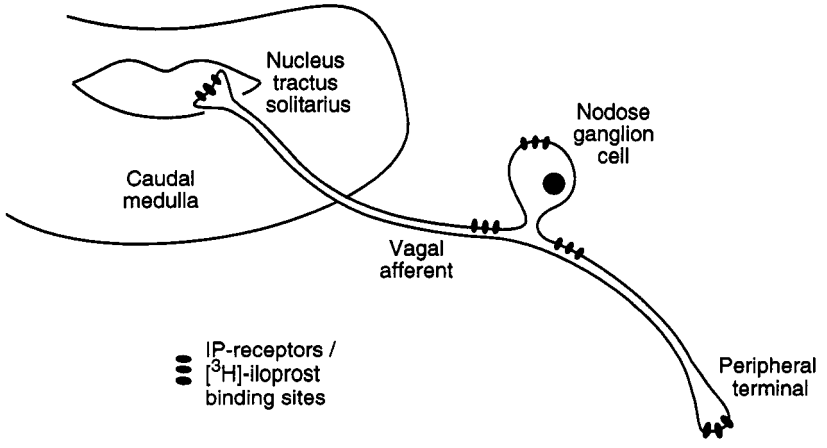

Figure 51. Distribution of IP-receptors in viscerosensory neurones.

In addition to [^{3}H]-iloprost, [^{3}H]-isocarbacyclin also bound strongly in the NTS and spinal trigeminal nucleus,[24] but its distribution in the CNS in general was otherwise quite distinct from that of [^{3}H]-iloprost. Together these results suggest that the classical platelet IP_1-receptor is the subtype involved in inflammatory pain.

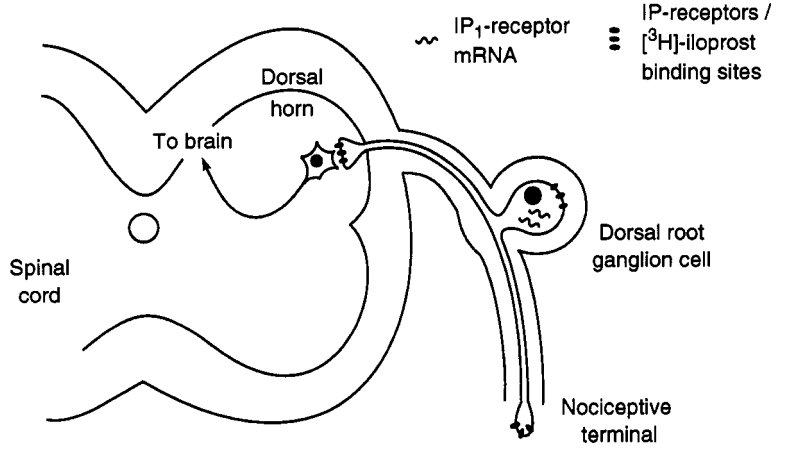

Figure 52. Distribution of IP-receptors and IP_1-receptor mRNA in primary nociceptive neurones.

2.2 Distribution of IP_1-receptor mRNA

Following from the successful cloning of the IP_1-receptor, Oida et al.[25] were able to probe various mouse organs to look for expression of IP_1-receptor mRNA. Matching the data from the autoradiographical studies described above (Section 2.1), they found little IP_1-receptor mRNA expression in mouse CNS and spinal cord, but the highest density of all signals was on neurones of the DRG (see Chapter 4, Table 7). IP_1-receptor mRNA was highly expressed in about 40% of the neurones in the DRG, including both small and large diameter neurones. The small diameter neurones in the DRG are capsaicin-sensitive and therefore belong to peripheral C- and Aδ--nociceptive fibres.[26] This was confirmed by the demonstration that about 70% of the DRG neurones co-expressed both IP_1-receptor mRNA and PPTA (substance P precursor) mRNA. Approximately 20 - 40% of the IP_1-receptor mRNA-positive neurones co-expressed EP_1, EP_3 and EP_4-receptor mRNA (EP_2-receptor mRNA was not examined).

Evidence presented elsewhere in this chapter would suggest that IP_1-receptors derived from this mRNA are expressed in both the peripheral and central endings of the primary afferent neurones (see Figs. 51 and 52). There is extensive evidence that prostacyclin acts in the periphery as a hyperalgesic agent (see Section 4.2). There is also evidence that intrathecal administration of COX inhibitors can block substance P and glutamate-induced hyperalgesia,[27] and that [^{3}H]-iloprost binding sites exist on the presynaptic terminals of nociceptive fibres terminating in the rat dorsal horn,[23] suggesting that prostacyclin or other prostaglandins may act within the spinal cord.

3. IP-RECEPTORS IN SPINAL CORD AND SENSORY GANGLIA

3.1 Spinal cord

While there is evidence for [^{3}H]-iloprost binding sites on the central terminals of primary afferent sensory fibres,[23] there have been few studies to determine the function of these IP-receptors in areas such as the superficial dorsal horn of the spinal cord. The neonatal rat spinal cord can be maintained in culture with a functionally connected tail and when bradykinin is applied to the tail, it activates capsaicin-sensitive peripheral fibres and thus evokes a depolarisation recordable from the spinal ventral root.[28] This

response to bradykinin is inhibited by COX inhibitors, mimicked by phorbol ester, is not affected by mepacrine (phospholipase A_2 inhibitor) and furthermore involves activation of pertussis toxin-insensitive bradykinin B_2-receptors. Responses of nociceptors in the rat tail, when activated by chemical or thermal stimuli, were enhanced in the presence of prostacyclin, cicaprost and cyclic AMP.[29] Results indicated that prostacyclin could sensitise peripheral nerve endings to noxious stimulation without directly activating the nociceptors. Despite these observations that prostacyclin and cicaprost could sensitise rat nociceptors to thermal stimulation,[29] IP_1-receptor knockout mice responded normally to the hot-plate and tail-flick tests,[1] suggesting that IP_1-receptors have no role in facilitating pain transmission at the spinal or supraspinal levels since the hot-plate test is a model of central nociceptive pathways.[30] Contrary to this evidence for a lack of role for IP_1-receptors in spinal processing, nonsteroidal anti-inflammatory drugs (NSAIDs) appear to exert a direct spinal action by blocking the heightened sensitivity to pain induced by activation of spinal glutamate and substance P receptors.[27] These results would then suggest that spinal prostaglandins are critical for the augmented processing of pain information at the spinal level, but of course this might be mediated by prostaglandins other than prostacyclin. Moreover, this study indicates that the analgesic effects of NSAIDs can be dissociated from their anti-inflammatory actions, and therefore that the spinal action of NSAIDs is independent of changes in peripheral terminal sensitivity.[27]

From studies on the release of CGRP from rat spinal cord following dorsal root stimulation, it was concluded that bradykinin acting on B_2-receptors in the spinal cord caused the formation of prostaglandins, which in turn caused an enhancement of neuropeptide release from primary afferent nerve terminals in the dorsal horn.[31] This enhanced release may be secondary to activation of adenylate cyclase as forskolin and phosphodiesterase inhibition also enhanced the bradykinin-evoked release of CGRP. Because B_2-receptors are mainly associated with primary afferent nerve terminals, it was suggested that prostaglandin production is also a function of these structures. In this example, neither prostacyclin nor PGE_2 were able to evoke neuropeptide release alone, but prostacyclin was much more potent than PGE_2 in potentiating bradykinin-evoked release. Although Wenrich[32] showed that bradykinin inhibits a Ca^{2+}-dependent, slowly developing and long-lasting spike afterhyperpolarisation (AHP_{slow}) in vagal afferent C-fibres, it was thought that inhibition of AHP_{slow} could not entirely account for the effect of bradykinin seen here on neuropeptide release.[31]

Although prostacyclin and PGE_2 alone failed to evoke the release of CGRP from rat spinal cord following dorsal root stimulation,[31] PGE_2 was able to release substance P from rat spinal cord slices, at 43% the response

evoked by KCl.[33] The PGE_2-evoked release of substance P was Ca^{2+}-dependent and similar to results obtained from embryonic rat DRG neurones in culture.[33] In rat spinal cord synaptosomes, PGE_2 increases glutamate and aspartate release by a Ca^{2+}-dependent mechanism, being maximally effective at 1 nM.[34] While this increased release of excitatory amino acids may be statistically significant, it was again small at 118% control compared to 162% control release for KC1. If we look further at the data available for PGE_2, we see that intrathecal PGE_2 itself may cause spinal sensitisation of the primary afferent neurones by the release of glutamate which in turn activates *N*-methyl-D-aspartate (NMDA)-receptors,[19] and in voltage-clamped neurones in rat NTS slices, PGE_2 increased the mean size of evoked excitatory postsynaptic currents, possibly by facilitating evoked and spontaneous release of glutamate vesicles.[35] We can also see that excitatory amino acids may in turn affect prostaglandin production, thus kainic acid can evoke the release of PGE_2 from rat spinal cord, and this release is inhibited by indomethacin.[36] In superfused rat spinal cord slices, capsaicin-evoked release of PGE_2 is partially blocked by COX inhibitors, results which further highlight the relative importance of prostaglandins in spinal nociceptive processing evoked by primary afferent activation.[37]

3.2 Dorsal root ganglia

Because of the difficulties of studying fine unmyelinated nociceptive fibres in vivo, studies of nociception have tended to rely more on the use of tissues and cells maintained in vitro. Sensory neurones from the DRG have been well studied and these can be divided into two broad populations: (1) neurones with large-diameter cell bodies and fast-conducting myelinated axons (A-fibres), and (2) neurones with small-diameter cell bodies and unmyelinated slowly conducting axons (C-fibres). Most of the small diameter neurones are activated by tissue-damaging stimuli whereas the larger neurones are principally involved in mechanonociception or proprioreception (although Aδ-fibres have a nociceptive role).[4,38]

In embryonic rat DRG neurones, carbacyclin causes a Ca^{2+}-dependent release of substance P and CGRP and potentiates capsaicin, bradykinin and KC1-evoked release of neuropeptides.[39,40] In addition, prostacyclin itself also potentiates capsaicin-evoked neuropeptide release. The dose-response relationship for carbacyclin suggests that low concentrations of prostacyclin would sensitise sensory neurones to other stimuli, while higher concentrations evoke release directly. Despite the evidence that bradykinin-evoked nociceptor responses are partially mediated via generation of prostacyclin (see Section 4.3), there is no evidence for cyclic AMP production by the capsaicin-sensitive population of cultured sensory

neurones in rat DRG in response to bradykinin; only cyclic GMP production.[41] In fact, only in the non-neuronal cells derived from DRG cell preparation did bradykinin increase cyclic AMP. In adult rat DRG neurones, cicaprost increases cyclic AMP production with an EC_{50} value of 14 nM, and is approximately 10-fold more potent than PGE_2.[42] At concentrations producing near maximal stimulation of adenylate cyclase activity, these agonists also stimulate the accumulation of [^{3}H]-inositol phosphates, again with cicaprost apparently more potent than PGE_2. In light of the coupling of the IP_1-receptor to G_s and G_q-proteins (see Chapter 4), it is interesting to note that the IP agonist-stimulated increase in phosphoinositide hydrolysis was not dependent on cyclic AMP accumulation since forskolin was unable to increase [^{3}H]-inositol phosphates despite increasing cyclic AMP to a greater extent than the prostanoids.

A few studies on F-11 cells (rat DRG x mouse N18TG2 neuroblastoma) indicate a cyclic AMP response to prostacyclin which increases 2-fold in cells differentiated to a more neuronal phenotype.[43] Although cyclic AMP elevations in response to prostacyclin can inhibit bradykinin-stimulated IP_3 release from F-11 cells, the inhibitory effect was never more than 50% and considered unlikely to be of functional significance.[44] With hindsight we can now question whether it was the DRG cell, the neuroblastoma cell, or both cell phenotypes which were responsible for the prostacyclin-mediated responses of F-11 cells. Neuroblastoma cells derive from cells of the neural crest,[45] as do the sensory neurones of the peripheral nervous system.[38]

In adult DRG neurones, PGE_2 induces a Ca^{2+}-dependent, N-type channel-dependent release of substance P,[46] yet its potentiation of bradykinin-induced release of substance P and CGRP was apparently not via L-, N- or P-type voltage-sensitive calcium channels.[47] Inhibition of a slow Ca^{2+}-dependent K^+ current (I_{KCa}) is generally thought to underlie the AHP_{slow}, resulting in nociceptor sensitisation, however most evidence comes from studies using the nodose ganglia. When examined in adult rat DRG, patch-clamp electrophysiology techniques suggest that PGE_2-induced sensitisation must involve the modulation of ionic currents in addition to that underlying AHP_{slow}.[48] Although PGE_2 could release substance P from adult DRG neurones, in neonatal rat DRG neurones PGE_2 has no effect alone yet potentiates bradykinin-induced release of substance P and CGRP,[49] and potentiates the slow depolarisation and burst of action potentials in response to bradykinin by a cyclic AMP-dependent mechanism.[50] When bradykinin, capsaicin or PGE_2 were applied to the processes of cultured mouse DRG cells, they all led to the generation of a tetrodotoxin-sensitive action potential in the cell body;[51] obviously it would be interesting to determine how prostacyclin compares with PGE_2 in this regard.

