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Biochemistry, Molecular Biology and Pharmacology of the Prostanoid DP Receptor

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ABSTRACT

The term prostanoids collectively describes prostaglandins, prostacyclin and thromboxanes. These compounds are products of arachidonic acid metabolism by the cyclooxygenase pathway. Prostanoids mediate various physiological and pathophysiological effects through their interaction with membrane-bound receptors. In this research, a thorough characterization of the recombinant human (h) PGD_2 receptor (DP) was performed, with respect to its radioligand binding and signal transduction properties using prostanoids and prostanoid analogues. The recombinant hDP receptor was then used along with other recombinant human prostanoid receptors to identify a novel specific agonist, L-644,698. This compound exhibits high affinity and potency at the hDP receptor. Moreover, L-644,698 demonstrates at least 300-fold higher selectivity for the hDP receptor than for any of the other seven recombinant human prostanoid receptors tested. Thus, L-644,698 is one of the most selective DP-specific agonists as yet described. Subsequently, the rat (r) DP receptor was cloned and functionally expressed. Pharmacological characterization using L-644,698 and other DP-specific ligands confirmed the identity of this protein as a homologue of human DP, and validated the use of the cDNA corresponding to rDP as a template from which to make rDP-specific riboprobes for *in situ* hybridization studies. mRNA corresponding to rDP was localized to the CNS and GI tract by the *in situ* hybridization technique. Within the GI tract, rDP-specific signals were observed repeatedly in the mucous-secreting goblet cells and, less often, in the adjacent epithelium of the stomach, duodenum, ileum, and colon. These observations corroborate prior data demonstrating an abundance of both hDP- and mDP-specific mRNA in GI tract tissues (especially in small intestine), and suggest a novel biological role for the DP receptor, namely the regulation of mucin secretion. DP-specific mRNA was then localized to the mucous-secreting goblet cells of the human colon, justifying the use of an established *in vitro* cell model of human origin for the study of mucin secretion, the LS174T colonic adenocarcinoma cell line. The endogenous expression of the hDP receptor on LS174T cells was confirmed pharmacologically using L-644,698 and other DP-specific ligands. Subsequently, activation of hDP was shown to

stimulate mucin secretion in this cell line, through the use of DP-specific agonists (PGD_2, L-644,698) and a DP-specific antagonist (BW A868C).

RÉSUMÉ

Les prostanoïdes regroupent les prostaglandines, la prostacycline et les thromboxanes. Ces composés sont dérivés de l'acide arachidonique, lequel est métabolisé par la cyclooxygénase. L'interaction des prostanoïdes avec les récepteurs membranaires induit de nombreux effets physiologiques et pathophysiologiques. Dans cette recherche, le récepteur PGD_2 humain (hDP) recombinant a fait l'objet d'une caractérisation approfondie au niveau de ses propriétés de liaison aux radioligands et de transduction du signal, et ce, à l'aide de prostanoïdes et des analogues de prostanoïdes. Le récepteur hDP recombinant a ensuite été utilisé, ainsi que d'autres récepteurs des prostanoïdes humains recombinants, pour identifier un nouvel agoniste spécifique, L-644,698. Ce composé présente une affinité élevée pour le récepteur hDP, auquel il se lie fortement. En outre, l'agoniste L-644,698 démontre une sélectivité au moins 300 fois plus élevée pour le récepteur hDP que pour chacun des sept autres récepteurs des prostanoïdes humains recombinants testés. L-644,698 est donc l'un des agonistes les plus spécifiques pour le récepteur DP connu à ce jour. Par la suite, le récepteur DP du rat (rDP) a été cloné et son expression a été confirmée de façon fonctionnelle. La caractérisation pharmacologique à l'aide du L-644,698 et d'autres ligands spécifiques au récepteur DP a confirmé que cette protéine constitue un homologue du récepteur DP humain. Elle a aussi validé l'utilisation de l'acide deoxyribonucléique complémentaire (ADNc) correspondant au récepteur rDP comme matrice afin d'élaborer des sondes d'acide ribonucléique complémentaire (ARNc) spécifiques pour le récepteur rDP utilisées dans les études d'hybridation *in situ*. L'acide ribonucléique messager (ARNm) correspondant au récepteur rDP a été localisé dans le système nerveux central et les voies gastro-intestinales au moyen des techniques d'hybridation *in situ*. Au niveau des voies gastro-intestinales, des signaux spécifiques au récepteur rDP ont été observés à plusieurs reprises dans les cellules caliciformes sécrétrices de mucus et, plus rarement, dans l'épithélium adjacent de l'estomac, du duodénum, de l'iléon et du côlon. Ces observations confirment les données précédentes qui démontraient l'abondance d'ARNm spécifique aux récepteurs hDP et mDP au niveau des tissus des voies gastro-intestinales (et plus particulièrement de l'intestin grêle) et suggèrent un nouveau rôle biologique pour le récepteur DP, soit la régulation de la

sécrétion de mucine. La présence d'ARNm spécifique au récepteur DP a ensuite été observée au niveau des cellules caliciformes sécrétrices de mucus situées dans le côlon humain, justifiant ainsi l'utilisation d'un modèle cellulaire *in vitro* d'origine humaine pour l'étude de la sécrétion de mucine, soit la lignée cellulaire d'adénocarcinomes coliques LS174T. L'expression endogène du récepteur hDP dans les cellules LS174T a été confirmée pharmacologiquement à l'aide du L-644,698 et d'autres ligands spécifiques au récepteur DP. Finalement, il a été démontré à l'aide d'agonistes spécifiques (PGD_2, L-644,698) et d'un antagoniste spécifique (BW A868C) que l'activation du récepteur hDP stimulait la sécrétion de mucine par cette lignée cellulaire.

Dedicated to my parents and my brother for their unwavering love, encouragement, understanding, and support. Thank you for believing in me.

Especially for Alastair, who acquires knowledge in the same manner that he lives other aspects of his life ... on his own terms.

" The things we know best are the things we haven't been taught ... "
Luc, Marquis de Vauvenargues (1715-47)

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ABBREVIATIONS

-/-	gene knockout
15-OH-PGDH	15-hydroxy-prostaglandin dehydrogenase
5-HT	5-hydroxytryptamine
AA	arachidonic acid
AMP-PNP	adenylyl-imidodiphosphate
AP-1	activator protein - 1
AR	adrenergic receptor
ASA	acetylsalicylic acid
ATII	angiotensin II
ATPγS	adenosine-5′-O-(3-thiotriphosphate)
BCA	bicinchoninic acid
BCG	bromocresol green
BK	bradykinin
BSA	bovine serum albumin
CaLB	calcium-dependent phospholipid binding domain
CHO	Chinese hamster ovary
CNS	central nervous system
COX	cyclooxygenase
CRE	cAMP responsive element
CSF	cerebrospinal fluid
cyclic AMP	adenosine 3′:5′-cyclic monophosphate
cyclic GMP	guanosine 3′:5′-cyclic monophosphate
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
diHET	dihydroxyeicosatrienoic acid
DMEM	Dulbecco's modified Eagle's medium
DP	prostaglandin D_2 receptor
EBNA	Epstein Barr virus nuclear antigen
EDTA	ethylenediamine-tetraacetic acid
EET	epoxyeicosatrienoic acid
EFA	essential fatty acids
EGF	epidermal growth factor
EP_1	prostaglandin E_2 receptor, subtype 1
EP_2	prostaglandin E_2 receptor, subtype 2
EP_3	prostaglandin E_2 receptor, subtype 3
EP_4	prostaglandin E_2 receptor, subtype 4
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FAP	familial adenomatous polyposis
FLAP	five lipoxygenase activating protein
FP	prostaglandin $F_{2\alpha}$ receptor

GABA	gamma aminobutyric acid
GalNAc	N-acetyl galactosamine
GI	gastrointestinal
GMP-PNP	guanylyl-imidodiphosphate
GPCR	guanine nucleotide binding protein-coupled receptor
GRE	glucocorticoid responsive element
GRK	guanine nucleotide binding protein-coupled receptor kinase
GSH	glutathione
GST	glutathione S-transferase
GTPγS	guanosine-5′-O-(3-thiotriphosphate)
h	human
HBSS	Hanks' balanced salt solution
HEK	human embryonic kidney
HEL	human erythroleukemia
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HETE	hydroxyeicosatetraenoic acid
HGF	hepatocyte growth factor
HPETE	hydroperoxyeicosatetraenoic acid
HUVEC	human umbilical vein endothelial cell
IBD	inflammatory bowel disease
ICG	indocyanine green
IFN	interferon
IL	interleukin
IP	prostacyclin receptor
IsoP	isoprostane
JAK	Janus kinase
LDL	low density lipoprotein
LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
m	mouse
MAPK	mitogen-activated protein kinase
Me_2SO	dimethylsulphoxide
MTSES	Na-(2-sulfonatoethyl) methanethiosulfonate
MUC	mucin
NANC	non-adrenergic non-cholinergic
NF-κB	nuclear factor kappa-B
NSAID	nonsteroidal anti-inflammatory drug
oatp	organic anionic transporter protein
ORF	open reading frame
PAF	platelet activating factor
PBS	phosphate buffered saline
PDA	patent ductus arteriosus
PDGF	platelet derived growth factor
PG	prostaglandin
PGDS	prostaglandin D synthase

PGES	prostaglandin E synthase
PGFS	prostaglandin F synthase
PGHS	prostaglandin H synthase
PGIS	prostacyclin synthase
PGT	prostaglandin transporter
PH	pleckstrin homology
PIP_2	phosophoinositol-4.5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptor
PTA	phosphotungstic acid
PTH	parathyroid hormone
r	rat
r.p.-h.p.l.c.	reverse-phase high performance liquid chromatography
REM	rapid eye movement
RT-PCR	reverse transcriptase – polymerase chain reaction
SAR	structure-activity relationship
SDS	sodium dodecyl sulphate
SRS-A	slow reacting substances of anaphylaxis
STAT	signal transducers and activators of transcription
SWS	slow wave sleep
TCA	trichloroacetic acid
TGF	transforming growth factor
TNBS	trinitrobenzene sulfonic acid
TNF	tumour necrosis factor
TP	thromboxane A_2 receptor
TR	tandem repeat
TX	thromboxane
TXAS	thromboxane A synthase
VLPO	ventrolateral preoptic area
vWF	von Willebrand factor
WT	wildtype

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1. INTRODUCTION

1.1. Historical perspective

The discovery of the prostaglandins (PGs) dates back to 1930 when *ex vivo* studies of human uterine tissue demonstrated relaxation and contraction upon the addition of human semen (1). In corroborating and extending this observation (2, 3), other investigators identified an active, lipid-soluble acid that they termed a "prostaglandin", since it was thought to be derived from the prostate gland. At the same time, independent research into the leukotrienes (LTs) was initiated when sputum from asthma patients was observed to stimulate smooth muscle contraction (4). More than 2 decades later, PGs were identified as a group of compounds and 2 family members, PGE_1 and $PGF_{1\alpha}$, were purified (5). Over the next 10-15 years, more members of the PG family were discovered and denoted alphabetically PGA_2 to PGH_2 (6, 7). Structural similarity between PGs and the essential fatty acids was realized and led to the biosynthesis of PGE_2 from arachidonic acid in the presence of sheep seminal vesicle homogenates (8). Subsequent identification of the endoperoxides PGG_2 and PGH_2 as unstable biosynthetic intermediates (9) was closely followed by the discovery of the unstable, PG-like compounds TXA_2 (10) and PGI_2 (11). Collectively, this growing family of PGs and PG-like compounds was termed the "prostanoids". The mechanism of action of aspirin and related drugs was demonstrated to be through inhibition of PG biosynthesis (12). Limited effort had previously been spent elucidating the receptors through which PGs acted, since many investigators believed that PGs dissolved through cell membranes due to their lipid-based structures and did not require receptors for activity. However, attention was eventually focused on the identification and classification of PG receptors in an attempt to rationalize the diversity of PG-mediated activities, the array of which was deemed to limit the usefulness of PGs for drug development. This work began with attempts to elucidate membrane specific-binding sites using radiolabeled derivatives of the endogenous ligands (13, 14, 15), and has been facilitated by the identification of synthetic, stable, selective ligands and the advent of recombinant DNA technology.

1.2. Eicosanoids

1.2.1. Eicosanoid precursors: essential fatty acids and arachidonic acid

The term eicosanoids (Greek: *eicosa* = "twenty") collectively describes prostanoids, leukotrienes, lipoxins, and related compounds [hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs)], and refers to the derivation of these compounds from 20-carbon essential fatty acids (EFAs). The EFA precursors, which contain 3, 4, or 5 sites of double unsaturation, are dihomo-γ-linolenic acid (also called 8, 11, 14-eicosatrienoic acid) (20:3ω-6); arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid) (20:4ω-6); and 5, 8, 11, 14, 17-eicosapentaenoic acid (20:5ω-6), respectively. When used as precursors, the 3, 4 or 5 series of EFAs correspond to the synthesis of the 1, 2, or 3 series of PGs, and the 3, 4, or 5 series of LTs, respectively (16, 17). These polyunsaturated essential fatty acids can be ingested directly (meat, fish or seaweed). Alternatively, arachidonic acid can be derived from dietary linoleic acid (9,12-octadecdienoic acid) (18:2ω-6) through metabolic processes involving desaturation and chain elongation to dihomo-γ-linolenic acid and subsequent desaturation (16, 18).

Arachidonic acid (AA) is the 20-carbon polyunsaturated fatty acid preferentially esterified into mammalian cell membranes and is efficiently incorporated into the sn-2 position of cell membrane phospholipids, making basal levels of free AA relatively low (16, 19). Since AA is the most abundant precursor for eicosanoid production, the 2-series prostanoids and the 4-series LTs are the predominating endogenous species of these eicosanoids. The biosynthesis of eicosanoids is a closely regulated process, depending on the liberation of AA by phospholipases and the re-esterification of AA by acyl transferases (16).

1.2.2. **Arachidonic acid release**

Since AA and its metabolites play such important roles in the regulation of intracellular and intercellular processes (20, 21), it is not surprising that the basal levels of liberated, unesterified AA are highly regulated. In fact, the net concentration of free AA under basal conditions is not known but its formation limits the synthesis of AA metabolites (22, 23, 24). Low-density lipoprotein (LDL) receptor-stimulated lipid hydrolysis, phospholipase-mediated hydrolysis of cell membrane phospholipids, and acyl transferase-mediated re-esterification of free AA into membrane phospholipids are all thought to contribute to the levels of free AA (19, 22, 25).

It has been demonstrated that the LDL receptor pathway can regulate the cellular levels of AA (26). Here, normal fibroblasts that had been pre-incubated with reconstituted LDL containing [^{14}C]arachidonic acid cholesterol esters and then challenged with platelet-derived growth factor (PDGF) produced radiolabeled PGs. However, PDGF challenge of fibroblasts from patients with familial hypercholesterolaemia that lack LDL receptors did not effect the incorporation of radiolabel in *de novo* PGs. Thus, LDL taken up by the LDL receptor pathway can feed the pathway of PG biosynthesis by producing AA, accompanied by the release of cholesterol.

In response to a large number of plasma membrane receptors, including guanine nucleotide binding (G) protein-coupled receptors (GPCRs) and tyrosine kinase receptors, phospholipases breakdown phospholipids and are considered to be major contributors to the cellular levels of liberated AA. Activation of phospholipase A_2 (PLA_2) is considered key in this regard and generates AA concomitantly with lysophospholipid, which is the precursor of the inflammatory mediator platelet-activating factor (PAF). Additionally, AA can be formed indirectly by phospholipase C (PLC) through the synthesis of acylglycerol intermediates (27), by phospholipase A_1 (PLA_1) through synthesis of a LDL intermediate, or by phospholipase D (PLD) through phosphatidic acid and acylglycerol

intermediates. The latter two enzymes are thought to play relatively minor roles in controlling free arachidonic acid levels (23, 24, 27).

PLA_2 is a rapidly growing family of enzymes whose activities control not only eicosanoid and PAF formation, but also membrane remodeling, microbial degradation, and general lipid metabolism (28). The PLA_2 family consists of multiple enzymes classified as numerous types denoted (with roman numerals) I-X. A recently updated classification of the PLA_2 family was proposed based on enzyme structure and biological characteristics (29) which facilitates their comparison. There are three main kinds of PLA_2: secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$). Additionally, there exist PLA_2s not believed to be involved in the arachidonic acid cascade. These include a 26 kDa lysosomal PLA_2 active in acidic pH (30) , a brush-border hydrolase known as phospholipase B (PLB) (31), and a class of enzymes known to degrade PAF, called PAF acetyl hydrolases (32, 33).

The most abundant and well studied of the PLA_2 family are the $sPLA_2$s. They are characterized by having low molecular mass (~14 kDa), a requirement of millimolar calcium concentrations for activity, little fatty acid selectivity *in vitro*, and high structural rigidity conferred by the presence of 5-8 disulfide bonds (34, 35). This stability makes them resistant to proteolysis and denaturation in their extracellular environment, but susceptible to reducing agents. Six mammalian $sPLA_2$ enzymes encoded by different genes have now been identified (34, 36): type I, the closely related group II $sPLA_2$s (types IIA, IIC, IID, and V), and the type X $sPLA_2$. The type I $sPLA_2$s can be divided into 2 groups (A and B) based on structure, and the human form is classified under group B ($sPLA_2$-IB). The human $sPLA_2$-IB is expressed in the pancreas, where it functions in the digestion of dietary phospholipids, and in several non-digestive organs where it may regulate cellular functions through the M-type $sPLA_2$ receptor. $sPLA_2$-IIA is the most abundant human isozyme and is induced by proinflammatory stimuli [such as interleukin (IL)-1 and tumour necrosis factor (TNF)] , as are $sPLA_2$-V (found in human heart and mast cells) and $sPLA_2$-IID (the most recently identified PLA_2, of murine origin). In contrast, $sPLA_2$-X is found in organs associated with the immune response. The $sPLA_2$-IIC is expressed in rodents, but is a pseudogene in humans (37). Considerable interest

has arisen around $sPLA_2$-IIA due to its increased levels in serum and inflammatory exudates during chronic inflammatory diseases (38, 39). Recently, $sPLA_2$ types IIA and V along with $cPLA_2$ (to be discussed below) have demonstrated activity as "signaling" PLA_2s that contribute to the cellular release of AA following agonist stimulation, depending on the phase of cell activation (35, 40, 41). In this regard, the activity of group II sPLAs appears to depend on their cell-surface proteoglycan-binding abilities (42, 43).

In contrast to the smaller $sPLA_2$ enzymes, the type IV $cPLA_2$ is an 85 kDa enzyme with wide tissue and cellular expression. In terms of mRNA distribution (44), human $cPLA_2$ is abundantly expressed in heart, spleen, lung and kidney, and to a lesser extent in hippocampus, eosinophils and neutrophils. It is also present in cell lines derived from monocytes, synovial fibroblasts, granulocytes, and one astrocytoma line (UC11) but not another (U373). $cPLA_2$ protein is found in human platelets (45), neutrophils (46), epithelial cells (47), the U937 monocyte cell line (48), the RAW 264.7 macrophage cell line (49), and in cells from other species (50). $cPLA_2$ is specific for phospholipids esterified with AA in the sn-2 position and its activity is highly regulated by receptor-mediated signal transduction and concomitantly produces lysophospholipid (28, 50). Increased $cPLA_2$ activity as a function of mitogenicity or stimulation is induced by many factors, including IL-1 (51) , TNF (47), macrophage-colony stimulating factor (52), thrombin (53), and lipopolysaccharide (LPS) (54). $cPLA_2$ is transcriptionally regulated by interferon (IFN)γ and glucocorticoid response elements (GREs) in its promoter region (55), by various factors (phorbol ester, PDGF, serum, and others) which increase the $cPLA_2$ transcript half-life, and by an adenosine-uridine rich sequence in the 3′-untranslated region which is thought to confer transcript instability (28). Alternatively, $cPLA_2$ can be posttranscriptionally regulated. Phosphatidylinositol-4,5-bisphosphate (PIP_2) has high affinity for and can activate $cPLA_2$, perhaps through a putative pleckstrin homology (PH) domain (56) based on a region of sequence homologous to the PH domain of phospholipase Cδ1. PH domains are known to be involved in the localization of proteins to membranes by binding phosphoinositides (57). The requirement of calcium by $cPLA_2$ is not related to its catalytic activity (like $sPLA_2$), but rather to its membrane and phospholipid binding capacity (58). Structurally, $cPLA_2$ contains an N-

terminal calcium-dependent phospholipid binding domain (CaLB), which shares homology with C2 domains of other proteins [including protein kinase C (PKC) and phospholipase Cγ] implicated in signal transduction and membrane trafficking, and a C-terminal catalytic domain. The CaLB and catalytic domains are structurally and functionally distinct (59). Calcium-mobilizing agonists are thought to induce translocation of $cPLA_2$ from the cytosol to the nuclear envelope and endoplasmic reticulum (60), where the cyclooxygenases (COXs) and 5-lipoxygenase are localized (61). Cellular localization of $cPLA_2$ translocation is also dependent on cell confluency (62). Phosphorylation of $cPLA_2$ on S505 by mitogen-activated protein kinase (MAPK) or p38 (a MAPK homologue), depending on the cell type, has also been shown to be important for its activation and is thought to be relevant *in vivo* (63). Direct phosphorylation by other kinases, such as PKC and protein kinase A (PKA), occurs but without a consequential increase in $cPLA_2$ activity (28). Increased phosphorylation and a consequential increase in $cPLA_2$ activity may (64) or may not (65) require a concomitant increase in intracellular calcium for AA release, depending on the agonist and the cell type under study. The contributions of $cPLA_2$ to early and late phase eicosanoid formation are well documented (35, 41, 47, 66). The critical role of $cPLA_2$ in the arachidonic acid cascade is highlighted in studies of mice having the $cPLA_2$ gene ablated ($cPLA_2$-/-) by homologous recombination (67, 68). Agonist stimulation of peritoneal macrophages from $cPLA_2$-/- mice did not produce arachidonic acid, eicosanoids (PGE_2, LTB_4, LTC_4), or PAF, in contrast to macrophages from wildtype (WT) mice. Additionally, $cPLA_2$-/- mice had lower allergic reactivity, fewer neurological deficits following ischemia, and compromised parturition, suggesting a nonredundant role in allergic responses, the pathophysiology of neuronal death following ischemia, and in reproductive physiology for $cPLA_2$.

The last of the PLA_2s to be discussed is the type VI $iPLA_2$. This ubiquitously-expressed enzyme, originally identified as a myocardial protein (69), exists as a homotetramer of 85 kDa subunits.(70) with no apparent AA specificity (71). The cellular localization of $iPLA_2$ is exactly the same as $cPLA_2$, and it is characterized by the presence of eight ankyrin-like domains (70) which may play a role in self-aggregation or interaction with other proteins. Evidence has been presented for a role for $iPLA_2$ in

phospholipid remodeling under basal conditions (72, 73). It has also been suggested that AA release by stimulus-activated $iPLA_2$ may occur in multiple cell types (74, 75).

Evidence also exists for the regulation of the reincorporation (reacylation) of AA into membrane phospholipids by PKC (76). The enzymes involved in reacylation (arachidonyl CoA synthetase and acyltransferase) can be negatively regulated by activators of PKC such as phorbol esters and synthetic diacylglycerol (DAG), as has been demonstrated in platelet homogenates. Therefore, following the production DAG by PLC or PLD, activation of PKC by DAG would lead to an inhibition of reacylation, increasing free levels of AA. Independent of this is evidence for AA-mediated activation of PKC (77), suggesting that free AA may mediate further AA liberation from membrane phospholipids.

1.2.3. **Arachidonic acid metabolism**

Once liberated from cell membrane glycerophospholipids, AA can be metabolized by non-enzymatic or enzymatic mechanisms. AA may undergo non-enzymatic free radical oxidation to form the isoprostanes. Much better studied, however, is the enzymatic conversion of AA by the cytochrome P-450/NADPH monooxygenase, lipoxygenase, or cyclooxygenase pathways.

Of recent discovery is the *in vivo* non-enzymatic free-radical induced peroxidation of AA leading to the synthesis of the isoprostanes (IsoPs), so called because of their structurally isomeric relationship to the prostanoids (78). The side chains of the IsoPs are predominantly oriented *cis* in relation to the ring structure, unlike the prostanoids in which the side chains are exclusively oriented *trans*. A variety of these compounds are generated based on the site of attack of molecular oxygen on the bicycloendoperoxide PG-like intermediates (79). Isoprostanes discovered to date include F_2-isoprostanes (F_2-IsoP), D_2-IsoP, E_2-IsoP and isothromboxanes. The importance of the isoprostanes to date includes their utility (especially F_2-IsoPs) as indicators of oxidative stress *in vivo* and their exertion of biological activities during oxidative stress which may mediate the associated oxidant injury, for instance potent renal vasoconstriction (80).

Cytochrome P-450 is a multigene family of enzymes involved in the monooxygenation of lipophilic substances (20). Metabolism of AA by the cytochrome P-450/NADPH monooxygenase system occurs in 3 ways: orefin epoxidation forming the 5,6-, 8,9-, 11,12-, or 14,15-epoxyeicosatrienoic acid (EETs) isomers which can be further hydrolyzed to dihydroxyeicosatrienoic acids (diHETs), allylic oxidation giving rise to the hydroxyeicosatetraenoic acids (HETEs), and hydroxylation forming 19,20-HETE and 20-carboxyl arachidonic acid. While the EETs exhibit vasodilatory effects, 20-HETE is a potent vasoconstrictor in a number of different species (81, 82). Due to their renal distribution, the P450 metabolites of AA demonstrate regional control over renal vascular tone. These effects are thought to contribute to the progressive elevation of the systemic

blood pressure during development *in vivo* (82), and inhibition of 20-HETE production by renal and cerebral arterioles attenuates blood vessel autoregulation (81).

The lipoxygenase products with biological activity can be subdivided into 3 general categories: (i) the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4), (ii) the non-peptide containing leukotrienes (LTA_4, LTB_4), and (iii) the hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins. Three groups of enzymes are responsible for generating the lipoxygenase products based on their positional specificity of polyunsaturated fatty acid oxygenation, namely 15-, 12- and 5-lipoxygenases (LOs). In addition, the recently described epidermal-LOs define their own group, although their positional specificity of polyunsaturated fatty acid oxygenation is heterogeneous (83, 84, 85).

The 15-LO pathway generates 15-HPETE with the addition of molecular oxygen at the carbon 15 position of AA (86). Subsequently, 15-HPETE can be metabolized to a variety of compounds, including 15-HETE by a peroxidase activity inherent to the 15-LO enzyme, the lipoxins (type A or B) by the 5-LO pathway (discussed below), and others (87). One human form of the 15-LO enzyme, found both in human reticulocytes (88) and airway epithelium (89) has been reported. A biological role for 15-LO products has recently been suggested in cell-specific differentiation (90), though these observations await further confirmation. The possible regulation of atherogenesis has been implicated for both 15- and 12-LO products (discussed below).

The 12-LO pathway generates 12-HPETE with the addition of molecular oxygen at the carbon 12 position of AA. Subsequently, the peroxidase activity of 12-LO is responsible for the reduction of 12-HPETE to its hydroxy analogue, 12-HETE (91). Distinct 12-LO enzymes have been isolated from platelets and leukocytes (92, 93). Less is know about the biological actions mediated by metabolites of the 12-LO pathway than those mediated by the 15-LO products. Specific 15- and 12-LO products have been found at the site of atherosclerotic plaques, however it is not clear whether these compounds act pro- or anti-atherogenically (94, 95). In fact, it is not even apparent if these compounds bind to cell-surface receptors or only act after incorporation into cell

membranes. Although some HETE binding sites have been documented (96, 97), no HPETE/HETE-specific receptor cDNAs have been cloned to date.

The 5-LO pathway leads to the formation of the LTs, so called because of their initial discovery in leukocytes (98) and the presence of a conjugated triene in their structure. LTs are anaphylactic and inflammatory mediators and were first identified in lung perfusates upon immunological challenge as the slow reacting substances of anaphylaxis (SRS-A) (99). Subsequently, SRS-A were found to be a mixture of LTC_4, LTD_4, and LTE_4. 5-LO catalyzes the first two steps in the biosynthesis of the LTs, the incorporation of molecular oxygen into AA at carbon 5 to generate 5-HPETE and the dehydration of this intermediate to LTA_4. Other enzymes are responsible for the conversion of LTA_4 to LTB_4 or the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4). Cellular leukotriene biosynthesis requires the translocation of 5-LO from the cytosol to the nucleus (100) and the presence of an accessory protein (5-lipoxygenase activating protein (FLAP)) which is responsible for presenting AA to 5-LO (101, 102). A single human 5-LO cDNA has been cloned, from multiple sources (103, 104). LTs mediate their biological actions through cell-surface receptors belonging to the GPCR superfamily of receptors (105). Recently, high-affinity receptors have been cloned which bind LTB_4 (106) and the cysteinyl leukotrienes LTD_4 and LTC_4 (107). As well as generating the LTs, 5-LO is responsible for converting 15-LO products to the lipoxins (subclasses A and B), so called because they result from lipoxygenase interactions (108). Leukocyte activation is required for the conversion of 15-HPETE or 15-HETE to lipoxin A or lipoxin B (109, 110). These compounds exhibit immunoregulatory and smooth muscle contractile properties (111).

Recently, a group of epidermal-type lipoxygenases have been discovered (85), of which 2 human forms have been cloned (112, 113). Biological activities for this group of lipoxygenases have yet to be described.