3.3 Viscerosensory ganglia

[^{3}H]-iloprost binding sites have been described on rat nodose ganglia, and these binding sites migrate in both directions away from the cell body by axoplasmic flow.[23] These [^{3}H]-iloprost binding sites presumably represent the IP-receptors which can be readily identified on desheathed vagus nerve preparations using extracellular recording techniques;[42,52,53] see Figure 53. Of the standard prostanoid agonists studied, prostacyclin and carbacyclin were clearly the most potent with EC_{50} values of 4 nM and 13 nM, respectively, for depolarising C-fibres of rat isolated vagus nerve.[42] The order of potency of prostanoids and high potency of cicaprost (EC_{50} 631 pM) is typical of an receptor.[42,53] Although prostacyclin-induced depolarisations were slow in onset when compared with those induced by 5-HT acting on ligand-gated ion channels (see Fig. 53A), the maximal response (400 μV) was quite comparable.[42,52] Responses to cicaprost were unaffected by either tetrodotoxin or removal of calcium from the bathing fluid, suggesting that they did not depend on the secondary release of another neurotransmitter.[53] We would expect these IP-receptors to couple to adenylate cyclase, and indeed forskolin can also depolarise the vagus nerve, and desensitise the nerve to the effects of prostacyclin.[42] Furthermore, phorbol dibutyrate displays similar effects as forskolin which could mean that we are also seeing one of the few examples of IP-receptor coupling to G_q in normal tissues. Although cyclic AMP contributes to primary afferent hyperalgesia for agents such as prostacyclin, no contribution of protein kinase C (PKC) was noted in the rat paw pressure response to prostacyclin.[54]

One important question to consider is why one can record IP agonist-mediated depolarisations along the length of the vagus nerve in vitro. To answer this question we can look at other receptors associated with sensory neurones. For example, primary sensory neurones respond to capsaicin along their entire extent (cell bodies, peripheral and central fibres and their respective endings) implying vanilloid receptor expression throughout these neurones.[26,55] In a discussion on the role of 5-HT3-receptors in emesis, Reynolds[56] concluded that the presence of apparently functional receptors along the length of the vagus nerve trunk was unlikely to provide a significant site of action for 5-HT_3-receptor antagonist drugs in vivo. After all, it is assumed that under pathophysiological conditions, 5-HT released from gastrointestinal mucosal enterochromaffin cells activates, and possibly sensitises, adjacent C-fibre terminals.[57] However for practical reasons, most studies on 5-HT have tended to concentrate upon the receptor populations located on vagal afferents and the visceral primary afferent cell bodies contained within the nodose ganglion, rather than those present at vagal terminals;[57] clearly the same conditions will apply to studies of IP-receptor

function in sensory systems. But if Preceptor activation depolarises the vagus nerve,[42, 52, 53] and if the bradykinin-mediated inhibition of AHP_{slow} is partially dependent on prostacyclin synthesis,[58] then it is conceivable that the intraneuronally-synthesised prostacyclin diffuses out of the neurone to activate IP-receptors located along the length of a sensory afferent fibre. The resulting depolarisation may serve to enhance the release of transmitter at the central terminals and thus facilitate nociceptive processing.

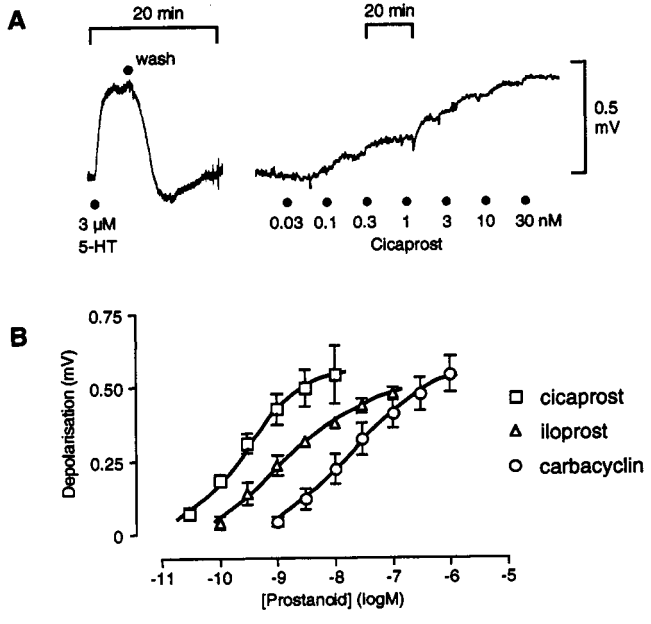

Figure 53. Depolarisation of isolated rat vagus nerve by IP agonists. A: Depolarisation responses to 5-HT and cicaprost. The undershoot on the 5-HT trace is a hyperpolarisation due to activation of $5\text{-}HT_{1A}$-receptors. Jones & Rudd, unpublished observations. B: Log concentration curves for depolarisation induced by IP agonists.[59]

In rabbit vagal afferent (nodose) neurones in vitro, bradykinin inhibits AHP_{slow} resulting in increased excitability of C-fibre neurones, which can be mimicked by cyclic AMP mimetics.[32] Furthermore, pretreatment of nodose ganglia cells with indomethacin completely prevented this bradykinin-induced inhibition of AHP_{slow}. The ability of indomethacin to block the bradykinin-mediated inhibition of AHP_{slow} was examined further by Weinreich et al.[58] in a quite demanding set of experiments. Using guinea-pig and rabbit nodose neurones they demonstrated that not only did bradykinin-mediated inhibition of AHP_{slow} depend on COX activation, it was also

inhibited by the prostacyclin synthase inhibitor tranylcypromine. Exogenous prostacyclin inhibited AHP_{slow} in the presence of either enzyme inhibitor (see Fig. 54). From these studies one can conclude that bradykinin stimulates the release of prostacyclin, but not PGD_2, PGE_2, $PGF_{2\alpha}$ or TXA_2, from nodose neurones via activation of the B_2 subtype of bradykinin receptor. Although bradykinin-evoked release of neuropeptides from DRG neurones is also inhibited by COX inhibitors, the primary prostaglandin mediating this particular response is less clear cut (see Section 4.3).

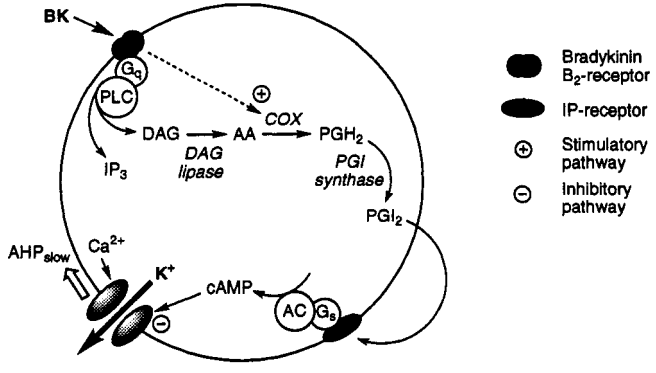

Figure 54. Schematic representation of bradykinin-mediated activation of an isolated nodose ganglion neurone.

Prostacyclin can stimulate vagal cardiac C-fibres in vivo.[60] When prostacyclin is injected into the coronary artery, it elicits vagal reflex bradycardia and hypotension due to withdrawal of sympathetic tone to peripheral vessels (see Chapter 6). When given intravenously to anaesthetised dogs prostacyclin, at doses which produce a substantial fall in systemic arterial blood pressure, also produced marked bradycardia instead of the expected reflex tachycardia in response to a hypotensive agent.[61] These vagal afferent receptors are termed "chemically sensitive receptors" and differ from the classical chemosensitive fibres which respond to changes in pO_2, pH, pCO_2 etc. It is also possible that the action of prostacyclin on cardiac receptors is indirect as epicardial or intracoronary prostacyclin can sensitise cardiac receptors to the actions of nicotine.[62] One interpretation of the data is that the action of prostacyclin involves a reflexly mediated increase in vagal efferent discharge to the heart, rather than a direct effect of prostacyclin on cardiac pacemaker cells, because the bradycardia induced by prostacyclin is abolished by atropine.[62] Alternatively, prostacyclin could sensitise the cardiac pacemaker cells to acetylcholine released from vagal postganglionic nerve endings; this effect would also be abolished by

atropine. This latter sensitising action of prostacyclin on cardiac receptors could relate to the production of hyperalgesia discussed in Section 4. When the heart is studied in isolation, PGE_1 can increase heart rate and contractile force, and independently cause the release of CRGP from sensory nerrones.[63]

4. NOCICEPTION

Activation of primary afferent C-fibres by noxious stimuli gives rise to spinal release of the excitatory amino acids, glutamate and aspartate, and neuropeptides including substance P and CGRP, and these mediators may facilitate the cascade of nociceptive processing.[64] Indeed the hyperalgesia induced by injection of PGE_2 into the mouse intrathecal space is blocked by NMDA antagonists and nitric oxide synthase inhibitors, and is lost in mice lacking either the ε1 or ε4 subunits of the ionotrophic NMDA-receptor.[65] At first it would seem surprising to find that PGD_2-induced hyperalgesia (hot-plate test) is maintained in these NMDA-receptor subunit knockout mice, but this is because substance P rather than excitatory amino acids appears to be the mediator of PGD_2 activity,[66] and despite the observation that the release of excitatory amino acids in the spinal dorsal horn is enhanced by substance P and CGRP.[67] Results such as these should perhaps caution against extrapolating too much from the actions of one prostaglandin to another, for even though PGD_2 and PGE_2 predominantly mediate their effects through activation of adenylate cyclase, their effector pathways in hyperalgesia are quite distinct. It would be fascinating now to see whether prostacyclin is more like PGE_2 or PGD_2 in this regard.

4.1 Source of prostacyclin in nociceptive pathways

A closer look at models of inflammation reveals that prostacyclin rather than PGE_2 is often the predominant prostaglandin produced. For example, zymosan injected intraperitoneally produces 20-fold more prostacyclin compared with PGE_2 in mice; both the prostaglandin production and consequent writhing response are blocked by COX inhibitors.[68] It is most likely that the resident peritoneal cells or the peritoneum itself, and not leukocytes, are the source of prostacyclin in response to zymosan injection because the concentrations of prostaglandins are already falling by the time inflammatory cells migrate into the peritoneal cavity. Similarly, in carrageenin-induced inflammation, the concentration of prostacyclin in the inflammatory exudate is already falling before infiltration by inflammatory cells occurs.[12] Besides, resident macrophages are a rich source of COX

metabolites, and elicited macrophages release much less prostacyclin than do resident macrophages (see Chapter 8). Certainly in kaolin-induced pleurisy, there is a biphasic exudation of plasma, peaking at 20 min and 3 - 5 h, with a rapid 12-fold increase in prostacyclin production during the first phase, and a smaller increase in PGE_2 corresponding to the second phase.[69] The exudate volume in carrageenin-induced pleurisy and in carrageenin-induced paw oedema were both significantly reduced in IP_1-receptor knockout mice, down to the level seen with indomethacin-pretreatment of wild-type mice, despite being no change in prostacyclin production.[1] Therefore, the pathway for prostacyclin production is not dependent on activation of IP_1-receptors.

If we look at prostaglandin levels in the spinal cord following induction of inflammation in the periphery, we see that intraplantar injection of rats with carrageenin led to hyperalgesia at 4 h with increased COX-2 mRNA in the lumbar spinal cord.[70] Although the COX-2 inhibitor DuP 697 was able to reduce the hyperalgesic response, it paradoxically potentiated COX-2 mRNA induction suggesting the existence of a potential regulatory mechanism to overcome blockade of enzyme activity. Messenger RNA of both COX isoforms is expressed constitutively in the rat spinal cord with COX-2 as the predominant form.[71] Injection of Freund's adjuvant into the rat paw led to swelling accompanied by increased expression of COX-2 mRNA, with no change in COX-1 mRNA, therefore COX-2 might be regarded as the COX isozyme responsible for spinal prostaglandin release in nociceptive processing under a peripheral inflammatory stimulus. Other studies have also noted an increase in COX-2 mRNA expression, but not in COX-1 mRNA, in the lumbar spinal cord 2 - 4 h after injection of Freund's complete adjuvant into the rat paw, with greater increases in prostacyclin compared to PGE_2.[22] This latter study showed that while selective COX-2 inhibitors attenuated allodynia (a state of discomfort and pain evoked by innocuous tactile stimuli), they had no effect on oedema or hyperalgesia, in contrast to the nonselective COX inhibitor indomethacin which attenuated both allodynia and the oedema response. So the surprising conclusion here was that prostaglandins are directly involved in the development of allodynia but not mechanical hyperalgesia.

In the rat, an intradermal formalin injection characteristically evokes a biphasic flinching behaviour but this does not seem to be paralleled by changes in the spinal release of PGE_2 when measurements are taken directly from the dorsal lumbar region using microdialysis methods.[72] NSAIDs tend to reduce nociceptive behaviour more during the second phase of response to formalin, therefore Scheuren et al.[72] have proposed that NSAIDs may in fact influence the flinching behaviour of rats during the second phase by inhibiting spinal release of prostaglandins during the first phase. Intrathecal COX-2 inhibitors did not alter the second phase of the formalin test or K^+-

evoked PGE_2 release from rat spinal cord in vitro - only nonspecific COX inhibitors such as (+)-S-ibuprofen were effective.[73] Therefore, Dirig et al.[73] suggested that COX-2 might not be associated with spinal prostaglandin synthesis acutely or with facilitated nociception which occurs within the limited time frame (1 h) of the formalin test. What is clear from all these studies is that there is a long way to go before we truely understand the sequential consequences of inflammatory stimuli.