Cyclooxygenase (COX), or prostaglandin H_2 synthase (PGHS) as it is more correctly called, is the enzyme responsible for the committed step in the conversion of AA to the bioactive prostanoids (see Figure 1.1.). In fact, there are two separate genes encoding PGHS proteins, PGHS-1 and PGHS-2. The cloning of PGHS-1 from sheep

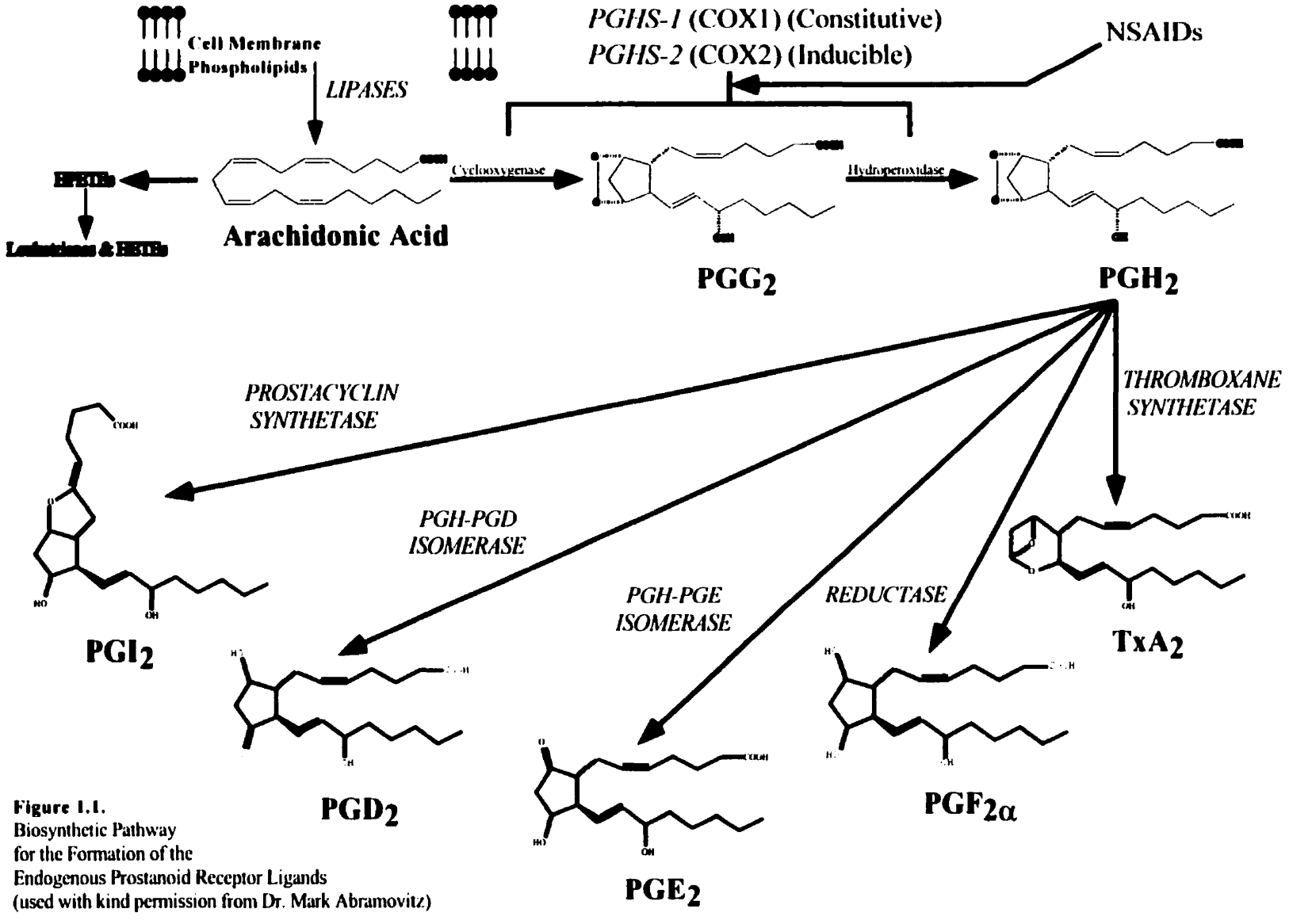

Figure 1.1.
Biosynthetic Pathway
for the Formation of the
Endogenous Prostanoid Receptor Ligands
(used with kind permission from Dr. Mark Abramovitz)

seminal vesicles was performed by three independent groups (114, 115, 116). However, knowledge of the enzyme's existence preceded this, and provided a basis for the seminal work of Vane et al (1971) which explained mechanistically the link between the activity of the non-steroidal anti-inflammatory drugs (NSAIDs) and the suppression of inflammation. Evidence for the inducible PGHS-2 became apparent with the cloning of PGHS-1. Investigators demonstrated that stimulation of both fibroblasts by mitogen (117, 118) and monocytes/macrophages by endotoxin (119) resulted in increased PGHS activity which was immunoprecipitatable with ovine PGHS antiserum. Concurrently, the glucocorticoid-mediated inhibition of prostanoid production, PGHS activity, and mitogen-inducible PGHS synthesis led to the suggestion of two "pools" of PGHS: i) a constitutive pool which appeared uninfluenced by cellular activators and steroid inhibitors, and ii) a ligand induced, steroid inhibited pool of PGHS (120). Subsequently, the isolation and sequencing of murine PGHS-2 (121, 122) led to the cloning of the human PGHS-2 cDNA (123, 124).

PGHS-1 and -2 are structurally similar but differ with respect to their localization and biology (125, 126, 127). Both enzymes are homodimeric, heme-containing, membrane-bound, glycosylated proteins with 2 catalytic sites. The human PGHS (hPGHS) isozymes share 63% identity and have roughly the same molecular weight (~71 kDa) and number of amino acids (~600), despite originating from very different genes. hPGHS-1 originates from a large, 22 kb gene with 11 exons, while the hPGHS-2 gene is a small, 8.3 kb, immediate early gene with 10 exons. The mRNA transcripts also differ in length for hPGHS-1 and -2, being 2.8 kb and 4.5 kb, respectively. As well, there exists an absence of 17 amino acids from the N-terminus and an insertion of 18 amino acids at the C-terminus of PGHS-2 in comparison to PGHS-1. The PGHS-2 isozyme has been shown to contain a number of enhancer sequences that mediate the inducibility of its expression. For instance, a cyclic AMP response element (CRE) regulates the effect of *src* on fibroblast expression (128). In addition to *src*, PGHS-2 can be induced by a number of signaling pathways, including PKA, PKC, tyrosine kinases, and LPS, as well as inhibited by corticosteroids. In contrast, very little is known regarding the regulation of PGHS-1 gene expression.

Both isozymes of PGHS catalyze the same reactions, the cyclooxygenase reaction in which AA is converted to the PGG_2 and the peroxidase reaction in which PGG_2 is reduced to PGH_2. The enzymes share similar enzyme kinetics, including cyclooxygenase turnover numbers, and K_m values for AA and O_2 (129, 130). As well, the key enzymes in catalysis are conserved and the crystal structures are essentially superimposable (126, 127). The cyclooxygenase reaction requires a hydroperoxide to initiate catalysis, which is provided by the peroxidase site of the enzyme, as follows (131). Oxidation of the heme group of PGHS by a hydroperoxide yields a peroxidase intermediate, which in turn oxidizes a neighbouring tyrosine (Y) residue. The cyclooxygenase then proceeds with the rate-limiting abstraction by this oxidized Y of hydrogen from carbon 13 of AA to yield an arachidonyl radical, followed by sequential oxygen additions at carbons 11 and 15, yielding PGG_2. Peroxidase activity generates PGH_2 with the reduction of the 15-hydroperoxide group of PGG_2 to an alcohol. Since cyclooxygenase activity of both isozymes can be inhibited by aspirin and other NSAIDS (12) leaving the peroxidase activity unaffected, the two catalytic sites for each enzyme are structurally and functionally distinct.

The classic NSAIDs non-selectively inhibit both PGHS isoforms (132), and are effective anti-inflammatory agents but have also been linked to ulcerogenesis in many NSAID users. The minimization of the inflammation resulting from PGHS-2 activity without the ulcerogenic effects that occur due to PGHS-1 inhibition is the rationale behind the development of inhibitors which are selective for PGHS-2 (133, 134, 135, 136). Two amino acid substitutions are apparent when comparing the catalytic sites and the immediately adjacent residues between the two isozymes, namely I in PGHS-1 at positions 434 and 523 for V in PGHS-2 at relative positions 420 and 509. Mutational analyses and the use of selective PGHS-2 inhibitors have shown that biochemical differences between PGHS-1 and -2 are attributed to these two substitutions (136, 137). Aspirin irreversibly acetylates the COX site of the PGHSs while other NSAIDs, such as ibuprofen or indomethacin, impart their reversible or irreversible inhibition by competing with AA for this site. Interestingly, acetylation by aspirin of PGHS-2 at S516 renders a change in enzyme activity, since subsequent oxidization of AA results in the formation of 15-HETE. However, aspirin acetylation of PGHS-1 at the analogous S, position 530,

renders the enzyme inactive (138). Another residue of interest is R120 in PGHS-1 (R106 in PGHS-2) which acts as the counterion for carboxylate-containing compounds, such as AA and the non-selective NSAIDs (139). Lastly, in terms of catalysis, PGHS activity is governed by suicide inactivation, where cyclooxygenase activity is limited not by product inhibition but by a mechanistic inactivation of the enzyme (140) which may result from an intramolecular cross-linking reaction (126).

PGHS-1 and -2 contain membrane binding domains that are thought to interdigitate with a single leaflet of the lipid bilayer (131). Both isozymes are targeted to the endoplasmic reticulum (ER) (141, 142) by an ER retention signal, which is encoded by the terminal 4 amino acids in each case (143). However, PGHS-2 is also localized to the nuclear membrane (144). Based on this differential localization, independent prostanoid biosynthetic systems have been hypothesized (132). PGHS-1, as part of the ER biosynthetic system, forms prostanoids for extracellular purposes that function through cell-surface GPCRs. PGHS-2, insofar as it is similarly localized, would support PGHS-1 in this role. However, PGHS-2 localized to the nuclear membrane would perhaps form prostanoids as part of a nuclear biosynthetic system for nuclear targets involved in cell differentiation and replication. The two PGHS isoforms have been shown to utilize different pools of AA (145) and different phospholipase systems (146). This, in addition to recent work with PGE_2 and metabolites of PGD_2 (discussed below), lends support to the notion of nuclear roles for prostanoids.

Typically, PGHS-1 can be detected in most tissues and in cultured cells it is expressed at constant levels throughout the cell cycle (132). Thus, PGHS-1 has been classically referred to as the constitutive enzyme. On the contrary, PGHS-2 is undetectable in most mammalian tissues, but its expression can be induced rapidly (2-6 h) in a variety of cells, including fibroblasts (147), endothelial cells (124), monocytes (148) and ovarian follicles (149). Regulation of PGHS-2 expression can be transcriptional (150) or post-translational (151). However, the classification of PGHS-1 and PGHS-2 as constitutive and inducible, respectively, is misleading since regulation of PGHS-1 expression [for instance, during development (152)] and constitutive expression of PGHS-2 [for instance, in brain (153)] has also been demonstrated.

The widely constitutive expression of PGHS-1 would suggest that it functions to maintain homeostasis. In the vascular system, PGHS-1 accomplishes this in platelets by providing precursors for the synthesis of thromboxane (a vasoconstrictor) and in endothelial cells by providing substrate for the synthesis of prostacyclin (a vasodilator) (154). PGHS-1 also regulates local vascular homeostasis in the compromised kidney and the stomach through compensatory vasodilation, and blockage of these systems by NSAIDs produces ischemia (155, 156). Products of this isozyme are also active in the brain, where they modulate complex integrative functions (153, 157) and in the uterus (158) where they function in early pregnancy. As mentioned previously, under some circumstances PGHS-1 is induced. An example of this occurs during the various stages of monocyte/macrophage differentiation and is driven by phorbol ester (159, 160).

In contrast, the inducible expression of PGHS-2 implies a more reactionary role, such as in inflammation. Thus, PGHS-2 upregulation occurs in response to salt deprivation (161) and salt reabsorption (162) in the kidney, and for parturition (158) in the uterus. In the brain, PGHS-2 levels are modulated by neural activity (153), by LPS to produce fever (163), and in response to inflammatory insult in the spinal cord (164). Emerging fields of study include those investigating roles for PGHS-2 in apoptosis (165), Alzheimer's disease (166) and cancer. PGHS-2 appears to be most prevalent in colorectal cancer (167), although its increased expression has also been associated with other cancers (168, 169). In a mouse model of familial adenomatous polyposis (FAP), a condition characterized by the progression of colorectal polyps to tumours, inhibition of PGHS-2 by selective inhibitors or genetic disruption markedly reduced the number of polyps present (170, 171).

Studies addressing the genetic disruption or "knockout" of PGHS-1 (PGHS-1-/-) (172) and PGHS-2 (PGHS-2-/-) (173, 174) often give markedly unexpected results, relative to what has previously been described. PGHS-1-/- mice demonstrated unresponsive platelets following AA challenge and no renal pathology, as would be expected. However, these mice had no gastric or intestinal ulcerogenesis, relative to WT mice, suggesting the presence of compensatory cytoprotective mechanisms in the PGHS-1-/- mice. Likewise, female PGHS-2-/- mice demonstrated anovulation and were infertile, as might be presumed. However, responses to acute inflammation induced by

AA or phorbol ester in PGHS-2-/- mice were unchanged from WT control mice. These results highlight the caution required when interpreting results from studies of genetic disruption.

1.3 Prostanoids

1.3.1. Chemistry, biosynthesis and metabolism

The prostanoids are derivatives of 20-carbon polyunsaturated fatty acid precursors, containing 2 aliphatic side chains and a cyclopentane ring. The primary PGs (including the related compound, prostacyclin) are individually designated A-I, based upon substitutions on the ring portion of their structure. PGA, PGB, and PGC are unsaturated ketones that arise non-enzymatically from PGE during extraction, and are not thought to occur *in vivo*. PGD and PGE are hydroxy ketones, and PGF_{α} is a diol. The subscript (α or β) is added to the F series of PGs to indicate the spatial position of the hydroxyl group on C-9 of the cyclopentane ring. PGG and PGH are cyclic endoperoxides. PGI is not a PG, but rather a PG-like prostanoid called prostacyclin, and is characterized by having a second ring structure attached to the cyclopentane ring common to the PGs. Thromboxanes are also PG-like prostanoids, but contain a six-membered oxane ring instead of the cyclopentane ring of the PGs. All prostanoids possess an α-hydroxyl at C-15 and a site of unsaturation between C-13 and C-14. As mentioned previously, AA is the predominant mammalian EFA and generates prostanoids of the 2-series. The metabolism of PGH_2 is the first committed step in the production of each of the primary bioactive prostanoids and occurs by way of individual synthesizing enzymes, or synthases (16).

There are currently three known PGD synthases (PGDSs), or enzymes that catalyze the conversion of PGH_2 to PGD_2; namely: i) glutathione (GSH)-independent PGD synthase (GSH-I-PGDS) (also called brain-type PGDS) (175), ii) GSH-dependent PGD synthase (GSH-D-PGDS) (also called spleen-type PGDS) (176), and iii) GSH S-transferase (GST) (177). In addition, serum albumin is known to catalyze this conversion (178). GSH-I-PGDS is active in the presence of various sulfhydryl compounds (for instance, β-mercaptoethanol), and does not specifically require GSH for catalysis (179). The human GSH-I-PGDS was cloned as a 27 kDa protein from brain, and sequence analysis revealed homology to the lipocalins, a superfamily of small secretory proteins

(for instance, retinol-binding protein) that all bind and transport lipophilic molecules (180). Thus, this protein is often referred to as the lipocalin type (L)-PGDS. The distribution of L-PGDS mRNA and protein has been studied (181, 182). *In situ* hybridization of rat brain revealed the mRNA to be highly expressed in the leptomeninges (the membranes enveloping the brain and spinal cord) and choroid plexus. Both mRNA and protein corresponding to L-PGDS are localized to oligodendrocytes, the glial cells responsible for axonal myelination. However, the highest L-PGDS activity is reported in the cerebrospinal fluid (CSF). Here, it is catalytically and immunologically identical to a protein that is present in large amounts whose function was previously unknown, called β-trace (183). Indeed, L-PGDS is a bifunctional protein that transports retinoids in addition to its PGD synthase activity (184). Its expression has been demonstrated in the normal human heart, as has its secretion from the stenotic sites and its accumulation in the coronary circulation of anginic patients (185). Genetic disruption of L-PGDS has implicated it in the regulation of allodynia (touch-evoked pain) mediated by PGE_2- and gamma-aminobutyric acid (GABA) A receptors (186). GSH-D-PGDS, the second type of PGDS, is similar in size (26 kDa) to L-PGDS, but in contrast has an absolute requirement of GSH for catalytic activity. This isozyme's wide distribution throughout peripheral tissues [including spleen (from where it was purified in the rat), thymus, bone marrow, digestive tract, mast cells, antigen-presenting cells] has led to the prediction that it functions in immune and allergic responses (187, 188). Since GSH-D-PGDS can also mediate GST activity, it is considered a member of the GST family of enzymes (189). Both the cloning and crystal structure determination of rat GSH-D-PGDS have recently been reported (190). This enzyme is the first vertebrate member of the sigma class of the GSTs. Other GSTs and serum albumin, independently, can also convert of PGH_2 to PGD_2. However, the major products of the metabolism of PGH_2 by GST are PGE_2 and $PGF_{2\alpha}$, rather than PGD_2 (177). The biosynthesis of PGD_2 by serum albumin in platelet-rich plasma requires no co-factors but is reportedly very low (191).

The catalysis of PGH_2 to PGE_2 occurs via PGE synthase (PGES), of which 2 human isoforms have been purified from human cerebrum (192). These enzymes demonstrated GST activity. As mentioned previously, some GSTs may also contribute to the formation of PGE_2 *in vivo*. It has been suggested from studies of rat PGES that GSH-

dependent and -independent isoforms are in existence (193). Recently, a GSH-dependent form of human PGES has been identified (194). This enzyme is membrane bound and is induced by IL-1β.

PGF synthase (PGFS) is a dual functioning enzyme that catalyzes the reduction of PGD_2 to 9α,11β-PGF_2 (a stereoisomer of $PGF_{2\alpha}$) and of PGH_2 to $PGF_{2\alpha}$ (195) at two distinct sites. It requires NADPH as a cofactor and reduces various other carbonyl compounds at the PGD_2 site (196). Two cDNA subtypes of PGFS have been cloned from bovine lung (197) and liver (198). The lung-type PGFS is also found in spleen, kidney and adrenal cortex (199). The liver-type PGFS differs by eleven nucleotides from the lung-type PGFS and has different Km values for PGD_2 (10 μM and 120μM, respectively). Sequence analysis of the liver-type PGFS suggests that it is a member of the aldoketo reductase superfamily (others include human liver aldehyde reductase, frog ρ-crystallin) (200). Recently, the amino acid sequence of liver-type PGFS has been shown to be identical to another human liver enzyme, isozyme 3 of the dihydrodiol dehydrogenase family (201).

Concurrent but independent investigations into the mechanisms of PGI_2 (202) and TXA_2 (203) synthesis led to the suggestion that the enzymes responsible for catalysis are analogous to P450 monooxygenases. Subsequent purification and cDNA cloning of the PGI_2 (204, 205) and TXA_2 (206, 207) isomerases verified these predictions. PGI synthase (PGIS) and TXA synthase (TXAS) are both membrane-bound proteins, contain the heme-thiolate catalytic site and other structural motifs that are characteristic of the P450 family, and exhibit the same spectroscopic properties. Neither synthase has greater than 16% sequence identity to any other P450 enzymes or to each other. Thus each constitutes its own subfamily, designated CYP8 and CYP5 for PGIS and TXAS, respectively. Human PGIS was cloned from aortic endothelial cells, but has a wide tissue expression including ovary, heart, skeletal muscle, lung and prostate (208). Recently, PGIS deficiency was noted in patients with severe pulmonary hypertension (209) and PGIS overexpression protected against the development of hypoxic pulmonary hypertension in a transgenic mouse model (210). TXAS is responsible for the production of TXA_2 from PGH_2 but yields 50-70% of its total products as compounds other than TXA_2 (specifically, malondialdehyde and hydroxyheptadecatrienoic acid) for unknown

reasons (211). There are actually two isoforms of the human TXAS enzyme, generated by alternative splicing of the gene (207). Human TXAS was cloned from platelets, but is also particularly abundant in peripheral blood leukocytes, spleen, lung, and liver (212). The TXAS gene is well-characterized and appears to be highly regulated (211).

Because of their biological potency, PGs must be efficiently inactivated and most tissues contain systems for their metabolic degradation. The prostanoids are quickly metabolized ($t_{1/2} < 5$ min) and the primary PGs (PGD_2, PGE_2, $PGF_{2\alpha}$) are highly extracted by the lungs after a single pass (213). The methyl end (β) side chain is usually metabolized first by conversion of the C-15 hydroxyl group to a keto moiety [by 15-hydroxy-PG dehydrogenase (15-OH-PGDH)] and enzymatic saturation of the Δ^{13} site, greatly decreasing the biological activity of the molecule (214). Other pathways of degradation include β- and ω-oxidation (which result in the shortening of the carbon chains) and spontaneous degradation reactions. There are often considerable species-specific differences observed in the metabolism of a given prostanoid.

The metabolic fate of PGD_2 is complicated compared to its synthesis and involves numerous pathways which often interconnect. One such route is its conversion to the unstable intermediate, 15-keto PGD_2, by the 15-OH-PGDH mentioned previously and subsequent metabolism by an NADPH-linked Δ^{13}-reductase to 13,14-dihydro-15-keto-PGD_2. The final products of this pathway are found in the urine as extensively β- and ω-oxidized metabolites of 13,14-dihydro-15-keto-PGD_2. However, the major products of PGD_2 metabolism in humans are PGF_2 ring derivatives (215) and predominantly include 9α,11β-PGF_2, catalyzed by PGFS as previously described. A third pathway of PGD_2 metabolism has been described which involves β-oxidation, or ω-and then β-oxidation, to produce dinor- and tetranor-PGD_1, or dicarboxylic acid and its derivatives, respectively (216). Alternatively, a fourth route of metabolism is the non-enzymatic conversion of PGD_2 to 9-deoxy-PGD_2 (PGJ_2), which is further metabolized in the presence of serum albumin to Δ^{12}-PGJ_2 and its metabolites (217, 218). The non-enzymatic conversion of PGD_2 to metabolites other than PGJ_2 has been described (219). Some of the products of PGD_2 metabolism are biologically active, as will be discussed.

The metabolism of PGE_2 is similar to PGD_2 in that 13,14-dihydro-15-keto metabolites accumulate in the blood with β- and ω-oxidation products subsequently found in the urine (220). An alternate pathway involves the reduction of the 9-keto group into F-series compounds, where the orientation (α or β) is species-specific (220). The non-enzymatic conversion of PGE_2 to dehydration products (PGA_2, PGB_2) is thought be an artifact of tissue processing, and obstructed attempts to use the unstable metabolite 13,14-dihydro-15-keto-PGE_2 as an indicator of *in vivo* PGE_2 release (221).

The metabolism of $PGF_{2\alpha}$ to the 13,14-dihydro-15-keto metabolite is followed by various rounds of β- and ω-oxidation to produce dinor and tetranor species (220). In humans, a predominant pathway of inactivation is the deoxygenation of C-15 (222). The end products excreted into the urine are tetranor compounds, the identity of which is species-specific. Another metabolic fate of $PGF_{2\alpha}$ is dehydrogenation of the 9α-hydroxyl to metabolites resembling the E-series, however humans do not use this pathway (220).

PGI_2 is quickly ($t_{1/2}$ = 2 min) and non-enzymatically converted to 6-keto-PGF_1 *in vivo* (223). Prominent pathways of 6-keto-PGF_1 metabolism include β- and ω-oxidation and 15-OH-PGDH with Δ^{13}-reductase. As well, biliary excretion metabolites are commonly observed, while dehydrogenation at C-15 occurs infrequently (223). The lung does not take up PGI_2, unlike the primary PGs. Blood vessel walls and the kidney exhibit a high capacity for PGI_2 metabolism (223).

Because of its chemically instability, TXA_2 is very quickly ($t_{1/2}$ < 1 min) hydrolyzed to TXB_2 which is more chemically stable than the parent compound but lacks biological activity. In turn, TXB_2 can be metabolized by 2 major pathways: β-oxidation or dehydrogenation at C-11, in the formation of 2,3-dinor-TXB_2 and 11-dehydro TXB_2, respectively (224). In addition, 13,14-dihydro-15-keto metabolites have been observed in the circulation (225).

1.3.2. **Release, transport, and transcellular metabolism**

Prostanoids are not stored following their synthesis but are instead released immediately (226). The specificity with which their production is controlled occurs at multiple levels in the AA cascade by numerous effectors, and depends on the species, cell type and (patho)physiological state of the system under observation. For instance, increases in intracellular cyclic AMP inhibit PG synthesis in cultured endothelial or renal tubular cells. In contrast, cyclic AMP stimulates the production of PGs in fibroblasts, neuroblastoma and glioma cells (227). Similarly, in rat kidney glomeruli angiotensin II (ATII) principally stimulates the production of PGE_2 and $PGF_{2\alpha}$, while in human glomeruli ATII activates the predominant synthesis of PGI_2. This exquisite control over prostanoid biosynthesis precludes an exhaustive list of the different regulators of prostanoid release in every different physiological setting. Rather, the different levels of control and some of the pertinent regulators at each level will be emphasized.

Regulating the liberation of AA and/or its conversion to the bioactive prostanoids can control prostanoid release. As previously mentioned, the control of the intracellular levels of AA is an important site of regulation and is a balance between PLA_2-mediated liberation and acyl CoA synthetase-mediated reacylation. $cPLA_2$ is dependent on calcium for substrate binding and fluxes in calcium are known to activate the enzyme leading to increased prostanoid production (63). This is the mechanism by which many extracellular receptor agonists [including ATII, ATP, bradykinin (BK), histamine and thrombin] are thought to stimulate prostanoid production, in blood vessels for example (228). This augmentation in activity is usually short-lived, over a period of seconds to minutes. In a similar (but calcium-independent) manner, the angiogenic protein angiogenin activates PLC to produce PGI_2 (229). Alternatively, cytokine-facilitated regulation of $cPLA_2$ activity has been observed at the translational level, such as in amnionic cells by IL-1β (230) and TNFα (231).

Theoretically, control of AA conversion to the bioactive prostanoids can occur through regulation of PGHS or prostanoid synthase activity. Of the two forms of PGHS, the constitutive type 1 and the inducible type 2, the latter is subject to much more regulation. The rapid induction of PGHS-2 by cytokines, growth factors, and other

mitogenic factors occurs over a period of hours (232). Accumulation of PGHS-2 mRNA transcripts is observed in response to tumour promoters, oncogene expression and growth factors in fibroblasts; following depolarization in neurons; in response to inflammatory cytokines in macrophages, endothelial and epithelial cells; following aggregation of IgE receptors in mast cells; and in response to hormones in mesangial cells, osteoblasts, and granulosa cells (233). Inducers in these cell types include LPS, IL-1β, EGF, PDGF, INF-γ, TNF-α, PTH, IL-3 and IL-10, to name only a few. The inducers transduce their signals via pathways (PKC, PKA, JAK-STAT, and others) which are downstream of tyrosine kinase receptors and GPCRs. The typically anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-β give mixed results, inhibiting PG production in some cells and stimulating it in others (234). Analysis of the promoter region of PGHS-2 demonstrates the presence of multiple elements (AP-1, NF-κB, and others) which regulate the nuclear expression of PGHS-2. Glucocorticoids block this induction of PGHS-2 mRNA. Post-transcriptional regulation can also occur, as observed in amnionic cells in response to TNF-α (235). For the most part, PGHS-1is not induced by these mechanisms. An increase in prostanoid synthesis through this enzyme occurs largely due to a modulation in substrate levels, such as by an increase in PLA_2 activity (228). However, induction of PGHS-1 with subsequent prostanoid (PGD_2) production has been observed by IL-3 and is enhanced with the inclusion of IL-9 or IL-10 (234). Much less is known about the regulation of the prostanoid-specific synthases.

Other factors such as mechanical stress (236), dietary influences (237), competing eicosanoid synthetic pathways (HETEs, for instance) (83) and prostanoids themselves (238) can also regulate prostanoid biosynthesis. As well, prostanoid biosyntheses can be altered under pathophysiological conditions (atherosclerosis, diabetes, and ischemia, for instance) (228).

Prostanoid catabolism regulates prostanoid activity rather than release. Both TNF-α and IL-β can promote a decrease in mRNA for 15-OH-PGDH, the enzyme responsible for generating the biologically inactive 15-keto metabolites of the prostanoids, thus prolonging the biological effects (239).

Being fatty acid derivatives, one might assume that prostanoids are highly membrane permeant, however this is not the case. Prostanoids are organic anions (pK_a = 5) that exist as charged species at physiological pH. Indeed, rabbit erythrocytes are relatively impermeable to the influx of various radiolabeled prostanoids (PGE_1, PGE_2, $PGF_{2\alpha}$, PGI_2, 6-keto-$PGF_{1\alpha}$, and TXB_2) (240). A similar impermeability to transmembranous PG transport (influx and efflux) has been observed using different types of cells, both native (Xenopus oocytes) and cultured (HeLa, canine kidney). Despite these observations, two lines of evidence are suggestive of PG transport. As discussed previously, the discovery of the two PGHS isoforms led to the suggestion of two independent prostanoid biosynthetic systems. The ER-bound PGHS-1 synthesizes prostanoids that eventually function in an autocrine or paracrine fashion through extracellular cell-surface receptors while PGHS-2, which is bound to both the ER and nuclear membranes, generates prostanoids that augment PGHS-1 activity and target the nuclear membrane (126). Extracellular activities mediated by prostanoids require their efflux across the plasma membrane. Following their extracellular activity, the prostanoids are subject to metabolism in order to abrogate any further effects. Metabolism by 15-OH-PGDH occurs in the tissues but not in the blood or plasma of multiple species including humans (213, 241). Therefore, the intracellular oxidative inactivation of prostanoids following their extracellular activation of cell-surface receptors necessitates their influx across the plasma membrane.

The transport of prostanoids by the lung is a well-characterized phenomenon, with the single-pass clearance of PGE_1, PGE_2, PGD_2, and $PGF_{2\alpha}$, but not PGI_2 following their infusion (213, 242, 243, 244, 245). The structural requirements for PG uptake in the rat lung have been elucidated: a carboxyl moiety at C-1, a hydroxyl group in the S-configuration at C-15, an oxygen group at C-11, and a site of unsaturation between C-13 and C-14 (246). In addition, compounds which inhibit the pulmonary clearance of PGs have been identified, namely indocyanine green (ICG) (247) and bromocresol green (BCG) (248). The predominant metabolites produced following pulmonary PG infusion are 15-keto PGs and 13,14-dihydro 15-keto PGs (249). The pulmonary carrier mechanism appears to facilitate entry of the bioactive PGs but not their metabolites (245, 249). Transport of PGs has also been demonstrated in other tissues such as liver (250)

and kidney (251). In hepatocytes, the uptake of PGs is followed by their metabolism by the P450 system (252). Renal uptake of PGE_2 occurs basolaterally in the proximal straight tubule, after which PGE_2 or its metabolites are secreted into the lumen (253). Not all tissues that transport prostanoids metabolize them. For instance, a carrier in the ciliary process removes PGs from the vitreous body and posterior chamber (254). The transport of $PGF_{2\alpha}$ from the cerebrospinal fluid to the venous blood (254) by a putative choroid plexus transporter is another example of this. In these tissues, carriers are thought to efflux PGs into the circulation for uptake and metabolism by other tissues (241).