4.2 Hyperalgesia

If agents which are released from tissue in response to injury are essential intermediaries in the pain response to an injury, then they should activate small diameter afferent fibres and produce pain when applied locally;[4] bradykinin' and capsaicin[26] are two such examples of chemical stimuli frequently used in models to detect hyperalgesia. Prostacyclin and PGE_2 are presumed to act directly on primary afferent nociceptors and can be considered as direct-acting hyperalgesic agents.[54] However unlike bradykinin and capsaicin, prostaglandins do not produce pain when applied locally.[6] This distinction between the hyperalgesic and nonalgesic action of prostaglandins is the theme of this section.

Hyperalgesia is measured in a variety of animal models, and it is clear that the role of prostaglandins in hyperalgesia is highly specific to the response being measured, and particularly to the time point at which the response is determined. Although a range of compounds produce hyperalgesia in the rat paw pressure test, prostaglandins tend to have the highest potency, and given that cyclic AMP mimetics also produce hyperalgesia, it was concluded very early on that the interactions of PGE_2 with a receptor at the peripheral nociceptive terminal activates adenylate cyclase.[74] Furthermore, intraplantar injection of morphine blocked this hyperalgesic response of prostacyclin and PGE_2 confirming a local effect of the prostaglandins.[75] Ferreira et al.[76] concluded that aspirin-like drugs prevent the triggering of hyperalgesia by inhibiting PGE_2 synthesis, whereas opiates increase the threshold of the nociceptive system lowered by prostaglandins. Further studies confirmed that injection of prostacyclin and PGE_2 directly into the rat paw induces rapid hyperalgesia,[5,77,78] and that this response is unaffected by COX inhibition, by depletion of transmitter in sympathetic postganglionic nerves or by removal of neutrophils.[78] The activation and sensitisation of the primary afferent nociceptive fibres by prostacyclin and PGE_2 appears linked to a cyclic AMP pathway,[54,79] and for PGE_2 is apparently mediated via G_s-proteins.[80]

Injection of PGE_2 decreases mechanical threshold and increases the number of action potentials elicited by the test stimuli in most C-fibre

nociceptors, resulting in sensitisation. Results from studies using single fibre electrophysiology of C-fibres innervating the dorsum of rat hind paw support the hypothesis that primary afferent sensitisation by PGE_2 is responsible for hyperalgesia, and is dependent on cyclic AMP.[81] It is possible that prostaglandins play a similar role in the CNS, directly activating and sensitising central neurones involved in nociceptive signalling. Indeed prostaglandins such as PGE_2 have been shown to activate neurones by causing increased cation influx.[82] PGE_2 is thought to block endogenous opioid-mediated analgesia systems by inhibiting the bulbospinal noradrenaline component of this analgesic pathway, as the hyperalgesia produced by intrathecally-administered PGE_2 is reversed by morphine.[83] From these studies it was concluded that tonic production of prostaglandins in the spinal cord can inhibit normal pain control circuits in the CNS by blocking the release of noradrenaline from spinal terminals of brainstem projection neurones.

PGE_2 has only weak excitatory effects on canine testicular polymodal receptors in vitro but both prostacyclin and PGE_2 can augment bradykinin responses.[84] Similarly, prostacyclin was able to increase the sensitivity of cat articular afferents to both mechanical and chemical stimuli.[17] Intra-arterial injection of bradykinin into the spleen produces a reflex increase in blood pressure (pseudo-affective response) and the release of prostacyclin, and this response has been used as a model of pain reflexes.[85] The response to bradykinin can be blocked by indomethacin, with prostacyclin being 3-fold more potent than PGE_2 in potentiating bradykinin-induced nociceptive responses in indomethacin-treated dogs, thus, prostacyclin may mediate the pain producing activity of bradykinin in this model.[86,87]

In addition to sensitising rat DRG neurones to release neuropeptides in response to capsaicin, bradykinin and KC1, carbacyclin also has a direct releasing action,[39] and in the isolated, perfused guinea-pig heart, prostacyclin and PGE_2 dose-dependently release CGRP from cardiac sensory neurones.[18] However in this latter example, the precise site of action of prostacyclin and PGE_2 is uncertain.

So far we have seen that there are many similarities between the hyperalgesic action of prostacyclin and PGE_2 yet they often display quite distinct profiles of activity when tested for direct effects using electrophysiological techniques. When studying the afferent discharge of rat ankle joint mechanonociceptors, it was found that prostacyclin and cicaprost, but not PGE_2, caused both sensitisation to mechanical stimulation and direct excitation of the majority of mechanonociceptors.[16] In normal rats, cicaprost excited more joint mechanonociceptors than PGE_2, and in rats with modified adjuvant arthritis, the response of mechanonociceptors was attenuated by salicylate.[88] It was thought likely therefore that IP agonists and PGE_2 act

differentially to sensitise joint mechanoceptors, since cicaprost, but not PGE_2, induced spontaneous activity in units from both control and inflamed joints. These results support the concept that endogenous prostacyclin plays an important role in lowering nociceptive thresholds.

But not all nociceptors behave similarly. Thus, although cicaprost, capsaicin, bradykinin and PGE_2 all excited chemosensitive units in rat ankle joint, only cicaprost and capsaicin excited the mechanonociceptors.[89] Bradykinin would normally be expected to excite mechanonociceptors, but in this preparation the isolated hindlimb was perfused with Krebs buffer and presumably lacked the necessary factors such as prostacyclin needed to potentiate the response to bradykinin. It was therefore concluded that prostaglandin-induced excitation of articular mechanonociceptors is mediated by prostacyclin activation of an IP-receptor. When extracellular recordings were made from single units dissected from the medial articular nerve of the cat knee joint, prostacyclin clearly both excited and sensitised (to bradykinin) a greater proportion of articular afferents than PGE_2.[17] Because all the afferents which were sensitive to prostacyclin were also sensitive to PGE_2, and not vice versa, it was suggested that prostacyclin binds both to its own receptor and with lower affinity to an EP-receptor. Furthermore, prostacyclin was able to increase the sensitivity of articular afferents to both mechanical and chemical stimuli.

If we look for prostacyclin-mediated activation of nociceptors in vivo, then we see that prostacyclin can produce a response in the absence of other stimuli, and this profile of response often differs from that of PGE_2. PGE_1 is 10-times more potent than PGE_2 in the mice writhing test; this pain response is blocked by morphine but not by aspirin, and is lost on the second challenge with prostaglandin.[90] Furthermore, bradykinin-induced writhing was blocked by indomethacin, clearly indicating a role for prostaglandins here. At this time it was assumed that PGE_1 and PGE_2 were acting at similar receptors and it was not understood that PGE_1, but not PGE_2, was an IP agonist. As a consequence, prostacyclin seemed to be ignored in favour of PGE_2 which then featured in many more studies on nociception. With the advent of more stable analogues of prostacyclin it became apparent that prostacyclin, and not PGE_2, was the significant prostaglandin here. In fact, carbacyclin is a potent inducer of writhing behaviour in mice, with as little as 10 $\mu g\ k^{-1}$ i.p. producing an immediate effects[30] Intraperitoneal injection of 2 μg prostacyclin produced a writhing response in 60% of mice tested, but less than 25% of mice responded to the same dose of PGE_2.[1]

Zymosan injected intraperitoneally produces 20-fold more prostacyclin compared with PGE_2 in mice; both the prostaglandin production and consequent writhing response are blocked by COX inhibitors.[68] However, it is only prostacyclin that can reverse the indomethacin-analgesia in zymosan-

treated mice, and only centrally-acting agents such as morphine and clonidine can block this nociceptive action of prostacyclin. Doherty et al.[68] therefore proposed that prostacyclin may have the ability to stimulate nociceptors directly in the mouse peritoneal cavity, in addition to its previously recognised ability to sensitise nociceptors to other pain-producing stimuli. In this study, PGE_2 failed to induce writhing in normal mice, and failed to potentiate writhing in zymosan-injected mice. While one could easily presume that prostacyclin injection is causing a direct nociceptive effect, it remains possible that the trauma of the injection process itself releases nociceptive factors whose action is then potentiated by prostacyclin to a now measurable level.

The writhing response in mice can be induced by intraperitoneal injection of a variety of agents, yet they may have different mechanisms of action. For example, kaolin-induced writhing depends on bradykinin production whereas zymosan-induced writhing depends directly on prostacyclin, with no involvement of bradykinin.[91] However, responses to both kaolin and bradykinin are inhibited by COX-inhibitors. Although prostacyclin production was unchanged in IP_1-receptor knockout mice, their writhing response to intraperitoneal injection of acetic acid was significantly reduced, to the same level seen in indomethacin-treatment wild-type mice;[1] further supporting the concept that prostacyclin production and the consequent activation of IP_1-receptors is the main cause for the writhing behaviour in response to intraperitoneal injection of inflammatory agents.

4.3 Prostacyclin as mediator of bradykinin activity

Bradykinin activates canine testicular polymodal nociceptors in vitro and this effect is clearly mediated by prostaglandins as it is blocked by aspirin.[84] Bradykinin-evoked responses in the rat tail-spinal cord preparation, but not the response to thermal stimulation, were also inhibited by COX inhibitors.[29] Results suggest a more important role for prostaglandins in mediating bradykinin-induced hyperalgesia to mechanical stimulation than in bradykinin-induced sensitisation to chemical and thermal stimulation.[5] Using the rat paw pressure test, it was thought that bradykinin hyperalgesia is mediated by PGE_2 whereas it is noradrenaline hyperalgesia which is mediated by prostacyclin.[92] The bradykinin-induced nociceptive response in dogs (reflex increase in blood pressure following splenic artery injection) is also blocked by indomethacin, and these studies noted that prostacyclin must be a major candidate involved in bradykinin-induced nociception.[86] Perhaps now therefore a more convincing case for prostacyclin as the prime mediator of bradykinin reponses can be made.

Uncertainty exists about the source of prostaglandins generated by bradykinin that are able to release sensory neuropeptides as there is conflicting evidence supporting sensory nerves as the source of these prostaglandins.[10] But when using isolated sensory neurones such as neonatal rat DRG neurones, we see that PGE_2 potentiates bradykinin-induced release of substance P and CGRP,[49] and that this response to bradykinin is inhibited by indomethacin; results which clearly suggest that bradykinin stimulation leads to activation of COX enzymes in sensory neurones with the consequent production of prostaglandins (see Fig. 55).

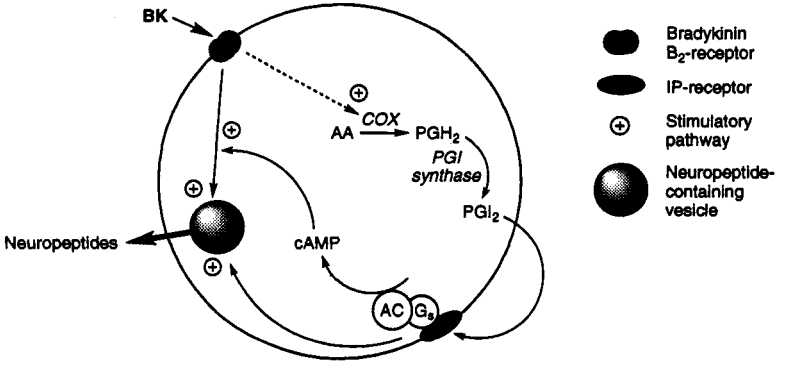

Figure 55. Schematic representation of neuropeptide release from an isolated dorsal root ganglion neurone.

In many tissues, bradykinin B_2-receptor activation leads to enhanced eicosanoid synthesis: potentially by release of arachidonic acid from the diacylglycerol (DAG) produced by inositol lipid hydrolysis.[93] In nonsensory tissues however, inositol lipid hydrolysis is pharmacologically independent of arachidonic acid release, which instead has been proposed to result from activation of phospholipase A_2. In addition, bradykinin B_2-receptor activation often leads to enhanced cellular accumulation of cyclic AMP, due indirectly to stimulation of prostaglandin synthesis and the subsequent activation of G_s-coupled receptors. In contrast, when we examine the effects of bradykinin in sensory tissues, we see a slightly different profile of activity. For example, when bradykinin is applied to the neonatal rat spinal cord-tail preparation, it activates capsaicin-sensitive peripheral fibres and thus evokes a depolarisation recordable from the spinal ventral root.[28] This response to bradykinin is inhibited by COX inhibitors but is not affected by the phospholipase A_2 inhibitor mepacrine. Bradykinin does appear to stimulate the production of arachidonic acid in sensory neurones, with the arachidonic acid possibly deriving from the breakdown of DAG by DAG

lipase. For example, in foetal rat DRG cells, mepacrine did not block the arachidonic acid release in response to bradykinin, suggesting that there might be a phospholipase A_1-lysophospholipase pathway which releases arachidonic acid.[94] In addition, and somewhat surprisingly, bradykinin does not cause an increase in cyclic AMP in rat DRG neurones.[41]

In visceral sensory neurones such as rabbit vagal afferent (nodose) neurones in vitro, bradykinin preferentially activates unmyelinated C-fibres and Aδ--myelinated fibres and indirectly increases excitability via inhibition of AHP_{slow}.[32] This effect of bradykinin allows the cell to fire repetitively following a stimulus and therefore may be one of the mechanisms underlying nociceptor sensitisation.[9] Bradykinin-induced inhibition of AHP_{slow} was mimicked by cyclic AMP mimetics, and was completely prevented by pretreatment of nodose ganglia cells with indomethacin.[32] The ability of indomethacin to block the bradykinin-mediated inhibition of AHP_{slow} has been examined further by Weinreich et al.[58] using guinea-pig and rabbit nodose neurones where they demonstrated that not only did bradykinin-mediated inhibition of AHP_{slow} depend on COX activation, it also depended on the activity of prostacyclin synthase, and that exogenous prostacyclin had a direct effect alone (see Fig. 54). From these studies they concluded that bradykinin stimulates the release of prostacyclin in preference to other prostanoids from nodose neurones following activation of the bradykinin B_2-receptor.