Carriers with various affinities towards the prostanoids have been identified. Fatty acid binding proteins (FABPs) are a multigene family of cytoplasmic lipid binding proteins with a marked affinity towards long-chain fatty acids and their derivatives (255). Tissue-specific isoforms of these proteins exist, and the liver (L)-FABP isoform has an affinity of 40 nM for PGE_1 (256). However, L-FABP is unable to bind other prostanoids such as PGE_2 and TXB_2 (257). Therefore, although FABPs may be important in AA transport they are unable to facilitate the transport of prostanoids. Subsequently, unrelated work that recognized homology between an organic anion transporter protein (oatp) and another rat (r) protein (matrin F/G) led to the identification of the latter as the first PG transporter (PGT) (258). Modeling and hydropathy analysis of rPGT suggest that it is a member of the 12-span family of transporter proteins (241). rPGT demonstrates a substrate specificity ($PGE_1 \equiv PGE_2 \equiv PGF_{2\alpha} > TXB_2 >>$6-keto $PGF_{1\alpha} \equiv$ iloprost (PGI_2 analogue) (258)) which is remarkably similar to the rank order of rat pulmonary prostanoid clearance ($PGE_1 \equiv PGE_2 \equiv PGF_{2\alpha} > TXB_2 > PGI_2$) (213, 242, 243). The measured affinities of rPGT for the prostanoids (K_m = 50-95 nM for PGE_1, PGE_2, and $PGF_{2\alpha}$; $K_m \approx 400$ nM for TXB_2) are physiologically relevant (259), and the structural determinants needed for uptake agree with those described previously (246). In addition, rPGT has marked affinity for the isoprostanes 8-IsoP-E_2 (62 nM) and 8-IsoP-$F_{2\alpha}$ (177 nM) (260). Both a cDNA (261) and a gene (241) have been cloned which correspond to human (h)PGT. The hPGT gene is $\approx$ 100 kb and is broadly expressed as multiple transcripts whose sizes are tissue-specific, suggestive of variable regulation (perhaps by alternative splicing or tissue-specific promoters). Sequence alignments identifying the

conservation of 3 charged residues (E77, R561, K614) between rPGT, hPGT, and members of the organic anionic transporter family suggest a relative importance of these residues in structure and/or function (241). Results from the use of chemical modifiers [anionic transport inhibitors, such as Na-(2-sulfonatoethyl) methanethiosulfonate (MTSES) (241), and amino acid modifying agents, such as lysine-specific trinitrobenzene sulfonic acid (TNBS) (241)] as well as site-directed mutagenesis of the cationic residues R561 and K614 (241) are highly suggestive of PGT acting as an anionic transporter. Some of the compounds previously identified to inhibit tissue prostanoid transport (ICG, BCG) inhibit the cloned PGTs. However, the observation that indomethacin and probenicid inhibit prostanoid transport by both of the PGTs, but are without effect on tissue prostanoid transport (258, 261), suggests that other PGTs have yet to be discovered.

Transcellular metabolism, the transfer of both substrate and enzymes between cells, can occur in the biosynthesis of prostanoids. The recent work of Reddy & Herschman (145) identified distinct intracellular and transcellular pathways of PG synthesis, in co-cultivations of fibroblasts and mast cells. For the intracellular route, ligand-activated intracellular phospholipases (such as $cPLA_2$) release AA that is converted to PGH_2 by PGHS-2, following PGHS-2 gene expression. The AA is not available to PGHS-1, suggesting compartmentalization of the substrate or the enzymes. The transcellular route involves the extracellular activities of $sPLA_2$. Release of $sPLA_2$ from proximal activated mast cells allows it to release AA in distal fibroblasts which is metabolized by PGHS-1 in the transcellular production of PGE_2. The investigators were careful to show that in this transcellular system it is the $sPLA_2$ enzyme, and not the AA substrate, which is the transcellular mediator. However, elegant studies with co-cultivated alveolar epithelial cells and macrophages differentially radiolabeled with [^{14}C]AA and [^{3}H]AA, respectively, demonstrate that AA can also be the transcellular factor (262). In this system, epithelial cells and macrophages exclusively synthesize PGHS products (PGE_2 and PGI_2) and 5-LO products (LTB_4 and 5-HETE), respectively. A23187 (calcium ionophore) stimulation favoured the transcellular flow of AA, since it promoted an increase in the measurable amount of [^{3}H]-labeled prostanoids and [^{14}C]-

labeled 5-LO metabolites. As well, enzyme inhibitors (indomethacin inhibits PGHS, MK 886 inhibits 5-LO) were employed to demonstrate the transcellular shunting of AA from an inhibited oxygenation pathway to an uninhibited one. This work is preceded by the observation of transcellular prostanoid metabolism in the vascular system (263). Here, TXA_2 and PGI_2 are exclusively produced by platelets and endothelial cells, respectively. Endoperoxides generated by platelets that had been stimulated with thrombin, collagen or A23187 could restore the capacity of aspirin-treated human umbilical vein endothelial cells (HUVECs) to synthesize PGI_2. The reverse has recently been shown (264), where the restoration of TX production by aspirin-treated platelets was shown to follow the stimulation of endothelial cells by thrombin. In this case, the instability of the transcellular mediator suggested that it was PGH_2, as did its recognition by PGH_2-specific antibodies. There are additional reports of platelet-endothelial transcellular prostanoid metabolism (265, 266). The prospect of transcellular prostanoid biosynthesis is not unexpected in light of the substantial literature documenting the transcellular production of the related eicosanoids, the LTs (267, 268).

1.3.3. **Physiological and pathophysiological effects**

The bioactive prostanoids facilitate a diverse number of physiological and pathophysiological effects in various systems. These effects are sometimes subject to species specificity or can be due to receptor cross-reactivity. PGD_2 is associated with pronounced vasodilation but can also mediate the relaxation of gastrointestinal, respiratory, and reproductive smooth muscle (269, 270). In contrast, some smooth muscle contractility has been observed (i.e. bronchoconstriction) upon PGD_2 challenge (271, 272). Platelet aggregation and shape change are both potently inhibited by PGD_2 (269). In the liver parenchyma, PGD_2 stimulates glycogenolysis (272). It is the most abundant prostanoid in the brain (273) and a number of PGD_2-mediated neuromodulatory effects in the CNS have been described such as sleep induction, hormone release, inhibition of sympathetic neurotransmitter release and the regulation of pain responses (272, 274). Systemic mastocytosis is a pathology characterized by excessive proliferation of tissue mast cells and extremely high plasma and urine levels of PGD_2, and is associated with various symptoms including tachycardia and life-threatening hypotension (274). Elevated PGD_2 levels may also induce or facilitate allergic responses such as skin reactions, asthma, allergic rhinitis and anaphylaxis. Some of the PGD_2 metabolites (J-series) exhibit anti-neoplastic effects.

In contrast to other prostanoids, PGE_2 can mediate seemingly contradictory biological actions within a given tissue or cell type. As will be discussed later, this is due to the existence of multiple receptor subtypes with opposing signal transduction pathways that are often coexpressed. PGE_2 can cause contraction or relaxation of vascular and non-vascular smooth muscle (269, 275). The non-vascular smooth muscle effected by PGE_2 includes gastrointestinal, respiratory, reproductive, urinary, and that in the eye. Inhibition and enhancement of neurotransmitter release result from application of PGE_2, as does the activation of neuronal depolarization (269, 276). At low concentrations, PGE_2 can cause platelet aggregation (277). In the kidney PGE_2 decreases vasopressin-induced sodium, and both increases and decreases water reabsorption (275). PGE_2 can stimulate the secretion of aqueous humour and the reduction of intraocular pressure in the eye (269). *In vitro*, PGE_2 has been shown to stimulate bone resorption (278). The

inhibition of gastric acid secretion, the enhancement of intestinal secretion, and the inhibition of intestinal Na^+ absorption result from PGE_2 administration (279, 280). Numerous pathophysiologies are also exhibited by PGE_2 (269, 275). Hyperemia, inflammation, and edema have all been associated with PGE_2. As well, it can modulate hyperalgesia, hypoalgesia, and pyrexia. In the immune system, a number of opposing roles have been documented for PGE_2 on B cells, natural killer cells and macrophages. An increased sensitivity of lymphocytes to PGE_2 has been noted with age. Contradictory actions of PGE_2 have also been noted in cancer and bone metabolism. PGE_2 is elevated in the urine of patients with some renal disease (systemic lupus erythematosus), contributes to various aspects of asthma (upper airway irritancy, mucous secretion), and can cause migraine.

Relative to PGE_2, the biological activity mediated by $PGF_{2\alpha}$ is much more discrete. $PGF_{2\alpha}$ mediates vascular smooth muscle contraction and, more predominantly, non-vascular smooth muscle contraction (269). It causes potent bronchoconstriction (281) and contraction of male and female reproductive non-vascular smooth muscle (269, 282). The contractile effect of $PGF_{2\alpha}$ on the sphincter muscle in the eye promotes uveoscleral outflow and reduces intraocular pressure, and is well documented (269). Roles for $PGF_{2\alpha}$ are also well substantiated in the reproductive system where its release from the endometrium is dependent on oxytocin. In the absence of pregnancy $PGF_{2\alpha}$ stimulates luteolysis, and during pregnancy it augments the myometrial contractions necessary for parturition (283). The most prevalent pathophysiology associated with $PGF_{2\alpha}$ is hyperalgesia, although its role is unclear since it may also induce hypoalgesia (269).

The primary physiological role of PGI_2 is the vasodilation of arterial smooth muscle (269, 284). PGI_2 can also inhibit sympathetic neurotransmitter release in the heart (269) and can enhance non-adrenergic non-cholinergic (NANC) transmitter release from enteric neurons (285). The ability of PGI_2 to inhibit platelet aggregation and shape change is well characterized (269, 285). In addition to its vasodilatory role, PGI_2 regulates renin release, diuresis and glomerular filtration rate in the kidney (269, 284). Both gastric and intestinal secretions are enhanced by PGI_2 (269). The pathologies associated with PGI_2-mediated activity include hyperemia and hyperalgesia (269). The

elevated levels of PGI_2 observed under conditions of ischemia and renal pathology are thought to be protective.

The role best documented for TXA_2 is in the vascular system where it causes smooth muscle contraction to balance PGI_2-mediated vasodilation (269, 286). However, it also mediates gastrointestinal, respiratory and reproductive smooth muscle contraction as well as that in the eye. TXA_2 can stimulate neuronal excitation and parasympathetic neurotransmitter release (287). The potent stimulatory effects mediated by TXA_2 on platelet aggregation and shape change are well documented (269, 286). TXA_2 may also regulate gastric secretion (288). The primary pathologies associated with TXA_2 are thrombosis and occlusive vascular disease (269, 286). Its vascular effects extend to various renal pathologies where it compromises renal blood flow and glomerular filtration rate. TXA_2 may balance the immunoregulatory functions of PGE_2. There is also a clear role for TXA_2-mediated bronchoconstriction in asthma.

1.3.4. Prostanoids in gastrointestinal physiology and pathophysiology

Prostanoid-mediated activity in the gastrointestinal (GI) tract received much attention when it was suggested that PG biosynthesis was inhibited by aspirin and stimulated by cholera toxin, causing gastric mucosal damage and diarrhea, respectively (12, 289). In fact, these landmark reports were preceded by observations of the antisecretory and antiulcer actions of the E-type PGs (290, 291). It is now appreciated that the prostanoids mediate various effects in the GI tract, both physiological (i.e. secretion and motility) and pathophysiological (i.e. ulceration, colitis and neoplasia).

Numerous studies have identified PGE_2, PGI_2, $PGF_{2\alpha}$, and TXA_2 as the predominant prostanoids synthesized in the GI tract (292, 293, 294). Although the precise cells that produce the prostanoids here remain to be established, contributions are expected from both vascular (i.e. leukocyte, platelet, endothelial) and non-vascular (i.e. epithelial, sub-epithelial) components (294, 295, 296). These findings are supported by studies documenting the GI expression of both PGHS-1 and PGHS-2, under various conditions. Constitutively, PGHS-1 is present and PGHS-2 is absent throughout the GI tract, at both the RNA (297) and protein (298) levels. However, PGHS-2 is upregulated upon GI injury, such as during erosion or ulceration of the stomach (299) and inflammation of the colon (300).

GI secretions (i.e. acid, bicarbonate, mucous) play a coordinated role that balances the provision of a milieu conducive to the ingestion and absorption of nutrients with the protection of the organs responsible for performing these functions. One of the most significant observations of prostanoid-mediated activity in the GI tract is gastric cytoprotection, an enhancement of the mucosal resistance to damage (301, 302). Damage to the gastric or duodenal mucosa by chemical agents (NSAIDs, strong acid, strong alkali, ethanol, and others), restraint, and even boiling water is reduced with prostanoid pretreatment (294). PGE_2 and $PGF_{2\alpha}$ are reportedly the most abundant prostanoids synthesized by the human gastric mucosa (303, 304). Suppression of endogenous prostanoids can induce acid secretion and stable analogues of both PGE_2 and PGI_2 can reduce it (305, 306, 307). Drug combinations of NSAIDs and PGE_2 analogues (such as Arthrotec™, which contains diclofenac and misoprostol) are commercially available for

the prevention of NSAID-induced gastric ulceration (308). In addition to suppressing gastric acid, E-type PGs can also augment gastric bicarbonate (309) and mucin secretion (310) although the latter may be subject to inter-species variability. Putatively, the EP_3 receptor is responsible for the antisecretory and cytoprotective aspects of PGE_2, while EP_1 is responsible for the diarrhea (311). The E- and I-type PGs enhance mucosal blood flow (presumably through the EP_2, EP_4 and IP receptors), while NSAIDs and TXA_2 inhibit the vasodilation induced by these PGs leading to gastric erosion (312).

The effects of the prostanoids in the intestine have received less attention, and there is marked variability among different species and different anatomic locations. PGs of the E- and F-type act as intermediaries in the secretion of Cl^- ion and are themselves released in response to many stimulants, depending on the animal model (i.e. IL-1and serotonin, in chicken intestine and rat intestine, respectively) (313, 314, 315). PGD_2 can both inhibit and facilitate Cl^- ion secretion, in rat colon and guinea-pig colon, respectively (316, 317). The targets for this Cl^- ion secretion may include enteric neurons and epithelial cells (318). PGI_2 and PGE_2 can mediate cytoprotection by closing epithelial tight junctions and thus restoring intestinal barrier function, following an ischemic insult (319). PGE_2 is well established as a secretagogue of intestinal mucins, the high molecular weight glycoproteins that impart the structural and functional properties of mucous (320, 321).

Prostanoids also regulate GI motility, the mixing and propulsion of luminal contents that is essential for digestion. These effects are also subject to inter-species variability. However, it has been repeatedly observed that PGE_2 relaxes the lower esophageal sphincter and $PGF_{2\alpha}$ constricts it (296). In the small intestine and colon, PGE_2 contracts the longitudinal muscle and relaxes the circular muscle while $PGF_{2\alpha}$ contracts both muscle layers. Again, there is some anatomic variability in these generalizations (296). PGD_2 also mediates contraction in a species-specific manner (274), however in human colon and stomach it had no effect (322).