Both arachidonic acid and bradykinin promote the release of CGRP from cardiac capsaicin-sensitive afferents of guinea-pig heart by a common mechanism involving prostaglandin production, and their mechanism is distinct from that activated by capsaicin.[18] For both arachidonic acid and bradykinin, the most likely candidate prostaglandin is prostacyclin as this was the only product of bradykinin infusion and was *per se* able to release CGRP. Geppetti et al.[18] considered that if there was an adequate stimulus to increase PGE_2, then this may also activate the "efferent" function of primary sensory neurones in guinea-pig heart. Thus, there is considerable evidence suggesting that prostacyclin mediates some of the nociceptive functions of bradykinin, but the intracellular pathways linking these two inflammatory agents remain to be clarified.

4.4 Mechanism of action of prostacyclin

Prostacyclin tends to have a different time course of action compared with PGE_2, but whether or not this reflects a different mechanism of action in sensory neurones is unclear. Prostacyclin has a more rapid onset of action and is more potent than other prostaglandins in producing hyperalgesia in animal models of pain.[15,77,86] Hyperalgesia induced by intradermal

prostacyclin is of shorter duration than that of PGE_2 in the rat paw pressure test,[92] and in the dog knee joint.[15] Data from such studies support the concept that prostacyclin, rather than PGE_2, participates in early inflammatory hyperalgesia seen in the carrageenin model of inflammation, with PGE_2 contributing later.

In contrast, the excitation of chemosensitive units by cicaprost and PGE_2 in the isolated rat hindlimb preparation tends to be delayed in onset, with cicaprost having a longer duration of action than PGE_2.[89] Prostacyclin and its mimetics have a direct depolarising action on the rat vagus nerve,[42 52,53,59] see Figure 53, and the slow onset of response suggests IP-receptor signalling via a G-protein. Prostacyclin also directly influences neuronal activity in rabbit and guinea-pig nodose neurones by inhibiting AHP_{slow}.[32] In vascular tissues IP-receptors may couple directly to ion channels, with iloprost-mediated hyperpolarisation of canine and rabbit carotid artery being partially inhibited by the K_{ATP} channel blocker glibenclamide.[95,96] Therefore, one possible mechanism for the hyperalgesic effect of prostacyclin is its ability to inhibit AHP_{slow} by a cyclic AMP-dependent K^+ conductance mechanism, thus allowing the cell to fire repeatedly.[11] Indeed recent evidence suggests that both carbacyclin and PGE_2 can suppress potassium currents (I_K) in rat embryonic DRG cells which may, in part, account for the increased membrane excitability underlying the hyperalgesic effect of these prostaglandins.[97]

Hingtgen & Vasko[39] remind us though that carbacyclin needs a 30 min incubation time to cause the direct release of neuropeptides from DRG neurones, which is much longer than needed to observe sensitisation by prostacyclin as measured by altered excitability of sensory neurones in situ.[17] Enhancing phosphorylation (by inhibiting phosphatase activity with okadaic acid) in DRG sensory neurones is an important component in augmenting neuropeptide release,[98] but it remains to be seen if phosphorylation contributes to the mechanism of action of prostacyclin. It is quite conceivable that this is indeed the case because we know that the IP-receptor can increase production of IP_3 (and presumably DAG) by coupling to G_q-proteins,[99,100] and we know that phorbol esters can mimic the depolarising action of IP agonists on the rat vagus nerve.[42]

In trying to understand the mechanism of action of IP agonists in rat DRG cells, Hingtgen & Nicol[101] noted that pretreatment with carbacyclin produced a three-fold enhancement in the number of neurones that were cobalt-labelled by capsaicin. The uptake of cobalt is via a receptor/ion channel complex that is activated specifically by capsaicin. Results suggest that the transduction pathway(s) activated by carbacyclin, which mediates the sensitisation, somehow modulates the permeability of the receptor/ion

channel complex to cobalt or alters the sensitivity of this complex so that it is now activated by lower concentrations of capsaicin.

The ability of carbacyclin and PGE_2 to potentiate bradykinin and capsaicin-evoked release of substance P and CGRP from rat DRG neurones is attenuated by inhibition of adenylate cyclase and is mimicked by cyclic AMP mimetics. Together with the marked ability of carbacyclin and PGE_2 to increase cyclic AMP production in these cells suggests that the cyclic AMP transduction cascade mediates the sensitising actions of prostaglandins on peptide release from sensory neurones.[40] Although we have seen before that IP agonists can directly release neuropeptides from DRG neurones, at least when used at higher concentrations than needed to potentiate bradykinin for example,[39,40] the cyclic AMP mimetics forskolin, 8-bromo cyclic AMP and cholera toxin had no effect alone despite clearly facilitating stimulus-evoked release. Bradykinin is probably one of the best studied algesic agents, and it is thought to release neuropeptides consequent to activation of phospholipase C (PLC) and subsequent stimulation of PKC by DAG in sensory neurones.[102] In addition to coupling to adenylate cyclase, IP1-receptors can also couple to PLC (see Chapter 4). It is therefore conceivable that the sensitising/potentiating action of IP agonists is mediated via adenylate cyclase, while any direct effect on neuropeptide release is mediated via PLC.

5. DISCUSSION

In a recent commentary on the landmark paper by Ferreira et al.,[85] Sir John Vane noted how this paper reinforced very strongly the idea that a prostaglandin sensitised sensory nerve endings to the nociceptive activity of bradykinin.[103] More importantly he stated "In those days we used PGE_1 but now know that the main prostaglandin released in inflammation is PGE_2". How correct this statement is in light of the IP_1-receptor knockout mice data, and the information presented herein, remains to be seen.

It should be apparent from the information presented above that prostaglandins are hyperalgesic agents and COX inhibitors are effective analgesic agents in part because of their ability to inhibit prostaglandin production. However questions concerning which particular prostaglandin mediates a particular hyperalgesic response remains complicated by the range of different experimental paradigms used. As it becomes clearer that prostacyclin has an additional direct action on nociceptors, it in turn becomes more difficult to interpret data from in vivo studies using multifactorial stimuli, such as arthritis induced by Freund's adjuvant or carrageenin injection. This is perhaps where we can make best use of the

IP_1-receptor knockout mice - at least until the advent of an IP antagonist. We should also remember that while the IP_1-receptor knockout mice studies indicate that the IP-receptor involved in inflammatory pain is the same as the classical platelet IP_1-receptor, the question of a role for IP-receptor subtypes remains largely unanswered. It is also worth noting that since prostacyclin and PGE_2 show many nociception properties in common, then deletion of the IP_1 -receptor gene may lead to compensatory overexpression of EP-receptors and the resulting possibilities of data misinterpretation. Despite these complexities, there is overwhelming evidence that IP-receptors are involved in pain responses, and that blocking these receptors should result in an effective analgesic agent for disorders involving inflammatory pain.

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Chapter 10

IP-receptors in the enteric nervous system

1. INTRODUCTION

The primary prostaglandins, including prostacyclin, have powerful effects on gastrointestinal function. Early studies in laboratory animals showed that PGE_2 and $PGF_{2\alpha}$ caused "enteropooling", that is, diarrhoea typified by secretion of a large volume of mucosal fluid.[1,2] On the other hand, prostacyclin,[3] its stable analogue iloprost,[4] and PGD_2[3] inhibited enteropooling. In man, prostacyclin and iloprost infusions tend to have stimulant effects, causing nausea, abdominal cramping and diarrhoea in some individuals.[5-8]

There is good evidence that the actions of IP agonists on the gut are mainly exerted through the enteric nervous system as opposed to directly on the end-organ (smooth muscle or mucosa). We will review this evidence and also discuss the subtypes of IP-receptors involved based on the potency rankings of prostacyclin analogues. In addition, the possible roles of endogenous prostacyclin in the normal and diseased enteric system will be described.

Four isolated enteric systems have received most attention: longitudinal muscle motility in guinea-pig ileum and rat colon, and mucosal secretion in guinea-pig and rat colon. The anatomical arrangements of the enteric nervous systems are shown in Figure 56, based on information in Jodal's review.[9] For convenience, neuronal prostanoid receptors have been assumed to exist on the soma and/or generator region of the axon as opposed to the axon proper and/or nerve terminals; in most cases the exact location has not

been demonstrated. In this context, it is important to appreciate the potential limitations of one of our main pharmacological tools, the Na^+-channel blocker tetrodotoxin (TTX). At concentrations between 10 nM and 1 μM, TTX selectively inhibits nerve impulse conduction thus permitting a distinction between neuronal (TTX-sensitive) and non-neuronal (TTX-resistant) drug actions. However, a false picture may arise in two situations. Firstly, modulation of transmitter release by a drug that acts on presynaptic receptors linked to Ca^{2+} influx channels may be resistant to TTX; for example, 5-HT activates 5-HT_3-receptors on juxtamucosal terminals to generate ω-conotoxin-sensitive, but TTX-resistant, action potentials.[10] Secondly, a drug whose sole action on the end-organ is enhanced or inhibited by tonic neuronal input will have a TTX-sensitive component.

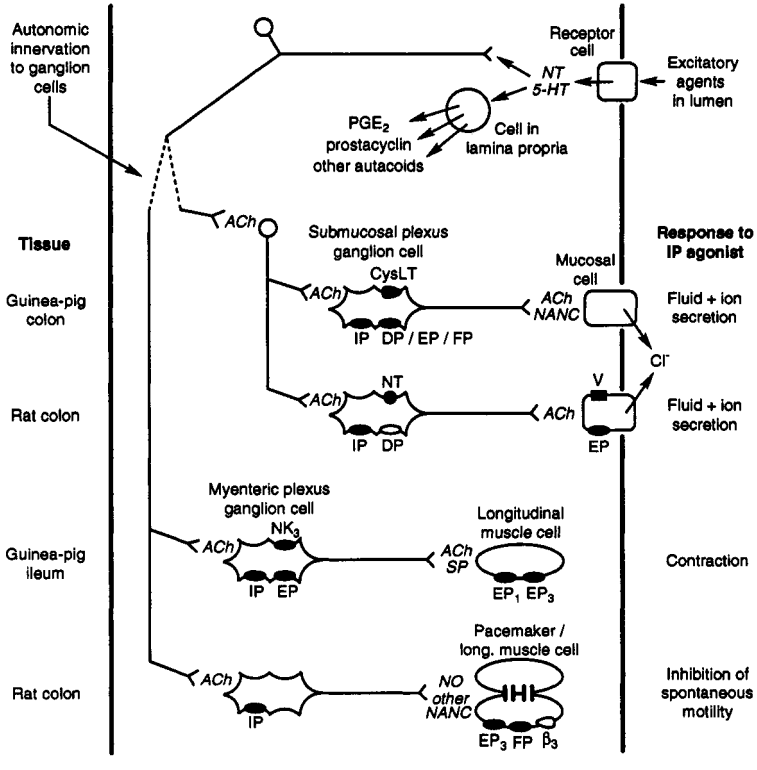

Figure 56. Scheme showing the proposed sites of action of IP agonists on four enteric neurone systems. Neurotransmitters are shown in italics; excitatory receptors (filled symbols), inhibitory receptors (open symbols). ‖ represents a gap junction linking the pacemaker cell to the smooth muscle cell. ACh (acetylcholine), β_3(β_3 -adrenoceptor), CysLT (cysteinyl leukotriene), NANC (nonadrenergic-noncholinergic), NK_3 (neurokinin-3), NO (nitric oxide), NT (neurotensin), SP (substance P), V (vasoactive intestinal peptide).

2. INTESTINAL MOTILITY

2.1 Guinea-pig ileum

Gaion & Trento[11,12] were the first to study the action of prostacyclin on the enteric nervous system of the guinea-pig ileum. They showed that contraction of the longitudinal muscle elicited by 20 nM prostacyclin was abolished by TTX and by the muscarinic antagonist atropine (30 nM), but was little affected by the ganglionic nicotinic blocker hexamethonium. In addition, prostacyclin enhanced twitch responses of the ileum to electrical field stimulation (EFS), but had no effect on direct contractions induced by acetylcholine. They concluded that prostacyclin activated cholinergic neurones in the myenteric plexus. A few years later, Jones & Lawrence[13] showed that the contractile action of cicaprost was abolished by either TTX or morphine (which activates presynaptic opioid receptors to suppress transmitter release), but only partially inhibited by atropine (200 nM). However, a combination of atropine and a selective neurokinin NK1-receptor antagonist (e.g. CP-96,345) produced complete block. Thus, IP-receptor activation induces the neuronal release of both acetylcholine and a substance P-like agonist; the NK3 agonist senktide acts on enteric neurones in a similar manner.[14]

A cicaprost-like profile on the guinea-pig ileum was also seen with another prostacyclin analogue, 10,l0-difluoro- 13,14-didehydro PGI_2 (see Chapter 2). In contrast, with iloprost, carbacyclin or high concentrations of prostacyclin (0.1 - 1 μM), TTX or morphine produced only partial block and the remaining contractile component was antagonised by the EP1 antagonist AH 6809.[15] It is likely that these three agents directly contract the longitudinal smooth muscle by activating EP_1-receptors, rather than IP-receptors as originally suggested by Gaion & Gambaratto.[16] EP_3-receptors are also present on the muscle cells, but it was not clear whether these were activated by the prostacyclin analogues.[15] In addition, TTX or morphine partially inhibited the contractile action of PGE_2 and abolished that of the selective EP_2 agonist butaprost, indicating the presence of excitatory EP-receptors on enteric neurones in the ileum. In the case of PGE_1, we would expect morphine to annul its neuronal EP_2 and IP agonist actions, leaving its direct (and potent) EP_1 and EP_3 actions intact. However, in an early study on the guinea-pig ileum,[17] the contractile effect of quite a high concentration of PGE_1(280 nM) was abolished by morphine.