The NSAID-induced inhibition of PGHS-1 not only causes gastric ulceration, but also upregulates PGHS-2 expression. The prostanoids synthesized by PGHS-2 may be involved in the repair process of these lesions (323). Although there is no evidence for prostanoids directly stimulating gastric epithelial cell growth, they may act indirectly

through cytokines such as hepatocyte growth factor (HGF). HGF is an established growth factor for gastric epithelial cells. The accelerated restitution of these cells is facilitated by PGs but completely inhibited by anti-HGF antibodies (324). Colitis is an inflammatory condition of the colonic mucosa, and is also associated with an upregulation in PGHS-2 expression (300). Prostanoids are thought to be involved in the repair process of colitis since PGHS-2 inhibitors cause exacerbation of this disease in a rat model. In contrast to these beneficial effects, epidemiological studies suggest that prostanoids may be involved in the pathogenesis of colorectal cancer. Colorectal cancer mortality rates of persons taking NSAIDs are 40-50% lower than those not taking NSAIDs (325, 326). These findings are further supported by studies addressing the efficacy of PGHS-2 inhibitors and the effect of PGHS-2-/- in mouse models of cancer (i.e. familial adenomatous polyposis) (170, 327). However, a role for prostanoids in colorectal cancer has recently been confirmed, as described previously. A decrease in the number of preneoplastic lesions in EP_1-/- mice relative to WT has been demonstrated, using a mouse model of colon cancer (328). The same effect has been demonstrated with a novel EP_1 antagonist in two different colon cancer mouse models. Thus, EP_1 antagonists may be useful chemopreventive agents in colon carcinogenesis.

1.4. Prostanoid receptors

1.4.1.A. Classification and nomenclature

Early investigations into prostanoid-mediated biological activity suggested the existence of prostanoid-specific receptors, even in the absence of direct evidence. Thus, different prostanoids were observed to have different effects on a single cell type. Additionally, the high potency of prostanoids (as low as 10^{-11} M) could be dramatically reduced following slight chemical modifications (329), suggesting that their structural recognition is implicit in the mediation of their biological activities (269). The earliest evidence of a prostanoid receptor classification system is based on the differential effects of PGE and PGF analogues on 3 isolated tissues (guinea pig uterus, human myometrium, and rabbit jejunum) (330). Subsequent reports supported directly (331) or indirectly (332) the existence of multiple receptor types. However, it was not until the work of Kennedy et al (1982) that a working hypothesis of prostanoid receptor classification was proposed.

Using a comparison of the rank orders of agonist potency in a range of smooth muscle preparations and prior evidence in the literature, these investigators hypothesized the existence of distinct receptors for each of the bioactive prostanoids (333). Thus, a finding in two or more different systems of similar relative potencies for a series of agonists suggests that both systems contain the same type of receptor while different relative potencies suggest the presence of a mixed receptor population (334, 335). Specifically, the prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and U-46619 (a stable TXA_2 analogue) were investigated for their contractile or relaxant effects on guinea-pig ileum, guinea-pig fundus, dog fundus, chick ileum, cat trachea, dog iris, cat iris, rat aorta, guinea-pig lung and dog saphenous vein. The former 5 tissue preparations defined a PGE_2-sensitive group that exhibited very similar rank orders of agonist potency ($PGE_2 > PGI_2 \equiv PGF_{2\alpha} > PGD_2 \equiv TXA_2$). In contrast, a very different rank order of potency defined the next 2 tissue preparations (dog and cat iris) which exhibited $PGF_{2\alpha}$-sensitivity ($PGF_{2\alpha} > PGD_2 > U46619 \geq PGE_2 > PGI_2$). U-46619/$TXA_2$ demonstrated marked potencies for the latter 3 tissue preparations (at least 140 times the other prostanoids), however the variation of the relative potencies of PGD_2, PGE2, $PGF_{2\alpha}$, and PGI_2

precluded a defining rank order of potency for these compounds. In the same study, these observations were extended by comparing the relative potencies in the presence of two putative prostanoid competitive antagonists that had previously been identified, SC-19220 and AH 19437. Thus, SC-19220 was without effect in the preparations defined as $PGF_{2\alpha}$- or U46619/TXA_2-sensitive. Additionally, it antagonized the PGE-sensitive receptors present in some preparations (guinea-pig ileum, guinea-pig fundus, and dog fundus) but not others (cat trachea, chick ileum), suggesting a subdivision of these receptors. In contrast, AH 19437 was without effect in the preparations defined as PGE_2- or $PGF_{2\alpha}$-sensitive but antagonized U46619/TXA_2-sensitive preparations. In addition to the results presented in this study, the authors cited evidence in the literature demonstrating that PGD_2 and PGI_2 both potently inhibit platelet aggregation (unlike other prostanoids) and do so at their own individual receptors (336, 337). The antagonist N-0164 could block the inhibition of platelet aggregation mediated by PGD_2, but not PGI_2. In light of this and other (338) evidence, the authors proposed a nomenclature for the prostanoid (P) receptors whereby the natural prostanoid to which each is most sensitive is indicated by a capital letter preceding the letter "P". Under this classification, the receptors for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are denoted DP, EP, FP, IP and TP, respectively. The identification of two groups of PGE-sensitive tissues differing in their susceptibility to antagonism by SC-19220 prompted a further division of the EP receptors into EP_1 (SC-19220 sensitive) and EP_2 (SC-19220 insensitive). The development of two other selective agonists (339), sulprostone and AY 23626, confirmed and extended the subdivision of the EP receptors by discriminating a third class denoted EP_3 (340). PGE receptors sensitive to AY 23626 but not to sulprostone are designated EP_2 receptors while those sensitive to both agonists are termed EP_3 receptors. More recently, an EP receptor was identified in piglet saphenous vein at which the available selective agonists for EP_1, EP_2, and EP_3 were weak or inactive (341). The selective antagonism of this receptor by AH23848B relative to other EP receptors suggested the existence of a fourth EP receptor subclass denoted EP_4.

The cloning of the prostanoid receptors (to be discussed) identified further heterogeneity, which arises as a result of alternative mRNA splicing. The molecular mechanism of alternative mRNA splicing involves the formation of multiple mRNA and

protein products from a single gene. Specifically, splice variants have been identified for the EP_1, EP_3, FP, and TP receptor homologues of various species. There are currently 9 known subtypes of the human EP_3 receptor: $EP_{3\text{-}1a}$, $EP_{3\text{-}1b}$, $EP_{3\text{-}II}$, $EP_{3\text{-}III}$, $EP_{3\text{-}IV}$, $EP_{3\text{-}V}$, $EP_{3\text{-}VI}$, $EP_{3\text{-}e}$, and $EP_{3\text{-}f}$ (342, 343, 344). Mouse (345, 346, 347), rat (348), bovine (349), and rabbit (350) counterparts have also been identified for some of these EP_3 subtypes. Human subtypes also exist for the TP receptor (351), specifically TP_α and TP_β. Two splice variants have been identified for each of the rat EP_1 (352) and ovine FP (353) receptors. These have been designated EP_1, $EP_{1\text{-variant}}$ and FP_A, FP_B, respectively. Human homologues to these subtypes have not been identified.

1.4.1.B. A subfamily of the rhodopsin-receptor family

Bacteriorhodopsin is a light-driven proton pump, not a cell-surface receptor. However, its functional relatedness to the visual protein rhodopsin (both proteins respond to light by way of a bound retinoid chromophore) suggested that it may also be related structurally (354). The cloning of the β_2-AR from hamster lung led to the recognition of its strong similarity to rhodopsin with regards to amino acid sequence, hydrophobicity, signal transduction mechanisms and post-translational modifications (355). This led to the notion of rhodopsin as the prototypical member of what is now a large family of related receptors, since it was the first to be cloned (356). This also justified the use of the high-resolution crystal structure available for bacteriorhodopsin (357) as a paradigm around which to model the related receptors (for which crystal structures are still lacking) (358, 359). One hallmark of these proteins is that reversible binding of their agonists leads to the activation of a restricted subset of heterotrimeric guanine nucleotide-binding (G) proteins, which transduce the signal to downstream effectors (such as enzymes and ion channels). The rhodopsin-type GPCRs are now considered the single largest family of cell surface receptors involved in signal transduction. Several hundred distinct members of this family of proteins are known to act as receptors for a variety of chemical transmitters (for instance, biogenic amines, amino acids, peptides, lipids, nucleosides, large polypeptides, light and odorants) in the regulation of a diverse array of physiological processes (such as neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, and inflammatory and immune responses) (for review (360, 361, 362)). All members of this receptor family putatively exhibit a 7-transmembrane spanning topology, with both extracellular domains (amino terminus and 3 loops) and intracellular domains (carboxyl terminus and 3 loops). The proper functioning of these cell-surface receptors requires several structural determinants that facilitate receptor folding, trafficking and transmembrane signaling. These will be discussed with respect to the prostanoid family of GPCRs.

Evidence suggested that the prostanoid receptors were rhodopsin-type receptors prior to their molecular cloning, such as the coupling of the TP receptor to G proteins (363) and its sensitivity to agonist-induced desensitization (364). The purification of the

TP receptor from human platelets (365) allowed its partial protein sequence to be identified, leading to the isolation of a cDNA for TP (366). Homology screening based on this sequence has established recombinant clones from various species for the eight individual prostanoid receptors previously defined pharmacologically. Thus, cloned receptors have now been reported for mouse and human DP (367, 368); mouse, rat and human EP_1 (369, 370, 371); mouse and human EP_2 (372, 373); mouse, rat, rabbit, bovine and human EP_3 (342, 343, 344, 345, 346, 347, 348, 349, 350); mouse, rat and human EP_4 (374, 375, 376, 377); mouse, rat, sheep, bovine and human FP (378, 379, 380, 381, 382); mouse, rat and human IP (383, 384, 385, 386, 387); and mouse, rat and bovine TP (388, 389, 390).

There are 28 amino acid residues conserved within all prostanoid receptor sequences, and 8 of these are shared with other GPCRs. These residues are believed to be particularly important in receptor structure and/or function. For instance, a D residue in the second transmembrane domain of various GPCRs is involved in ligand binding and signal transduction (391). The role of this residue has not been studied directly in any prostanoid receptors but indirect observations suggest that it may be required for DP receptor functional expression, as will be discussed later. Two conserved C residues (one in each of the first and second extracellular loops) are suggested to form a disulfide bond and contribute to the stabilization of GPCRs in the membrane (392). In the rabbit EP_3 receptor, an A residue substituted for the C residue in the second extracellular loop had no effect on binding (393). This is in contrast to studies of the human TP receptor, where substitution of S for the analogous C completely abolished ligand binding (394, 395). These studies also showed a similar effect by mutating the C of the first extracellular loop, which corroborates evidence demonstrating a loss of agonist binding to TP following chemical perturbation of these C residues (with dithiothrietol or sulfhydryl alkylation) (396).

As well as containing conserved residues, prostanoid receptors share several other characteristics with other members of the GPCR family. They contain consensus sites for N-glycosylation of N residues (N-X-S/T) in their extracellular domains. These sites are requisite for ligand binding at the human TP receptor (397) and mutation of the N residues or deletion of their carbohydrate moieties abolishes binding. The amount of

glycosylation can be significant as demonstrated when purified TP receptors of molecular mass ~57 kDa were shifted to their predicted molecular mass (based on primary structure) of ~37 kDa upon treatment with N-glycanase (398). GPCRs are often post-translationally modified at their cytoplasmic domains. Phosphorylation of S and T residues occurs as part of the process of receptor desensitization, which is the waning of receptor response following persistent stimulation (399, 400, 401). Heterologous desensitization involves feedback inhibition by the second messenger kinases that these receptors activate (i.e. PKA and PKC). Stimulation of these pathways can cause phosphorylation and subsequent desensitization of any GPCR containing the appropriate PKA or PKC consensus site(s) (S/T-X-K/R and R-X-X-S, respectively). Homologous desensitization is an alternate pathway that involves receptor phosphorylation by a specific GPCR kinase (GRK) and its subsequent binding by arrestin, a protein that sterically restricts signaling to the G protein. Desensitization of all prostanoid receptor types except DP has been demonstrated. Many reports identify the importance of heterologous desensitization in the regulation of prostanoid receptor activity, and a role for homologous desensitization has only recently been suggested.

The prostanoid receptors define their own subfamily within the GPCR family of receptors (402, 403). Prostanoid receptor-specific motifs include sequences in the second extracellular loop (G-R-Y-X-X-Q-X-P-G-T/S-W-C-F), in addition to the third and seventh transmembrane domain (M-X-F-F-G-L-X-X-L-L-X-X-X-A-M-A-X-E-R and L-X-A-X-R-X-A-S/T-X-N-Q-I-L-D-P-W-V-Y-I-L, respectively). These regions are conserved between receptors of different species and are thought to play fundamental roles in the structure of the prostanoid binding domains. A R residue in the seventh transmembrane domain conserved between all prostanoid receptors was proposed to be the binding site for the carboxyl moiety of the prostanoids (404, 405), although the conserved motif in the second extracellular loop may also function in this regard (393). Residues are also conserved among prostanoid receptors for signal transduction. For instance, R in the first intracellular loop is conserved between all prostanoid receptors. A mutation of this residue to L in the TP receptor was found to be associated with a hereditary bleeding disorder (406). The mutant TP receptors demonstrated unperturbed ligand binding but compromised signal transduction.

1.4.1.C. Phylogenetic and genomic organization

The eight known types of prostanoid receptors are each encoded by an individual gene. Phylogenetic analyses indicate that receptors sharing a common signaling pathway have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand (368, 373, 407). The effects of prostanoid receptors reflect this relationship. For instance, DP, IP, EP_2, and EP_4 induce smooth muscle relaxation and are more closely related to each other than to the other prostanoid receptors. Similarly, EP_1, FP, and TP receptors cause smooth muscle contraction and form another group based on sequence homology. The EP_3 receptors also stimulate smooth muscle contraction and define a third group. The signal transduction pathways underlying these mechanisms of prostanoid action are also shared within these groups and will be explained in a subsequent section. Based on these phylogenetic analyses, it has been suggested that the PGHS pathway may have evolved from PGE_2 and an ancestral EP receptor (402). The evolution of the different EP receptor types from this ancestral prostanoid receptor would have linked PGE_2 to different signal transduction pathways. The receptors for the other prostanoids would have then evolved by gene duplication of these different EP receptor subtypes.

Chromosomal mapping has been used to determine the localization of many of the genes encoding the mouse (408, 409) and human (410, 411) prostanoid receptors. Of the 8 genes encoding the prostanoid receptor types, only that encoding the human DP receptor has yet to be mapped. The genes for the EP_1, EP_4, IP, and TP receptors are localized to chromosomal segments previously identified to be homologous between the mouse and the human. The structure for the human TP receptor gene was the first prostanoid receptor gene to be determined (411). It contains three exons separated by two introns, one in the 5′-noncoding region and the second at the end of the sixth transmembrane domain. This exon-intron relationship is conserved in other prostanoid receptors across various species such as mouse and human DP (367, 368), mouse EP_1 (412), human EP_3 (344), human EP_4 (413), mouse FP (414), and human IP (415). An interesting exception to this occurs in the mouse EP_4 receptor gene, where the first intron occurs 16 bp downstream of the site for translation initiation rather than in the 5′-

noncoding region (416). The position of the second intron is unchanged. Further exception is taken with the mouse (417) and human (418) homologues of the EP_2 receptor. These receptor genes consist of two exons separated by a single intron at the end of the sixth transmembrane domain.