There are no reports of the actions of IP agonists on guinea-pig ileum studied by intracellular electrode recording on a conventional longitudinal muscle-myenteric plexus preparation. In the case of PGE_2 (1 nM - 1 μM),

this technique showed slow membrane depolarisations in both AH- and S-type neurones, which were unaffected by TTX, accompanied by bursting fast excitatory post-synaptic potentials in S neurones, which were TTX-sensitive (i.e. due to input from adjacent neurones).[18] Rengel et al.[19] showed binding sites for PGE_1 accompanied by elevation of cyclic AMP in both somata-enriched and nerve terminal-enriched fractions from the myenteric plexus of the guinea-pig ileum, but whether these represented EP- or IP-receptors was not determined. Interestingly, μ-opioid receptors were also identified on both somata and nerve endings, but only the former were linked to inhibition of adenylate cyclase.[19]

There is however some data on the role of endogenous prostacyclin and PGE_2 in maintaining the excitatory sensitivity of the soma of cholinergic neurones in the ileum.[20,21] In the plexus preparation, the cyclo-oxygenase (COX) inhibitor indomethacin suppressed the spontaneous release of acetylcholine and that induced by nicotine and substance P acting on acetylcholine and NK3-receptors on the soma of enteric neurones.[22] Interestingly, indomethacin did not inhibit acetylcholine release induced by either EFS or 5-HT. The authors proposed that these stimuli activate axons/nerve terminals, rather than somata, and prostanoids have little excitatory action on axons/nerve terminals; alternatively, it is possible that nicotine and substance P-induced acetylcholine release is mediated by prostanoids, whereas acetylcholine release induced by EFS and 5-HT is not. The inhibitory action of indomethacin could be almost completely overcome by 15-cyclopentyl-ω-pentanor carbacyclin (OP-41483, 100 nM), whereas PGE_2 (150 nM) was only partially effective, and PGD_2, $PGF_{2\alpha}$ and the TP agonist STA_2 (all 100 nM) had no significant effect.[21] In view of the fact that prostacyclin generation by the plexus preparation was about four times higher than that of PGE_2 and also better maintained throughout the course of the experiment, the authors concluded that prostacyclin is an important modulator of the excitability of cholinergic enteric neurones.

2.2 Rat colon

Prostacyclin and its analogues can abolish the spontaneous motility of the rat isolated colon,[23,24] and in our laboratory, we were using this preparation to screen for potential IP-receptor antagonists using the partial agonism shown by the nonprostanoid prostacyclin mimetic BMY 42393 as a lead (see Chapter 3). However, we obtained highly variable effects with some highly lipophilic analogues which led us to question whether the IP-receptors were located on the pacemaker/smooth muscle system as had been long assumed. When we found that the inhibitory action of cicaprost was abolished by TTX, a neuronal location seemed likely.[25] In fact, IP agonists activate

nonadrenergic-noncholinergic (NANC) neurones in the colon. One inhibitory transmitter is nitric oxide (NO); the identity of the second remains unknown, but its action is abolished by apamin, a blocker of small-conductance calcium-activated K^+-channels.

3. MUCOSAL SECRETION

3.1 Guinea-pig colon

On submucosa-mucosa preparations of the guinea-pig colon set up in Ussing chambers, prostacyclin (10 nM to 10 pM) increased the short-circuit current (I_{sc}), indicative of an increase in electrogenic $C1^-$ secretion.[26] The $I_{,,}$ response was virtually abolished by TTX, markedly suppressed by atropine, but unaffected by hexamethonium and the COX inhibitor piroxicam. The authors concluded that prostacyclin activates submucosal neurones, resulting in the release of ACh and a noncholinergic transmitter onto epithelial cells. PGD_2, PGE_2 and $PGF_{2\alpha}$ also induce TTX-sensitive increases in I_{sc}[26-28] and it is likely that other prostanoid receptors besides the IP-receptor are present on the submucosal neurones.

3.2 Rat colon

Using the Ussing chamber technique, prostacyclin induced a TTX-sensitive increase in I_{sc} of rat colon submucosa-mucosa (EC_{50} = 10 nM), again indicative of $C1^-$ secretion, and was much more potent the PGE_2 (EC_{50} = 500 nM).[29,30] In later experiments, it was shown that PGD_2 (5 pM) markedly inhibited increases in $I_{,,}$ induced by prostacyclin, iloprost, neurotensin and DMPP (a nicotinic agonist), without affecting responses to PGE_2 and vasoactive intestinal peptide.[31-33] It was concluded that the former agonists excite mucosal / submucosal neurones and are susceptible to inhibition by PGD_2, which also has a neuronal site of action (Fig. 56). In contrast, the latter two agonists activate receptors on the colonocyte, which are linked to $C1^-$ secretion through adenylate cyclase.[34]

The increase in I_{sc} in rat colon induced by leukotriene D_4 (LTD_4), a cysLT-receptor agonist, is completely inhibited by the COX inhibitor piroxicam, and it is likely that the prostanoid biosynthesis occurs in the lamina propia rather than the epithelial layer.[35] However, neither TTX nor atropine affected the LTD_4 response and this suggests that if prostacyclin mediates the action of LTD_4 it is not via activation of enteric neurones. An

involvement of PGE_2 acting on the epithelium is more likely, although one still has to explain the observation that 5-HT_2 and 5-HT_3 antagonists also blocked the LTD_4 response.

4. IP-RECEPTOR CHARACTERISATION

Accurate comparisons of the potencies of prostacyclin analogues at neuronal IP-receptors in the gut are confounded by activation of EP-receptor systems on neurones and smooth muscle, which oppose or add to/synergise with the IP action.[15,24] We can however obtain a crude measure of IP agonist potency by using the concentration range corresponding to the lower portion of the concentration-response curve (say 10 - 30% of the cicaprost maximum), where the effect is TTX/morphine-sensitive and interference by the EP agonist action is likely to be small (Fig. 57, inset). Figure 57 shows this approach applied to the previously discussed motility measurements in guinea-pig ileum and rat colon. There are two rankings on guinea-pig ileum, one for PGE analogues defining the neuronal EP_2-receptor, the other for prostacyclin analogues defining the neuronal IP-receptor. We have also included a ranking for potentiation of EFS responses in guinea-pig vas deferens, where IP agonists enhance transmitter release from sympathetic nerve endings. It should be noted that interference from presynaptic EP_3-receptors linked to inhibition of transmitter release results in bell-shaped log concentration-response curves for many prostacyclin analogues.[36] Finally, a ranking for depolarisation of the rat vagus nerve is shown;[37,38] this is a simple system to analyse since it is likely that only IP-receptors are present.

Not all prostacyclin analogues were tested on the four preparations, but several important trends can still be seen. Firstly, cicaprost is a highly potent agonist. Secondly, the potency ranking is cicaprost $\geq$ iloprost $\geq$ prostacyclin $>$ carbacyclin/isocarbacyclin. This would strongly suggest the presence of IP1-receptors in all four neuronal preparations. The IP_2-receptor has been shown to have a completely different ranking: isocarbacyclin > iloprost > cicaprost, with cicaprost only showing activity in the micromolar range.[39]

Turning to nonprostanoid prostacyclin mimetics (see Chapter 3), BMY 42393 and BMY 45778 proved to be unexpectedly weak at inhibiting the spontaneous motility of the rat colon (<10 and 15% inhibition at 10 μM). This contrasted with their higher potencies as inhibitors of rat neutrophil activation, and we have suggested that this within-species difference is more likely due to the existence of different IP-receptor subtypes as opposed to efficacy-related phenomena.[40] However, when we subsequently found that BMY 45778 and other nonprostanoids were moderately potent, full agonists for depolarisation of the rat isolated vagus nerve, which is highly responsive

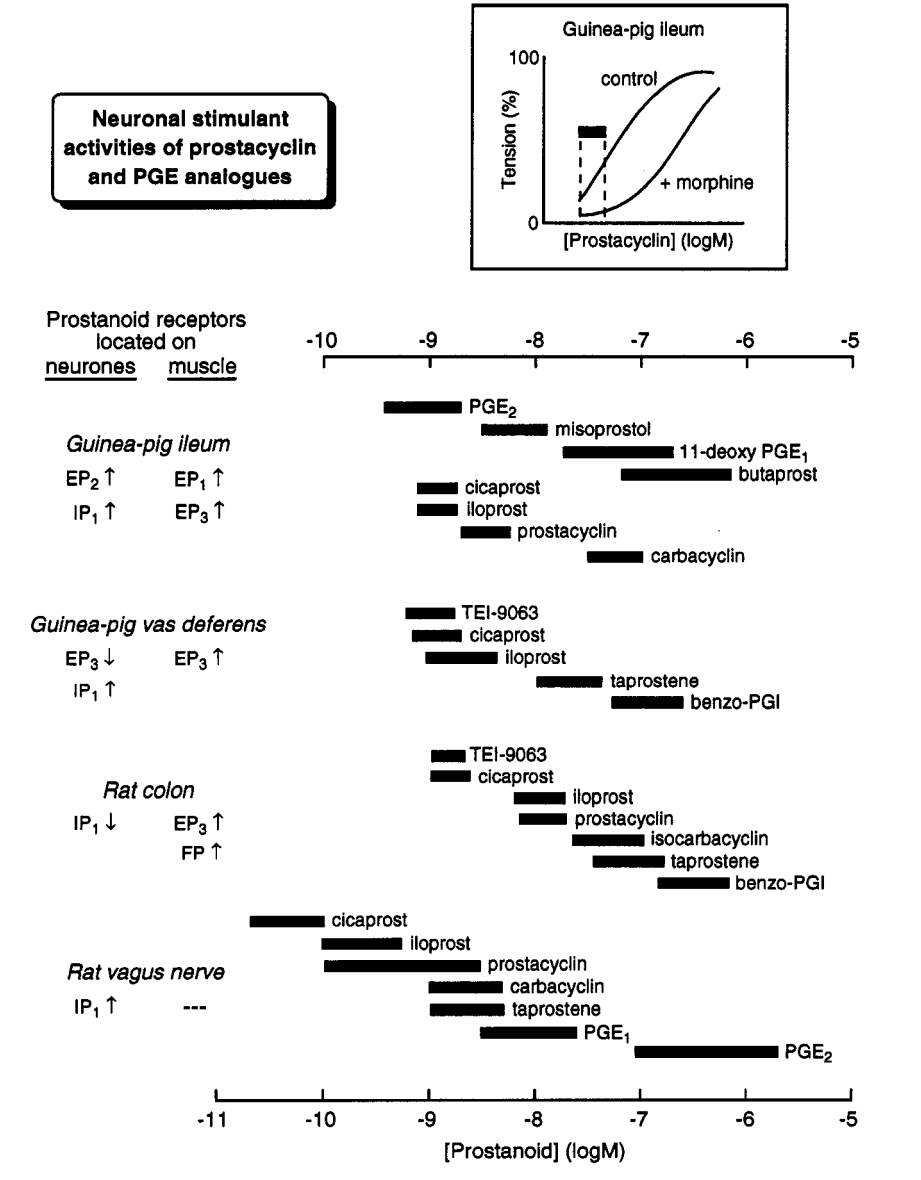

Figure 57. Rankings for agonist potency at neuronal prostanoid receptors. The solid bars show the lower portion of the concentration range over which each prostanoid shows neuronal stimulant activity (see inset). On the left ↓ and ↑ represent inhibitory and excitatory responses of each preparation respectively. TEI-9063 = 17α,20-dimethyl isocarbacyclin, benzo-PGI = benzodioxane prostacyclin. Based on data in Ref. No. 15,24,36-38,60.

to cicaprost (EC_{50} = 0.23 nM; Fig. 57)[38] (see Chapter 9), we reinvestigated the rat colon. We found that the neuronal stimulant actions of the nonprostanoids on the colon are surprisingly slow in onset. After 30 - 60 min exposure, BMY 45778 shows IP partial agonist activity between 0.1 and 3 μM with a maximum inhibition of about 35%.[38] Thus the difference in the profiles of BMY 45778 on the rat vagus and rat colon may simply reflect the low efficacy of BMY 45778; on the higher sensitivity preparation it behaves as a full agonist, on the lower sensitivity preparation as a partial agonist. Although the rat neutrophil profile is still different, our original proposal for IP-receptor subtypes is now less secure.

5. MODULATION OF IN VIVO FUNCTIONS

Modulatory influences on the enteric nervous system may come from the gut lumen (food products and mechanical stress), the vascular supply and the autonomic innervation. Enteric reflexes are initiated by activation of receptor cells in the villous surface causing release of 5-HT and neurotensin onto sensory afferent nerve endings (Figure 56). These agents may also induce prostanoid biosynthesis in the lamina propria and submucosa, potentially leading to activation of enteric neurones or end-organ cells.

5.1 Physiological functions

In guinea-pig colon submucosa/mucosa preparations, distension by reduction of pressure in the submucosal half of the Ussing chamber induced both rapid and longer-acting increases in I_{sc}.[41] Both phases were partially inhibited by TTX and atropine. However, the COX inhibitor piroxicam did not affect either the full distension response or the TTX-resistant component.