Alternative splicing of the exon encoding the seventh transmembrane domain occurs at a position approximately 9-12 amino acids into the carboxy terminus of the EP_3, FP, and TP receptors of various species. The rat EP_1 receptor is also subject to alternative splicing, but instead diverges midway into the sixth transmembrane domain. The variant form ($rEP_{1\text{-variant}}$) contains none of the amino acids that are highly conserved within the seventh transmembrane domain of the other prostanoid receptors. Generally, prostanoid receptor isoforms exhibit similar ligand binding but differ in their signaling pathways, their sensitivity to agonist-induced desensitization, and their tendency towards constitutive activity, as will be discussed. Whereas there is homology between the EP_3 receptor isoforms of different species, the human and mouse TP receptor isoforms demonstrate no homology. This may be indicative of other TP isoforms (402). The receptors that are subject to alternative splicing (EP_1, EP_3, FP, and TP) are phylogenetically related, perhaps suggesting the evolutionary conservation of the sequence(s) involved in this process. The rEP_1, $EP_{3\text{-II}}$, FP_A and TP_α splice variants are all generated by the failure to utilize a potential splice site (419). The splicing out of various introns and the use of downstream exons generates the other alternatively spliced forms of EP_3. The regulation of the process of alternative splicing with respect to the prostanoid receptors has yet to be studied.

Prostanoid receptor expression is regulated by numerous factors through action on *cis*-acting regulatory elements on their respective genes. The regulation of the EP_2 (417), EP_3 (343), EP_4 (412, 413), TP (411), and IP (415) receptor expression in various species has been studied. The 5′-flanking region and first intron of many of these receptor genes have basal promoter motifs (such as a TATA box) as well as several responsive motifs, including those for proinflammatory agents (such as NF-κB). Although many different motifs have been identified, less information is available regarding the actual regulation of receptor gene expression. The studies to date suggest that species or cell-type differences may play a role. For instance, the TP receptor gene contains a phorbol ester

response element and TP receptor expression can be stimulated in human erythroleukemia (HEL) cells (420). However, despite the presence of response elements for glucocorticoids and IL-6 in the TP gene these factors are insufficient to induce TP expression in HEL cells (421). However, they can induce TP expression in rat cultured vascular smooth muscle cells (422). *In vivo* regulation of prostanoid receptor expression has not yet been studied directly.

Despite the high homology between the prostanoid receptors of various species, there are differences in the translation initiation sites of some receptor types which effect the amino-terminal extracellular domain of the receptor (402). Thus, the human, bovine and rabbit EP_3 receptor is 20 residues longer than the rat and mouse homologues. The human IP receptor is 30 amino acids shorter than the mouse and rat homologues. In contrast, the human DP receptor is only 1 residue longer than its mouse and rat counterparts.

1.4.2.A. Ligand binding selectivity

Each of the eight prostanoid receptor types exhibits a distinct profile of selectivity in its ability to specifically bind ligand. Indeed, it is the differential selectivity for the naturally occurring prostanoids and some of their early synthetic analogues that facilitated the original classification of the prostanoid receptors (333), as previously discussed. However, the interpretation of the results from many of the early studies are fraught with complexities since: (i) many of the compounds used display receptor cross-reactivity, (ii) many of the tissues used contain heterogeneous populations of receptors, and (iii) the tissues used to define a given receptor sensitivity often have different degrees of responsiveness. For these reasons, efforts have been made to simplify the systematic analysis of prostanoid ligand-receptor interactions by: (i) synthesizing ligands of increased selectivity, and (ii) cloning the cDNAs for the prostanoid receptors in order to express them in unambiguous environments. Two recent reports describe the analysis of ligand selectivity by competition binding in recombinant systems of the 8 types of prostanoid receptors from human (423) and mouse (424).

The rank order of affinity of ligands reported from competition binding assays for the cloned human DP receptor is BW245C = PGD_2 = BW A868C >> PGE_2 > $PGF_{2\alpha}$ = iloprost > U46619, where the DP-specific ligands (PGD_2, BW245C, and BW A868C) demonstrate inhibitor constant (K_i) values of approximately 1 nM. In contrast, mouse DP has a 10-fold higher affinity for PGD_2 (K_i = 21 nM) than for both the antagonist BW A868C and the agonist BW245C (424). PGD_2 demonstrates high cross-reactivity with the FP receptor in both the human (K_i = 6.7 nM) and mouse (K_i = 47 nM) recombinant systems. It has been suggested that PGD_2-induced bronchoconstriction is mediated by the FP receptor, as observed in the anesthetized dog (269). The current work addresses the selectivity of the DP receptor for prostanoids and synthetic prostanoid analogues in further detail.

The rank order of affinity of PGE_2 for the cloned human EP receptors is: $EP_{3\text{-III}}$ (the only EP_3 subtype used in the study) > EP_4 >> EP_2 > EP_1 (K_i values of 0.33, 0.79, 4.9, 9.1 nM, respectively) (423). PGE_2 is actually quite selective for the EP receptors, and demonstrates 10-fold lower affinity for the human FP receptor. No highly selective

agonists have been reported for the EP_1 receptor. All the agonists with high affinity for human EP_1 (carbacyclin, sulprostone, and enprostil with K_i values of 23, 107 and 82 nM, respectively) have higher affinity for human $EP_{3\text{-III}}$ (423). The three antagonists SC-51322, SC-51089 and AH6809 display varying affinities for human EP_1 (K_i = 14, 1332, 1217 nM). However, SC-51322 has 50-fold less affinity and SC-51089 has 10-fold less affinity for $EP_{3\text{-III}}$ and TP. AH6809 has similar affinity for EP_2, EP_3 and DP. In contrast, the mouse EP_1 receptor has similar affinity for both PGE_2 and sulprostone (K_i = 20 nM), but no affinity for AH6809.

Butaprost is known to be selective for the EP_2 receptor and displays a 3-fold higher affinity for the human EP_2 receptor (K_i = 3513 nM) relative to the human $EP_{3\text{-III}}$ receptor (423). Butaprost is normally used as a methyl ester but was also studied in its free acid derivative since this is thought to be the active form *in vivo*. The free acid form of butaprost has 40-fold higher affinity and improved selectivity for EP_2 relative to its methyl ester derivative. The mouse EP_2 receptor has only a 10-fold higher affinity for PGE_2 over butaprost (12 and 110 nM, respectively) (424). Butaprost did not show affinity for any of the other mouse prostanoid receptors. Compounds such as PGE_1, 16,16-dimethyl PGE_2 and 11-deoxy-PGE_1 bind to EP_2 but demonstrate various profiles of cross-reactivity at the other EP receptors. There are no selective antagonists for EP_2, only the non-selective activity of AH6809 mentioned earlier.

Both the free acid and methyl ester derivatives of misoprostol were studied against the cloned human $EP_{3\text{-III}}$ receptor, for the reason described above (423). The rank order of affinity of ligands at human $EP_{3\text{-III}}$ is M&B 28767 = PGE_2 = sulprostone > GR63799X = misoprostol free acid = enprostil = carbacyclin > misoprostol methyl ester. Sulprostone is the most selective agonist reported at the human $EP_{3\text{-III}}$ receptor, where its affinity (K_i = 0.35 nM) is 300-fold higher than that for EP_1 (423). A similar rank order of affinity of sulprostone = M&B 28767 = PGE_2 = GR63799X > carbacyclin > misoprostol (methyl ester) was observed at the mouse EP_3 receptor (424). At mouse recombinant receptors, sulprostone displays 30-fold higher affinity for EP_3 (K_i = 0.6 nM) relative to EP_1 (K_i = 21 nM). Again, there are no selective antagonists of EP_3, only the non-selective action of AH6809 described earlier.

Generally compounds that show affinity for EP_4 have little selectivity. Of the compounds used in both studies, the rank order of affinities for ligands at the cloned human and mouse EP_4 receptors are comparable (PGE_2 > M&B 28767 = misoprostol and PGE_2 > misoprostol > M&B 28767, respectively) (423, 424). However all these compounds show equal or higher affinity for the EP_3 receptor in the respective systems. The putative EP_4 antagonist AH23848B has higher affinity for TP (25-fold), $EP_{3\text{-III}}$ (3-fold) and DP (10-fold) in the human system of recombinant prostanoid receptors (423). This compound was not studied in the system of cloned mouse prostanoid receptors.

The human FP receptor exhibits a rank order of affinity of cloprostenol ≥ $PGF_{2\alpha}$ = fluprostenol > PGD_2 which agrees well with the rank order observed at the mouse FP receptor of $PGF_{2\alpha}$ = fluprostenol > PGD_2 of the similar compounds tested (cloprostenol was not investigated at mouse FP) (423, 424). In addition, fluoprostenol demonstrated marked selectivity for the FP receptor in both the human (300-fold over $EP_{3\text{-III}}$) and mouse (no detectable cross-reactivity) systems. Latanoprost is a highly selective FP agonist used for the treatment of glaucoma and demonstrated a selectivity of 600-fold for human FP over EP_1 (423). $PGF_{2\alpha}$ has a 10-fold lower affinity for the human $EP_{3\text{-III}}$ receptor relative to the FP receptor. It was not tested in the mouse recombinant system of prostanoid receptors. There have been no reports of a FP selective antagonist.

The use of prostacyclin is inhibited by its chemical instability. The human IP receptor exhibited a rank order of affinity of iloprost = cicaprost > carbacyclin which paralleled the rank order observed at the mouse IP receptor (423, 424). Iloprost and cicaprost had affinities of approximately 10 nM in both cases. Both receptor systems demonstrated the cross-reactivity inherent to iloprost, where it shows similar affinity for the EP_1 and EP_3 receptors. Cicaprost showed 2-fold less affinity toward human EP_4 relative to IP, but was not tested against the mouse EP_4 receptor. Carbacyclin demonstrated cross-reactivity with mouse EP_3 where it has 3-fold higher affinity. In the human recombinant system of prostanoid receptors, carbacyclin has a higher affinity for $EP_{3\text{-III}}$ (20-fold), EP_1 (10-fold), and DP (2-fold) relative to its affinity for IP. In fact, carbacyclin is highly cross-reactive and has affinities of less than 500 nM for all prostanoid receptors except EP_2 (K_i = 1 μM) and TP (K_i = 20 μM). There are no known antagonists for the IP receptor.

In contrast to the other prostanoid receptors, there are many selective antagonists for the TP receptor and fewer selective agonists. The instability of TXA_2 is well documented and U46619 is often used as a substitute agonist. The TP receptor has an affinity (K_i = 67 nM) 10-15 fold higher than its affinity for the FP receptor, depending on the system (423, 424). The agonist STA_2 was not tested at the human recombinant prostanoid receptors but had affinity for the mouse TP receptor (K_i = 14 nM) equal to its affinity for EP_3. I-BOP is a high affinity agonist (K_i = 0.6 nM) of the TP receptor with marked selectivity (> 150-fold) over FP, EP_2 and EP_3. The most common TP antagonist is SQ 29548, which has high affinity for both the human (K_i = 4 nM) and mouse (K_i = 13 nM) receptors and displays no cross-reactivity at the other prostanoid receptors.

1.4.2.B. Signal transduction and desensitization

Early studies of the second messengers downstream of the prostanoids focused on cyclic nucleotides (425). For instance, PGE_2 and $PGF_{2\alpha}$ were reported to stimulate cyclic AMP (426) and cyclic GMP (427), respectively. Since then, other signal transduction pathways have been suggested by the observation of prostanoid-mediated activation of second messengers such as free Ca^{2+} and inositol phosphate (402). The molecular cloning of the prostanoid receptors, their confirmation as GPCRs, and the study of the heterotrimeric G proteins which carry their signals have facilitated this. These heterotrimeric G proteins are composed of three structural subunits designated α, β, and γ, of which numerous subtypes exist for each (428). Functionally, G proteins are comprised of two subunits, since receptor activation provokes the dissociation of the $G\alpha$ subunit from a complex of the $G\beta\gamma$ subunits. Both the $G\alpha$ and the $G\beta\gamma$ subunits can act as effectors in signal transduction.

As indicated previously, prostanoid receptors sharing a common signaling pathway have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand (368, 373, 407). Thus, three clusters of related receptors were defined: (i) DP, IP, EP_2, and EP_4; (ii) EP_1, FP, and TP; and (iii) EP_3. Prostanoid receptors in group (i) are linked to heterotrimeric G proteins that are comprised of a $G\alpha$ subunit that stimulates adenylate cyclase (designated $G\alpha_s$) to produce cyclic AMP. Thus, an increase in intracellular cyclic AMP concentration is observed following stimulation of the recombinant human DP (368), IP (383, 384, 385), EP_2 (373), and EP_4 (374, 375) receptors in addition to their species homologues. The results obtained with recombinant receptors corroborated those obtained previously in isolated tissues. For instance, DP stimulates cyclic AMP formation in bovine embryonic trachea cells (429), EP_2 activates cyclic AMP production in rat cortical collecting tubule cells (430), and IP receptors on mouse mastocytoma cells induce cyclic AMP synthesis (431). PGD-, PGE-, PGI-responsive receptors cause the stimulation of cyclic AMP production in platelets (337) and in human colonic mucosa (432). However, the recombinant human IP receptor can also mediate inositol phosphate production and increases in free Ca^{2+} levels (386). Receptor activation was shown to be insensitive to toxins that inactivate $G\alpha_s$ or another

family of Gα subunits, called $G\alpha_i$. G proteins composed of $G\alpha_i$ subunits couple to the inhibition of adenylate cyclase and to the activation of ion channel conductance, which may be dependent or independent of the stimulation of phospholipase C in the production of inositol phosphates. These results suggested the participation of members of a third family of Gα subunits that also activate inositol phosphate production through the stimulation of phospholipase C, designated $G\alpha_q$.

Prostanoid receptors in group (ii) couple to increases in intracellular free Ca^{2+} through the activation by $G\alpha_q$ of phospholipase C, with subsequent inositol phosphate liberation. This pathway has been demonstrated for FP using anti-$G\alpha_q$ antibodies (433) which corroborates earlier results demonstrating inositol phosphate turnover in isolated luteal cells upon $PGF_{2\alpha}$ administration (434). In the case of TP, $G\alpha_q$ activation is the primary effector pathway (435) as shown during stimulation of native TP receptors in platelets (436). However, the previously described TP receptor splice variants TPα and TPβ also signal through $G\alpha_i$ and $G\alpha_s$ to inhibit and stimulate adenylate cyclase, respectively (437). Contraction of guinea pig trachea occurs following activation of native EP_1 receptors and is absolutely dependent on extracellular Ca^{2+} (438). Downstream of the recombinant mouse EP_1 receptor a similar extracellular Ca^{2+}-dependent response is observed which is accompanied by an almost undetectable inositol phosphate response (369), suggesting the modulation of a Ca^{2+} channel putatively by $G\alpha_q$ (275). There was no change in cyclic AMP in these cells. The splice variant of the rat EP_1 receptor described earlier ($rEP_{1\text{-variant}}$) binds ligand but does not mediate a change in free Ca^{2+} levels (352). In this way it is thought to interfere with the potency of any co-expressed EP receptors by lowering the effective concentration of PGE_2 (419).