Everted sac preparations of the rat colon are much more sensitive than Ussing chamber preparations to the inhibitory action of PGD_2, and this has been attributed to better preservation of the neuronal plexus.[33] It has been further suggested that endogenous PGD_2 functions to suppress colonic secretion in vivo, and this would account for the observation that iloprost does not induce net secretion in the rat colon unless prostanoid biosynthesis is first inhibited by indomethacin.[42]

5.2 Contribution to purgative action

The well-known purgative action of senna anthraquinones is due to stimulation of both intestinal fluid secretion and motility.[43] In the rat,

indomethacin alone delayed the onset of and partially inhibited rhein anthrone-induced diarrhoea. The combination of indomethacin and the Ca^{2+}-channel blocker nifedipine completely inhibited the diarrhoea, whereas indomethacin / verapamil was less effective.[44]

5.3 Disease states

Diarrhoea is a common finding in infectious and inflammatory conditions of the gut. The watery diarrhoea typical of some enteropathogen infections may be due to luminal stimuli (heat-stable *E. coli* toxins, cholera toxin) dramatically accentuating the release of 5-HT and other transmitters. Inflammatory cytokines may be crucial intermediaries through their ability to induce the biosynthesis of prostanoids and other mediators in the lamina propria and submucosa, with resident lymphocytes and phagocytes playing an important role.[9,45-48]

In isolated systems, the final secretory responses to the various stimuli are often inhibited by TTX and/or lignocaine, indicating a neuronal involvement.[9] For example, in a myenteric plexus-longitiudinal muscle preparation from the rat jejunum, a combination of interleukin-1 (IL-1β) and IL-6 produced a delayed decrease in [^{3}H]-noradrenaline release from sympathetic nerves.[49] The effect was largely suppressed by the COX inhibitor piroxicam and partially inhibited by cycloheximide. Since PGE_2 levels were raised by the cytokine mixture, it was suggested that induction of COX leads to an inhibitory (EP_3) action of PGE_2 on sympathetic nerve endings, resulting in enhanced contractility. These observations do not however rule out a contribution from prostacyclin acting to enhance excitatory nervous pathways; the appropriate experiments have yet to be done. In human isolated colonic mucosa, the increase in I_{sc} (mainly C1-secretion) induced by tumour necrosis factor-α (TNF_α) was abolished by indomethacin and partially inhibited by removal of subepithelial tissue.[50] However, TTX did not affect the TNF_α response and the investigators concluded that a neuronal action of prostacyclin was unlikely. A direct action of PGE_2 generated in subepithelial cells seemed more probable since its levels were raised six-fold following TNF_α challenge.

Turning to disease of the human gut, there are comparable increases in the levels of PGE_2, TXB_2 (metabolite of TXA_2) and 6-oxo PGF_α (metabolite of prostacyclin) in inflamed duodenal mucosa tissue from patients with active coeliac disease (gluten-sensitive enteropathy)[51] and in cultured rectal mucosa cells from patients in the active, but not remission, phases of ulcerative colitis.[52] In a more detailed study, Zifroni et al.[53] showed that cultures of intestinal mononuclear cells from patients with Crohn's disease synthesised more prostanoids than control cultures; in mononuclear cell

cultures from ulcerative colitis patients, prostanoid levels were only elevated in the one patient who was not being treated with high doses of corticosteroid. More recently, Balint et al.[54] reported that 6-oxo PGF$_{1\alpha}$ levels in the colonic mucosa of newly developed, untreated ulcerative colitis patients were not different from controls. From these studies, a unique pathogenic role for prostacyclin is not apparent. Indeed, indomethacin offers no clinical benefit in ulcerative colitis[55] and has been associated along with other COX inhibitors with flare-up of the colitis leading to hospital admission.[56-58] Perhaps we should be thinking of an anti-inflammatory or cytoprotective role for prostanoids, including prostacyclin, in these conditions.

In the acute phase of human cholera, aspirin, indomethacin and ibuprofen have little effect on the massive purgation present.[59] This is not to say that prostanoids are not involved; Jodal[9] has suggested that the local release of prostanoids is so vast that incomplete inhibition of COX would still leave a substantial stimulus, and this could interact with the high levels of other secretory mediators which are almost certainly present.

6. CONCLUDING REMARKS

Prostanoids are clearly able to modulate activity of the gastrointestinal tract, but identifying their site of action and inferring a physiological/pathophysiological role presents a considerable challenge. To date, IP-receptors would appear to be located primarily on enteric neurones, but their precise location on these neurones and the signal transduction pathways involved have yet to be investigated in detail.

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Chapter 11

IP-receptors in the central nervous system

1. INTRODUCTION

Although relatively little attention has been paid in the past to the role of prostacyclin in the central nervous system (CNS), we already know a great deal about the regulation of IP-receptors and their coupling to adenylate cyclase from studies using neuronal cell lines. At the present time though our attention is strongly drawn to the role of prostacyclin in the CNS following the discovery by Takechi et al.[1] of a novel IP-receptor subtype present in rat CNS; referred to here as the IP_2-receptor. One of the aims of the following chapter is to clarify why this CNS receptor remained unknown for so long, and to discuss its possible significance.

2. IP-RECEPTORS IN NEURONAL CELL LINES

2.1 Neuroblastoma cells

Regulation of adenylate cyclase activity by prostacyclin has been demonstrated in many tissues with several of the early detailed studies employing neuroblastoma cell lines,[2-4] (see Table 18). Additionally, some of the first studies using radiolabelled IP agonists were performed using neuroblastoma cell lines such as NCB-20[5,6] and NG108-15 .[7] But whether these so-called neuronal cell lines truely represent neuronal cells in vivo is a crucial factor in our correct interpretation of the potential contribution of IP-

receptors to neuronal activity in the CNS. Twenty years ago it was noted that prostacyclin was a much more potent stimulator of adenylate cyclase activity in neuroblastoma cells (EC_{50} 3 nM) than in astrocytoma cells (EC_{50} 10 μM).[2] The neuroblastoma cells were of mouse origin while the astrocytoma cells were human, and although we should remain cautious when interpreting data across species, the difference in EC_{50} values is more likely to represent a lack of functionally significant IP-receptors on astrocytoma cells, rather than a difference between mouse and human IP-receptors. The NCB-20 cell line has featured in many of the early studies of "neuronal" IP-receptors. This cell line derives from N18TG2 mouse neuroblastoma and foetal Chinese hamster brain cells, thus both parent cell lines are neuronal in origin and the hybrid expresses numerous functions of differentiated nerve cells.[3] However, data in Table 18 and our current knowledge on the relative lack of [^{3}H]-iloprost binding sites in the CNS[1,8] might suggest that the adenylate cyclase stimulatory activity of prostacyclin in NCB-20 cells derives primarily from their neuroblastoma rather than their neuronal heritage.

Table 18. comparison of the adenylate cyclase stimulatory activity of IP agonists in neuronal cell lines

Cell line	*Adenylate cyclase activity* EC_{50} *or* K_{act} *(nM)*	
	Prostacyclin	*Iloprost*
1321N1 human astrocytoma[2]	10,000	
SK-N-SH human neuroblastoma[9]		16
N4TG3 mouse neuroblastoma[2]	3	
N18TG2 mouse neuroblastoma (parent of NCB-20 and NG108-15 cell lines)[4]	22	
NCB-20 mouse neuroblastoma x foetal hamster brain cells[3,6,7,10,11]	25,28, 40, 64, 76	79
NG108-15 mouse neuroblastoma x rat glioma hybrid[7]		24

K_{act} is the concentration of agonist giving half-maximum enzyme activation.

The NCB-20 cells usually display a single class of [^{3}H]-iloprost binding sites with a K_d of 5 - 30 nM and B_{max} of 183 - 347 fmol mg protein^{-1}, equivalent to about 80,000 sites per cell.[6,12] It is interesting to note that very similar B_{max} values had been recorded earlier using [^{3}H]-PGI $_2$ as the radioligand.[5] In contrast, Leigh & MacDermot[11] demonstrated the presence

of two binding sites for [^{3}H]-iloprost in NCB-20 cells; there was a single population of high affinity receptors (K_d 9.55 nM and B_{max} 431 fmol mg protein^{-1}) and an additional second low affinity, nonsaturable, nonstereospecific site which appeared of no biological significance. It is likely that this second site is not often seen in other studies due to the need to use relatively high concentrations of radioligand.[7]

Iloprost and carbacyclin show similar potencies for competing with [^{3}H]-iloprost binding to NCB-20 cells (30 and 21 nM respectively in a study by Hall & Strange,[6] and 100 and 210 nM respectively in a study by Armstrong et al.[13]). Although Hall & Strange[6] concluded that there was a broad agreement between data from NCB-20 cells and human platelets, in hindsight we see the same signs of a species difference for carbacyclin compared to iloprost as seen in cells transfected with IP_1-receptor cDNA (see Chapter 4, Table 5).

NG108-15 cells (mouse N18TG2 neuroblastoma x rat-glioma hybrid) are another neuroblastoma cell line frequently used in the study of IP-receptors. As seen with NCB-20 cells, the K_d value for [^{3}H]-iloprost binding to membranes of NG108-15 cells is also approximately 5 - 15 nM,7 which again is similar to the affinity of [^{3}H]-iloprost binding in the nucleus tractus solitarius (NTS) rather than in the thalamus,[1] (see Table 20) suggesting that we are looking at the platelet-like IP1-receptor.

Early studies using NCB-20 cells clearly showed the high potency of prostacyclin in stimulating cyclic AMP production (EC_{50} 25 nM), suggesting a direct neuronal effect of this prostanoid.[3] By using 6P-PGI_1, a stable analogue of prostacyclin, Blair et al.[3] showed that the increase in adenylate cyclase activity was mediated by a single receptor population; data which were later supported by radioligand binding studies.[5,11] The parent cell line to NCB-20 and NG108-15 cells, i.e. N18TG2, shows the conventional order of potency for an IP-receptor of prostacyclin > PGE_1 >> PGE_2 for stimulation of adenylate cyclase activity, but at the time it was not known whether these agonists bound to the same or different receptors.[4] Later that year (1981), Blair & MacDermot[5] suggested that PGE_1 receptors should really be considered as prostacyclin receptors on the basis of [^{3}H]-PGI_2 binding properties to NCB-20 cell membranes because PGE_1 displayed only 8-fold lower affinity than prostacyclin, whereas PGE_2 was 800-fold less potent. Nonprostanoid prostacyclin mimetics (see Chapter 3) frequently behave as partial agonists compared to cicaprost or iloprost when studied as activators of adenylate cyclase, as seen with EP 157 and EP 035 in NCB-20 cells and BMY 42393 and BMY 45778 in SK-N-SH cells (see Table 19).

Table 19. Comparison of the adenylate cyclase stimulatory activity of IP agonists in mouse/hamster (NCB-20) and human (SK-N-SH) neuroblastoma cells

Drug	*NCB-20 cells*[6]	*NCB-20 cell*[13]		*SK-N-SH cells*[9]	
	EC_{50} (nM)	*EC_{20} (nM)*	*Maximum response (% iloprost)*	*EC_{50} (nM)*	*Maximum response (% cicaprost)*
Prostacyclin	76				
Cicaprost				12	100*
Iloprost	79	12	100**	16	103
Carbacyclin	100	95	78		
BMY 45778				18	48
BMY 42393				1905	79
Octimibate				1479	34
EP 157		1660	49		
EP 035		7240	24		

*30 -fold increase, ** 15-fold increase.

NG108-15 cells are currently the favoured cell line for studying IP-receptor regulation. The IP-receptor in NG108-15 cells is present at approximately 100,000 copies per cell, whereas there are some 1,250,000 copies of $G_{s\alpha}$ but only 17,500 copies of adenylate cyclase per cell.[14] Thus the amount of adenylate cyclase present is thought to form the rate-limiting component in the signal transduction pathway following IP-receptor stimulation in these NG108-15 cells. Iloprost causes a concentration-dependent increase in the formation of the complex between $G_{s\alpha}$ and adenylate cyclase (as measured by specific high affinity binding of [^{3}H]-forskolin) in NG108-15 cells, with an IC_{50} value of 5 nM.[15]

Incubation of NCB-20 cells with 1 μM carbacyclin for 16 h increased K_{act} (concentration of agonist giving half-maximum enzyme activation) and decreased the maximum rate of cyclic AMP production as seen by a decrease in both the K_d and B_{max} for [^{3}H]-PGI_2 binding.[10] Later studies failed to demonstrate any change in K_d,[7,11] and now it would be accepted that downregulation of IP-receptor-mediated responses is due to loss of receptors rather than a change in receptor binding affinity. However in these and other studies,[5] [^{3}H]-PGI_2 was used as the radioligand and it exhibits a high level of nonspecific binding which may complicate data interpretation; therefore later studies have concentrated on the more useful radioligand [^{3}H]-iloprost.

Cyclic AMP production by NCB-20 cells was downregulated following exposure to IP agonists such as prostacyclin, iloprost, carbacyclin and PGE_1, but unlike the effect of carbacyclin pretreatment in NG108-15 cells, there was no evidence for heterologous desensitisation.[7,11,16] This is perhaps surprising given that preincubation of N18TG2 mouse neuroblastoma cells (i.e. one of the parental cells of both NCB-20 and NG108-15 cell lines) with

1 μM PGE_1 produced heterologous desensitisation.[4] Therefore, why prolonged incubation of NCB-20 cells with IP agonists should only produce homologous desensitisation remains a mystery.

In the heterologous form of desensitisation seen in NG108-15 cells, there is loss of adenylate cyclase responsiveness not only to IP agonists, but also to adenosine A_2-receptor agonists and NaF.[7] It was suggested that this homologous and heterologous desensitisation was most likely mediated by a single process such as co-internalisation of receptors and G-proteins.[16] Kelly et al.[7] proposed that the loss of responsiveness in NG108-15 cells is probably related to the functional loss of $G_{s\alpha}$ in these cells. In NG108-15 cells, IP agonists induce a biphasic desensitisation of IP-receptor-stimulated adenylate cyclase with an initial phase coinciding temporally with loss of receptors and $G_{s\alpha}$ whereas the second component appears to occur independently of $G_{s\alpha}$ loss.[17] The role of IP-receptor internalisation in the process of IP-receptor downregulation is still far from clear,[18-20] although a major component may be due to loss of IP-receptors and $G_{s\alpha}$ 17 and the increased breakdown of existing receptors.[21] In opposition to IP-agonist-mediated downregulation of IP-receptors, the cyclic AMP response of NG108-15 cells and human neuroblastoma SH-SY5Y cells to PGE_1 (presumably via activation of IP-receptors) can be increased in opioid-tolerant/dependent cells due to enhanced coupling efficiency between the receptor and G_s.[22,23]

Despite some confusion over the status of IP-receptors in neuronal cells, we should perhaps acknowledge here the major contribution which NCB-20 neuroblastoma cells have made to our understanding of the critical groups in the prostacyclin molecule which allow binding to the IP-receptor,[3] see Chapter 4, Section 3.1. The IP-receptors present in neuroblastoma cells have been further characterised using some of the nonprostanoid prostacyclin mimetics related to octimibate.[9] These studies showed that the IP-receptor coupled to adenylate cyclase in the human neuroblastoma cell line SK-N-SH was indeed similar to the human platelet IP_1-receptor. This is perhaps not unexpected because neuroblastoma cell lines derive from neural crest cells which ordinarily give rise to spinal ganglionic cells,[24] and as noted in Chapter 9, the dorsal horn and dorsal root ganglia (DRG) are well endowed with mRNA for the IP_1-receptor. Exactly what are the consequences of IP-receptor mediated increases in cyclic AMP in NG 108- 15 cells remains to be clarified. We know that iloprost partially inhibits both L- and N-type calcium channel currents, and this effect can be attenuated with protein kinase A inhibitors such as Rp-cAMPS and H89, and by downregulation of G_s,[25] thus one of the consequences of IP-receptor activation in NG108-15 cells is cyclic AMP-dependent inhibition of calcium channel currents.

2.2 Glial cells

Whether or not IP-receptors are important in the functioning of glial cells is unclear. The presence of EP-, FP- and TP-receptors has been detected on cultured glial cells, but no mention was made of IP-receptors.[26] Messenger RNA for EP_3-, FP- and TP-receptors is present in cultured rat astrocytes and oligodendrocytes, but only EP_3- and TP-receptor mRNA was found in microglia.[27] More recently, Oida et al.[28] found no evidence of IP_1-receptor mRNA in mouse glial cells. With the IP_1-receptor being the last of the prostanoid family of receptors to be cloned, we will have to wait a while to gain more information on the expression of IP_1-receptor mRNA in isolated cultures of glial cells.

Purified primary cultures of rat type-1 rather than type-2 astrocytes respond well to PGD_2 and PGE_2, but there is only a minimal cyclic AMP response to iloprost.[29] In conjunction with the low potency of prostacyclin in human astrocytoma cells (EC_{50} 10 μM) [2] it is therefore probable that astrocytes lack functionally significant IP-receptors.

Microglial cells are the resident macrophages of the brain and, like peripheral macrophages, can be stimulated by lipopolysaccharide (LPS) or cytokines to release prostanoids. LPS induces expression of cyclo-oxygenase-2 (COX-2), but not COX-1 mRNA in microglial cultures from neonatal rat cerebral cortex, producing PGE_2, PGD_2 and TXA_2 in greater amounts than in astrocytes.[30] In contrast, LPS-stimulated LTB_4 production was much greater in astrocytes than in microglial cells; sadly no measurements of prostacyclin production were made in this study.

3. IP-RECEPTORS IN NEURONAL TISSUES

3.1 Distribution of IP-receptors in the CNS

The expression of IP_1-receptor mRNA was not detected in mouse or human brains;[31,32] these data match the relative lack of [^{3}H]-iloprost binding in the higher centres of rat CNS and contrast with the distinct distribution of IP_1-receptor mRNA and [^{3}H] -iloprost binding in the nucleus tractus solitarius (NTS), spinal trigeminal nucleus, dorsal horn and DRG.[8,28] Although [^{3}H]-iloprost shows little binding in the CNS, [^{3}H]-isocarbacyclin binds well, Thus, in addition to binding in the rat NTS and spinaltrigeminal nucleus, [^{3}H] -isocarbacyclin binding was observed in the thalamus, lateral septal nucleus, hippocampus, cerebral cortex, striatum, and dorsal cochlear nucleus.[1] [^{3}H]-Isocarbacyclin did not appear to discriminate between IP-

receptors in the NTS and thalamus, whereas [^{3}H]-iloprost bound with 23-fold lower affinity in the NTS compared with the thalamus (Table 20). Furthermore, the distribution of [^{3}H]-isocarbacyclin was quite different from that of [^{3}H]-PGD_2, [^{3}H]-PGE_1, [^{3}H]-PGE_2 and [^{3}H]-$PGF_{2\alpha}$. Thus [^{3}H]-isocarbacyclin recognises a distinct form of the IP-receptor in the CNS, and one that is poorly recognised by iloprost.

Table 20. Scatchard analysis of [^{3}H]-iloprost and [^{3}H]-isocarbacyclin binding in rat brain

Brain region	*[3H]-iloprost*		*[3H]-isocarbacyclin*	
	K_d *(nM)*	B_{max} *fmoI mg protein^{-1})*	K_d *(nM)*	B_{max} *fmol mg protein^{-1})*
Nucleus tractus solitarius (NTS)	6.8	194	3.9	199
Thalamus	159	163	7.8	230

Reprinted with permission from Wise[33] using data from Takechi et al.[1]

Earlier studies from Watanabe's group[8] had revealed that [^{3}H]-iloprost binding sites in sensory neurones were distributed presynaptically (see Chapter 9). In contrast, hemilesion studies of striatal neurones lesioned by kainate or of dopaminergic afferents lesioned by 6-hydroxydopamine revealed that the binding sites for [^{3}H]-isocarbacyclin existed postsynaptically on neuronal cells in the striatum, but not on the presynaptic terminals of afferents or on glial cells.[1] Therefore Watanabe proposed to designate this new IP-receptor subtype in the CNS, i.e. the [^{3}H]-isocarbacyclin binding site, as the IP_2-receptor and refer to the classical platelet IP-receptor as the IP_1-receptor;[1] a system of nomenclature that we are also using in this monograph.

Having identified 15R-TIC (15R-16-*m*-tolyl isocarbacyclin, see Fig. 18) as a selective ligand for this neuronal IP_2-receptor (see Section 3.2 below), the next step was taken by Watanabe's group to prepare [^{3}H]-15R-TIC and examine its binding distribution in rat brain. [^{3}H]-15R-TIC binding in frozen sections of rat brain was similar to that of [^{3}H]-isocarbacyclin,[1] being high in thalamus, striatum, hippocampus, and piriform, entorhinal and some other cortices.[34] Two components of high affinity binding were found in the rostral regions with K_d values of < 1 nM and approximately 30 nM. In a positron emission tomography (PET) study, the methyl ester of [^{11}C]-15R-TIC showed higher uptake into normal rhesus monkey brain than did the free acid form, and also showed a similar distribution to that seen in rat brain.[34] Once the methyl ester form gets into the brain, it is deesterified and then binds to the IP_2-receptor, with the highest uptake in the thalamus, and lesser uptake in the striatum and some cortical areas.

3.2 Characterisation of IP-receptors in the CNS

Further support for the idea that [^{3}H]-isocarbacyclin recognised a novel IP-receptor in the CNS came from detailed characterisation studies and the discovery of 15R-TIC. The origin of 15R-TIC lies in attempts to convert isocarbacyclin into a ligand suitable for PET studies.[35] However, 15R-TIC turned out to be a selective ligand for the IP_2-receptor, able to compete with [^{3}H]-isocarbacyclin binding in rat thalamus and NTS with IC_{50} values of 31 nM and 1.2 μM respectively.[35] Specificity of [^{3}H]-isocarbacyclin binding in rat brain was distinctly different in the NTS compared with the thalamus; the order of potency for competition with [^{3}H]-isocarbacyclin binding in the NTS was isocarbacyclin = cicaprost = iloprost > carbacyclin > PGE_1> 15R-TIC > PGE_2 > PGD_2 = $PGF_{2\alpha}$ compared with the thalamus where isocarbacyclin = 15R-TIC > carbacyclin > iloprost > PGE_2 > PGE_1 = cicaprost>PGD_2=$PGF_{2\alpha}$[1].

In agreement with these binding experiments, 15R-TIC was approximately 100-fold less potent than cicaprost and isocarbacyclin in inhibiting platelet aggregation.[35] Although the 15 S-isomer also showed high affinity binding in the thalamus (IC_{50} = 38 nM), it did not discriminate between the platelet IP_1-receptor and neuronal IP_2-receptor (IC_{50} = 23 nM in NTS).[35] Another distinction of this neuronal IP-receptor is that it does not appear to elicit changes in cyclic AMP production, phospholipid turnover, or calcium concentrations, but the authors make a cautionary note that further experiments are needed to make sure that this is not simply a reflection of low sensitivity assays in cell systems which may have a low level of receptor expression.

3.3 Function of IP-receptors in the CNS

In the 1960's, Avanzino et al.[36,37] observed that PGE_1 had a selective excitatory action on brainstem neurones of the cat, but noted that the functional significance of these observations was unknown; amazingly we can still say the same thing some 30 years later. In terms of behavioural effects, PGE_1 tends to be depressant,[38] although this effect could be due to activation of EP-receptors. In addition, the effect of IP agonists is highly species dependent, e.g. prostacyclin injected into the third cerebral ventricle (i.c.v.) will increase locomotor activity in rabbits.[39] Although prostacyclin injected i.c.v. can cause a surprisingly long-lasting hyperthermic response, it is considerably less potent than PGE_2 and would not appear to be involved in the pathogenesis of fever.[39]

Beraprost (0.3 mg kg^{-1}, orally) suppresses spontaneous activity and reduces body temperature in mice with similar pharmacological effects as

prostacyclin when administered intravenously.[40] Although this behavioural suppression by IP agonists was described as a CNS effect, it is unlikely that the decrease in spontaneous activity is truely a neuronal effect of beraprost. This is because the dose-response relationship for the hypothermia response, the decrease in spontaneous activity and the potentiation of hexabarbitone-induced sleeping time were similar, and the latter two responses could well be ascribed to the hypothermic effect of beraprost. Because flushing of the skin was noticeable at even the lowest dose of beraprost studied (0.3 mg/kg, orally), then the hypothermia could be due to the vasodilator action of beraprost. Cerebral blood flow will depend on the relative dilator capacities of other vascular beds (i.e. the steal phenomenon; see Haberey et al.[41]) and the ability of the heart to maintain cardiac output under conditions of arterial hypotension and reduced venous return.

The pharmacology of the isomeric pair FCE-22176 and FCE-22177 has already been described in Chapter 4 (Section 5). It is however worth pointing out here that while the 5E-isomer (FCE-22177; natural configuration) was depressive in mice following intravenous injection (5 - 400 μg kg^{-1}), the 5Z-isomer (FCE-22176) was stimulant.[42] Because FCE-22176 had been claimed to be a potent IP antagonist,[43] it was suggested that FCE-22 176-induced excitation was due to antagonism of the endogenous (depressive) prostacyclin, or indeed, that there was a different site of action.[42] It will be interesting to see how this theory survives the recognition of a novel IP2-receptor in the rat CNS.

While the distribution of [^{3}H]-iloprost binding sites in the CNS readily supports an involvement of IP-receptors in sensory pathways (see Chapter 9), a functional correlate with the distribution of [^{3}H]-isocarbacyclin is harder to make. Therefore when considering the possible functions of neuronal IP_2-receptors, perhaps we should start to concentrate on their distinct cellular distribution. Thus the classical IP_1-receptor appears to be localised presynaptically in DRG neurones and visceral afferents,[8] (as does the peripheral neuronal IP-receptor present in guinea-pig vas deferens[44,45]) whereas the neuronal IP_2-receptor of Takechi et al.[1] is located postsynaptically. Electrophysiological studies in the CA1 region of the rat hippocampus revealed that prostacyclin itself, and some prostacyclin analogues, have a facilitatory effect on excitatory transmission through this novel IP_2-receptor, but that cicaprost shows little or no activity matching its inability to compete with [^{3}H]-isocarbacyclin for binding in the rat CNS.[1]

The blood-brain-barrier permeability of isocarbacyclin and related analogues can be increased by incorporating the prostanoids into lipid micros heres,[46,47] and when the methyl ester form of isocarbacyclin (TEI-9090)[48] is incorporated into lipid microspheres, the resulting drug (TTC-909) can pass through the blood-brain-barrier where it changes to its active

metabolite isocarbacyclin (TEI-7165). When given intravenously 10 min after transient forebrain ischaemia in stroke-prone spontaneously hypertensive rats, TTC-909 dose-dependently protected against pyramidal cell layer death in the hippocampus.[46] Although the attentuation of ischaemic neuronal cell death by TTC-909 may be due to improvement in post-ischaemic events in the local cerebral blood flow and blood-brain-barrier permeability, the authors speculate that there may also be a direct effect of TTC-909 on the CNS itself. This conclusion is looking increasingly attractive given that 15R-TIC and its more potent derivative, 15-deoxy-TIC, have an inhibitory effect on apoptosis of neuronal cells at nanomolar concentrations.[49]

Furthermore, when patients with chronic cerebral infarction were treated with this lipid isocarbacyclin preparation, a significant improvement in neurological and mental symptoms was noted after merely seven days.[50] However, because lipid microspheres containing prostacyclin analogues tend to accumulate at the arteriosclerotic lesions in the vascular wall,[50] and because isocarbacyclin is nonselective for IP_1-receptor and IP_2-receptors, it is possible that we are primarily seeing a beneficial cardiovascular effect of IP agonists, rather than an effect mediated by neuronal IP_2-receptors.

When considering the potential role of the prostacyclin in the CNS, it is worth noting the results from clinical investigations of patients with Alzheimer's disease. There appears to be a beneficial effect for patients taking nonsteroidal anti-inflammatory drugs (NSAIDs) on the progression of the disease which may indicate an important role for prostanoids in neurodegenerative diseases;[51] only time will tell if prostacyclin is one such prostanoid.

3.4 Cyclo-oxygenase activity in the CNS

We have already noted in the preceeding chapters that prostacyclin is involved in many biological functions in addition to simply inhibiting platelet aggregation. Perhaps what is surprising when trying to solve the puzzle of the role of prostacyclin (if any) in the CNS is the range of psychotropic drugs able to inhibit COX activity. Thus, monoamine oxidase inhibitors such as phenelzine and tranylcypromine, and the tricyclic antidepressant doxepin have comparable potency with indomethacin when tested in vitro,[52] Additionally, tranylcypromine inhibits prostacyclin synthase activity.[53] Whether or not any of the prostanoids themselves play a role in depression though is another question altogether.

If we consider the sensory afferent nerves which respond to IP agonists, we can see that the source of endogenous prostacyclin is both the initial inflammatory event, e.g. prostacyclin generated at the site of inflammation,

and by prostacyclin generated intraneuronally as in the case of bradykinin-activation of nodose ganglia neurones (see Chapter 9). But what is the source of prostacyclin in the CNS which will be needed to activate the postsynaptic IP_2-receptor? Sympathetic postganglionic neurones can produce prostacyclin in response to noradrenergic stimulation,[54,55] therefore it has been presumed that prostacyclin production by other neuronal cells is possible, Definite proof of this though has been difficult to obtain due to the low level of prostanoid production by cultured neuronal cells,[56] which of course may not truly reflect the situation in vivo.

Eicosanoids, including 6-oxo $PGF_{1\alpha}$, can be released in micromolar concentrations from brain tissues under hypoxic stress.[57,58] 6-Oxo $PGF_{1\alpha}$ is detectable in the brain and is increased by pathological conditions such as convulsion,[59] and prostaglandins are released during tonic-clonic convulsions, possibly as part of an anticonvulsive action mediated primarily by PGD_2.[60] There are also a number of studies showing the release of prostaglandins from the CNS and from cultured neuroblastoma and glial cells, and in general the rate of release correlates with the level of neuronal activity/stimulation.[60-62] However the profile of synthesis reported varies markedly among tissues and species.[63] For example, the small blood vessels, choroid plexus, and the leptomeninges tend to synthesise more prostacyclin than PGE_2, $PGF_{2\alpha}$, or TXA_2, whereas brain tissue makes mostly PGD_2, PGE_2, or $PGF_{2\alpha}$ depending on the species; PGD_2 appears to be the major prostaglandin metabolite in rat brain.[57] On this basis it would seem that the neuronal IP-receptors are more likely to be activated by prostacyclin released from non-neuronal tissues, but more studies are needed here.

In inflammatory events, COX-2 is the inducible enzyme, but COX-2 mRNA in the CNS is constitutively expressed in rat telencephalic neurones even under normal unstimulated conditions.[64] Indeed, the expression of the COX-2 gene in the CNS appears to be activity-dependent, and increases in response to electroconvulsive seizure activity and natural synaptic activity.[65] Furthermore, COX-2 is a member of a growing family of immediate early genes (IEG) that are rapidly regulated in brain neurones by synaptic activity but do not appear to encode transcription factors. And as with other immediate early genes, COX-2 mRNA shows a distinct developmental pattern which parallels that of excitatory synapse formation in the rat brain.[65] Because the increased expression of COX-2 would be expected to increase the generation of PGH_2, we clearly need a better picture of the distribution of prostacyclin synthase in the brain to help understand the role for prostacyclin here. As pointed out by Yamagata et al.,[65] PGH_2 is highly lipid soluble and could diffuse from neurones to more remote regions of the same neurone, or to adjacent neurones and glial cells. Thus, PGH_2 might diffuse to adjacent oligodendroglia and be converted to PGD_2, or to microvessels and be

converted to prostacyclin. Additionally, one could speculate that PGH_2 might diffuse intraneuronally to be converted to prostacyclin elsewhere in the neurone.

In experimental seizures in rats, COX-2 mRNA (but not COX-1 mRNA) is up-regulated in neurones, an effect which is blocked by *N*-methyl-D-aspartate (NMDA)-receptor antagonists such as MK801.[65] COX-2 expression also increases in response to high frequency stimili applied in the hippocampus (blocked by MK-801), and is induced by acute stress.[65] Excitotoxin injection into the rat nucleus basalis also causes a marked induction of COX-2 mRNA which is attenuated by MK-801 and by inhibition of glutamate release by lamotrigine.[66] This suggests that COX-2 expression may be induced by activation of NMDA-receptors as glutamate is released during cerebral ischaemia. There is also a correlation between the extent of COX-2 mRNA induction in cortical regions at 4 h (attenuated by MK-801) and the severity of tissue damage at 24 h following cerebral ischaemia resulting from permanent middle cerebral artery occlusion in the rat.[67] COX-2 immunoreactivity (COX-2-i.r.) was particularly prominent at the medial edge of an ischaemic area, and its absence in glial fibrillary acidic protein (GFAP)-positive cells suggested that COX-2 is located neuronally.[68] Furthermore, the COX-2 inhibitor NS-398 reduced cerebral ischeamic damage at the periphery of the infarct suggesting that COX-2 is involved in mechanisms of delayed neuronal death at the infarct border.

COX-2-i.r. is selectivity expressed in subpopulations of excitatory neurones in neo- and allocortices, hippocampus and amygdala, and is compartmentalised to dendritic arborisations.[69] Moreover, COX-2-i.r. is present in dendritic spines, which are specialised structures involved in synaptic signalling, and since COX-2 produces diffusible prostanoid products, it may play a role in postsynaptic signalling of excitatory neurones in cortex and associated structures. Since COX-2 expression is tightly regulated by neuronal activity, Kaufmann et al.[69] inferred that neurones regulate the production of prostanoids by controlling the availability of cox-2.

Double-labelling analyses demonstrated a lack of co-localisation of COX-2 and GFAP, suggesting that COX-2 is not present in glial cells, but is localised in neuronal cells.[68,69] Despite these observations, there is some evidence for COX-2-i.r. induced by LPS in isolated glial cells.[30] Furthermore, when studied in isolation, microglial cultures display a distinct response to activation of adenosine A_{2a}-receptors which leads to an increase in COX-2 mRNA levels and the synthesis of PGE_2.[70] However when studied in mixed primary cultures, prostaglandin endoperoxide synthase protein (i.e. COX) was found predominantly in neurones, not in glial cells, and the results suggest that COX is induced mainly in the neurone during the

developmental stage with the aid of a certain factor(s) derived from proliferating glial cells, and then is located in both neurones and glial cells in the mature brain.[71]

Injection of LPS in rats can induce COX-2 expression in nonparenchymal cells of blood vessels and leptomeninges, and in neuronal cells restricted to telencephalic areas. Thus Cao et al.[64] concluded that prostaglandins derived from induced COX-2 in the inner surface of blood vessels and leptomeninges were most likely responsible for fever generation in response to LPS, and later went on to identify endothelial cells as the major source of COX-2 induced in response to interleukin-1β.[72] In a model of closed head injury in rats, the 6-oxo $PGF_{1\alpha}$ concentration in cortical slices taken from the injured zone doubles after 15 min and returns to basal levels between 4 and 10 days following injury.[73] The most marked changes in cerebrospinal fluid prostacyclin and TXA_2 occur during the first 4 h after stroke onset, whereas they were undetectable in cerebrospinal fluid samples from non-neurologic patients,[74] and samples of ventricular fluid from patients with head injuries yield some of the highest prostanoid values, especially for prostacyclin.[63] Therefore, it is possible that vascular-derived prostacyclin is crucial in injuries to the head, with additional prostanoids being generated by activity-dependent COX-2 in neurones under conditions resulting in high glutamate concentrations.[75,76]

In glutamate-induced cytotoxicity of primary cultures of rat cortical neurones, PGE_1 and EP_2 agonists were cytoprotective, but since PGE_1 was much less potent than PGE_2, it is probable that PGE_1 is not acting via IP-receptors to mediate this cytoprotective action.[77] In contrast, prostacyclin has a direct cytoprotective effect on rat brain cells subject to hypoxia/reoxygenation or glutamate-induced injury.[78] Curiously, this cytoprotective effect of prostacyclin and prostaglandins in general in the CNS may not be mediated by cell surface receptors,[79] or by cyclic AMP.[78] Given that carbacyclin can also function independently of a cell surface IP-receptor in adipocytes, possibly by activation of the nuclear peroxisome proliferator-activated receptor (PPAR) family,[80] then it would be interesting to look for a comparable relationship in the CNS.

NMDA stimulates phospholipase A_2 activity by a Ca^{2+}-dependent mechanism in a variety of neuronal cell populations resulting in arachidonic acid release, and COX inhibitors block NMDA induction of *c–fos* mRNA.[75] Further studies demonstrated that NMDA receptor activation triggers synthesis of PGE_2 and $PGF_{2\alpha}$, but not PGD_2 in rat cerebral cortical neurones in vitro, with $PGF_{2\alpha}$ being particularly important for the NMDA induction of *c-fos* mRNA in dentate gyrus cells.[76] Sadly for the current discussion, no measurements of prostacyclin were made in this study. Now *c–fos* is an intermediate early gene and as such is crucial in the development of activity-

dependent modifications in neuronal structure and function.[76] NMDA receptors are enriched in dendrites and are preferentially localised to dendritic spines of neurones,[75] which is exactly what we see for the localisation of COX-2-i.r.[69] With COX-2 now also recognised as an intermediate early gene,[65,69] we await the future with keen interest once prostacyclin is included in such studies as these.

In line with the comments made by Yamagata et al.[65] concerning PGH_2, Lerea et al.[76] also noted that while these current studies have measured the release of prostaglandins from NMDA-stimulated neuronal cells, the concentration of intracellular prostaglandins remains unknown. Therefore in this particular example looking at $PGF_{2\alpha}$, it is still possible the $PGF_{2\alpha}$ acts as an intracellular diffusible signal (intracrine) travelling from the site of synthesis to the nucleus and in this way it could serve a pivotal role in the spatial translocation of a signal from the surface NMDA-receptor located on distal dendrites to the nucleus. Alternatively, prostaglandins could diffuse to act extracellularly (autocrine/paracrine), or the arachidonic acid released after NMDA receptor activation could diffuse from neurone to glial cells for conversion. All these possibilities need careful consideration given the non-cell surface receptor activity of carbacyclin in adipocytes,[80] and the cytoprotective action of IP agonists in the CNS.[78,79]

4. DISCUSSION

The information presented in this chapter both challenges our understanding of neuronal IP-receptors and also begs the question: what makes an isolated cell line truely neuronal? For many years, IP-receptor function and regulation at the biochemical level has been studied using the so-called neuronal cell lines NCB-20 and NG108-15. With the currently available evidence, the IP-receptor expressed by these neuroblastoma cells has the characteristics of the platelet IP_1-receptor, i.e. it has high affinity for cicaprost and iloprost, and responds to these IP agonists by increasing cyclic AMP production. In contrast, the neuronal IP_2-receptor subtype described by Takechi et al.[1] shows low affinity for cicaprost and iloprost, and does not appear to respond by increasing cyclic AMP production. Until definitive proof is provided by studies of 15R-[^{3}H]-TIC binding to neuroblastoma cells, then it might be prudent to consider these cells as models for studying platelet rather than neuronal IP-receptors. Therefore, if a neuronal cell line has any neuroblastoma cell heritage, then we must be cautious in extending the results to a CNS site of action. Sadly this does limit the ease with which the neuronal IP_2-receptor subtype can be studied.

Another major implication from these studies on rat CNS tissue is our interpretation of data obtained using the standard IP agonists, cicaprost and iloprost. To date, activation by low concentrations of cicaprost has always been used as evidence for the role of IP-receptors in mediating a biological response. However, given the low potency of cicaprost at this neuronal IP_2-receptor, we will now have to be more cautious in dismissing the role of IP-receptors if cicaprost shows little activity. Under these circumstances, it would be advisable to check the activity of prostacyclin or carbacyclin as these are currently the only commercially available agonists for both IP_1 and IP_2-receptors. Prostacyclin in the CNS has long been overlooked on the assumption that its role is simply a cardiovascular one. It is time now to consider the potential role in the CNS for prostacyclin, in addition to other prostanoids, resulting from synaptically-induced COX-2 expression.

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