The EP_3 subtypes constitute group (iii) of the prostanoid receptor family and employ as their primary effector pathway the inhibition of adenylate cyclase through the $G\alpha_i$ family of G proteins, as demonstrated in bovine medulla (439). However, the molecular cloning of the bovine EP_3 receptor splice variants demonstrates the array of second messengers to which these receptors are coupled. Four subtypes of bovine EP_3 have been cloned (designated A, B, C, and D) and all show identical agonist binding properties (349). However, EP_{3A} acts through $G\alpha_i$ to inhibit adenylate cyclase, EP_{3B} and

EP_{3C} signal through $G\alpha_s$ to activate adenylate cyclase, and EP_{3D} is coupled to $G\alpha_i$, $G\alpha_s$, and $G\alpha_q$, resulting in the inhibition and activation of adenylate cyclase as well as the activation of phospholipase C. A novel type of G protein regulation has also been reported for the EP_{3B} and EP_{3C} receptors. In addition to their stimulatory effects on $G\alpha_s$, they are thought to negatively regulate G protein activity by specifically inhibiting the GTPase activity of $G\alpha_o$, a member of the $G\alpha_i$ family (440). The EP_3 receptor subtypes may also differ in their levels of constitutive activity, the agonist-independent activity of the receptor. This evidence comes from studies of the mouse isoforms of the EP_3 receptor (designated α, β, and γ). The $EP_{3\beta}$ demonstrates no constitutive activity while $EP_{3\gamma}$ is almost fully constitutively active, with respect to the $G\alpha_i$-mediated inhibition of adenylate cyclase (441). The demonstration of levels of activity similar to $EP_{3\beta}$ upon treatment of $EP_{3\gamma}$-transfected cells with pertussis toxin (which inactivates $G\alpha_i$) confirms that the $EP_{3\gamma}$-$G\alpha_i$ is constitutively active.

Information on the desensitization of the prostanoid receptors is not as complete as other aspects of their biology. Many of the receptors are known to be susceptible to the second messenger kinases that were discussed earlier. However, much less is known about their sensitivity to the previously defined GRKs. PKC regulates the short-term (5 min) and long-term (24 h) desensitization of the mouse EP_1 receptor, observed as a suppression of the agonist-mediated dose-response and a reduction in EP_1 mRNA levels, respectively (442). The mouse EP_2 receptor undergoes long-term agonist-induced desensitization in the form of receptor downregulation following a 12 h exposure to PGE_2 but is insensitive to short-term desensitization (30 min) (443). Short-term (1 h) desensitization of human EP_2 was observed upon PKC activation (444). Splice variants of a given species homologue of EP_3 differ in their sensitivity to desensitization. The mouse $EP_{3\alpha}$ exhibits sequestration following short-term (30 min) PGE_2 exposure and downregulation upon long-term (24 h) PGE_2 exposure, while mouse $EP_{3\beta}$ does not undergo agonist-induced desensitization (346, 445). Similarly, the human $EP_{3\text{-}II}$ demonstrates slow persistent desensitization in contrast to the rapid transient desensitization exhibited by the $EP_{3\text{-}III}$ and $EP_{3\text{-}IV}$ receptors (446). Desensitization of EP_4

is perhaps the most intensely studied to date of the EP receptors. The mouse EP_4 receptor is sensitive to both short-term (30 min) and long-term (12 h) desensitization (443). The rapid agonist-induced desensitization of human EP_4 is independent of second-messenger kinases, and is instead regulated by GRKs (445). This was the first demonstration of prostanoid receptor desensitization facilitated by GRKs. Another recent study has mapped the S sites in the carboxyl-terminus responsible for desensitization of the human EP_4 receptor (447). Even though the desensitization of the IP receptor has been studied for the native receptor in a single cell type (neuroblastoma-glioma cell hybrid, NG108-15), the results are contradictory. Long-term (17 h) desensitization has been observed as receptor sequestration and downregulation with no change in agonist affinity (448), where the kinases involved appear to depend on the agonist used (449). In contrast, another group has identified that long-term desensitization is specifically accompanied by a concurrent downregulation of the $G\alpha_s$ subunit (450). PKC is observed to mediate short-term (45 min) desensitization of the native FP receptor in bovine iris sphincter (451). Short-term desensitization *in vivo* of the FP receptor also occurs in the ovine corpus luteum, where its temporal nature has been suggested to influence the oxytocin-mediated pulsatile release of $PGF_{2\alpha}$ (452). The TP receptor has been shown to undergo desensitization, specifically as short-term (10 min) agonist-dependent phosphorylation which could be blocked by antagonist (453). The *in vivo* desensitization of the TP receptor has been demonstrated in the pulmonary vascular system of late gestation rabbits as a decrease in receptor number and affinity (454). The desensitization of the phospholipase C effector pathway downstream of TP has also been shown in human platelets (455). A recent report demonstrates that the TP splice variants exhibit different sensitivities to desensitization. $TP\beta$ is susceptible to agonist-induced desensitization in a GRK-dependent manner, while $TP\alpha$ does not desensitize (456). In contrast to the other prostanoid receptors, there are no direct reports of desensitization of the DP receptor.

1.4.2.C. **Gene disruption**

Targeted gene ablation or disruption (also called gene knockout) involves the development of a targeting vector and its subsequent introduction into pluripotent embryonic stem cells (457, 458). This technique may be especially useful for the prostanoid receptors because of the lack of suitable antagonists for this receptor family. The disruption of all of the prostanoid receptor genes has now been reported, with the exception of DP.

There are three individual reports of the genetic disruption of the EP_1 receptor gene (EP_1-/-) (328, 459, 460). A gender-specific effect on blood-pressure homeostasis was observed. Male (but not female) EP_1-/- mice exhibited hypotension with elevated heart rate and plasma-renin activity, as well as blunted ATII-mediated vasoconstriction, relative to WT mice (460). An independent group has recently reported a decrease in the number of preneoplastic lesions in EP_1-/- mice relative to WT using a mouse model of colon cancer. The same effect has been demonstrated with a novel EP_1 antagonist in two different mouse models of colon cancer and suggests that EP_1 antagonists may be useful chemopreventive agents in colon carcinogenesis.

Of all of the prostanoid receptors, the ablation of the EP_2 receptor gene (EP_2-/-) has been studied most intensely (461, 462, 463, 464). Contradictory effects of the EP_2-/- phenotype are apparent for blood pressure homeostasis. Both hypertension (461) and hypotension (462) are reported for EP_2-/- mice relative to WT. The vasodepressor responses of PGE_2 in female EP_2-/- mice are compromised, while in males the EP_1 receptor contributes predominantly to this response (463). Salt-sensitive hypertension is observed in EP_2-/- mice suggesting a role for EP_2 in the regulation of sodium in the kidney (461, 462). The EP_2 receptor plays an important role in reproduction since female EP_2-/- mice exhibit a reduced litter size, a decrease in ovulation number and a reduced fertilization rate (461, 462, 464). An EP_2-dependent role was demonstrated in the expansion of the follicular granulosa cells surrounding the oocyte, which is a process known as cumulus expansion. These results suggest that the incomplete cumulus expansion observed in the absence of EP_2 contributes to the reduced ovulation and the failure of fertilization (464).

The knockout of the EP_3 receptor gene (EP_3-/-) has also been reported (459, 465, 466). EP_3 is clearly involved in PGE_2-induced pyrexia since EP_3-/- mice fail to mount a febrile response to exogenous (i.e. LPS) and endogenous (i.e. IL-1β) pyrogens (459). PGE_2 also functions through the EP_3 receptor to concentrate urine, however these effects are deemed unessential for the normal regulation of urinary osmolality (465). There is a recent report of EP_3-/- mice being unable to secrete duodenal bicarbonate upon luminal perfusion with PGE_2 relative to WT (466). Because these EP_3-/- mice subsequently exhibit susceptibility to acid-induced injury, this receptor may function in maintaining mucosal integrity.

Individual reports of the effects of ablation of the EP_4 receptor gene (EP_4-/-) concur that it has various functions in the vascular system (460, 463, 467, 468). In the neonate, EP_4 is responsible for the closure of the patent ductus arteriosus (PDA), an arterial connection in the fetus that directs deoxygenated blood toward the placenta (467, 468). Compensatory pathways for the closure of the PDA were demonstrated when a mouse strain of mixed genetic background was used to generate the EP_4-/- mice (460). The EP_4 receptor may also control blood volume homeostasis. Adult male EP_4-/- mice demonstrate low plasma renin activity despite systolic hypotension, but exhibit hypertension when fed a high sodium diet (460). However, these results are in contrast to others reporting the changes in vascular tone with female, but not male, EP_4-/- mice relative to WT (463).

FP knockout (FP-/-) mice have been generated and studied for their reproductive function (469). Female FP-/- mice are fertile, carry their litters to term, but fail to undergo parturition. This is because progesterone levels remain elevated, since $PGF_{2\alpha}$ is responsible for the luteolysis that subsequently reduces progesterone and signals parturition. $PGF_{2\alpha}$ is also responsible for up-regulating the uterine oxytocin receptors that facilitate parturition (470), and therefore the onset of labour can not occur in FP-/- mice.

The genetic knockout of the IP receptor (IP-/-) produced a phenotype exhibiting both expected and unexpected results (471). As expected, the susceptibility of IP-/- mice to thrombosis was increased relative to WT, and IP-/- mouse platelets and vascular smooth muscle were unresponsive to IP agonists. However, the pain and inflammation

responses of IP-/- mice in multiple models were similar to those observed in PGHS inhibitor (indomethacin)-treated WT mice. These observations suggest a role for the IP receptor in pain perception and the inflammatory response.

Targeted disruption of the TP receptor gene (TP-/-) confirms a role for TP in vascular responses and homeostasis (472). The hemodynamic collapse observed upon AA infusion into WT mice is absent from TP-/- mice. Platelet responses are also changed. WT mouse platelets respond to collagen and to TP agonists by aggregating. TP-/- mouse platelets are completely unresponsive to TP agonists and responses to collagen are delayed. Also, TP-/- mice exhibit a prolonged bleeding time, reinforcing the function of TP in maintaining hemostasis.

1.5. Gastrointestinal mucins

1.5.1. Structure and biosynthesis

Mucins are high molecular weight glycoproteins (200 – 2000 kDa) (473). The mucin family can be subdivided into secretory and membrane-associated forms. Secretory mucins oligomerize through the disulfide linkage of mucin monomers to form the mucous present in the tracheobronchial, gastrointestinal, and reproductive tracts. In contrast, membrane-associated mucins contain a hydrophobic membrane-spanning domain and are not observed to oligomerize (474). This section will focus on the gastrointestinal mucins, which are predominantly of the secretory type.

Mucins confer the viscoelastic and lubricant properties of the mucous that covers the lumenal surfaces of epithelial organs. In this capacity, mucins play a protective role against mechanical stress and luminal irritation. Additionally, the high mucin content of O-linked oligosaccharides and the extraordinary diversity of these carbohydrate structures provides lectin-binding capacity. In this way, mucins may also function as a host defense system against invading pathogens (473, 474, 475, 476).

Mucins are characterized as glycoproteins that contain a large core peptide (called apomucin) and a high carbohydrate content (50-80% dry weight). To date, nine human mucin genes encoding different apoproteins have been identified, denoted MUC1-4, MUC5AC, MUC5B, and MUC6-8. The complete nucleotide sequences are reported for MUC1 (477), MUC2 (478), MUC5AC (479, 480, 481, 482), and MUC7 (483), and partial sequences are reported for the others. Human chromosome 11p15 contains a mucin gene cluster known to include MUC2, MUC5AC, MUC5B, and MUC6 (484). It is now clear that a given mucin gene is expressed in more than one human tissue and many tissues express more than one mucin gene (485, 486).

The apomucins contain tandem repeating (TR) sequences within their core regions that are rich in S, T, and P residues. Each apomucin has a unique TR sequence (MUC2 has two TRs). The length of the TR sequences in human mucins can vary in size from 8 to 169 residues (479, 487). The number of iterations of these TR sequences for a given mucin can also vary, which has complicated the cloning of the full length mucin

cDNAs. The S and T residues represent O-glycosylation sites, while the P residues are thought to govern the specificity of the initial galactosamine transferase responsible for mucin oligosaccharide synthesis (488, 489). The S and T content of the TR sequences varies significantly between different mucins (i.e. 25% for MUC1, 75% for MUC5AC), which may effect the physical and biological properties of the corresponding mucins (476). In contrast to the mucin core regions, the N- and C-terminal regions are C-rich. These residues are thought to mediate the intermolecular interactions that facilitate the process of oligomerization (490, 491).

MUC2 is the most prominent secretory mucin found in the intestinal tract, where it is expressed predominantly within the goblet cells of the small intestine and colon (492, 493). It is a highly repetitive gene encoding a protein of ~5100 residues. As previously noted, MUC2 contains two TR domains. They are rich in T and P, and act as sites for O-glycosylation (478, 494). The N- and C-terminal portions of MUC2 display homology to the serum glycoprotein pro-von Willebrand factor (vWF). Both MUC2 and pro-vWF contain D-domains involved in polymerization, three in the N-terminus and one in the C-terminus (478).

MUC3 is also found in the intestinal tract, but is expressed in both the goblet cells and absorptive cells (493). This gene contains a single TR sequence rich in S and T, and the mature mucin is heavily O-glycosylated (495). The functional relevance of MUC3 is questionable, since it has been suggested to not contribute to the mucin blanket (496).

MUC5AC is a predominant secretory mucin expressed by the surface mucous cells of the gastric glands of the stomach (497). This gene gives rise to two different cDNAs, designated MUC5A and MUC5C (479, 498). The cloning of the MUC5AC gene has recently been completed and it contains the D-domains identified previously in MUC2 and pro-vWF, which are thought to be involved in mucin oligomerization (482). In addition, MUC5AC contains a leucine zipper motif which has been hypothesized to facilitate oligomerization by stabilizing the monomers during this process.

MUC6 is found in the antral mucous cells of the stomach (499). This partially cloned mucin contains a TR region 169 amino acids in length, the longest yet identified in a human mucin gene (487). Recently, cloning of the C-terminus of the gene has

identified homology to MUC2, MUC5AC, and pro-vWF (500). This mucin is hypothesized to exist as a disulfide-bonded oligomer.

Mucin biosynthesis has been studied in particular for MUC2 (501, 502, 503). Whether these observations are true for all mucins remains unclear. Synthesis of nascent mucin peptides occurs on membrane-bound ribosomes, which are subsequently transported to the rough endoplasmic reticulum (RER) (475). The MUC2 apomucin is cotranslationally N-glycosylated with mannose oligosaccharides and also undergoes limited O-glycosylation with N-acetyl galactosamine (GalNAc). Oligomerization (or at least dimerization) of mucin monomers by disulfide bonding occurs in order for the mucins to enter the Golgi apparatus, probably under the influence of the RER enzyme disulfide isomerase (504). Dimerization of MUC2 monomers is thought to occur through their C-termini (tail-to-tail), based on homology to pro-vWF whose biosynthesis and oligomerization has been studied in detail (476). Chain elongation of the O-glycans with GalNAc, galactose, N-acetylglucosamine, and sialic acid occurs within the trans-Golgi. Mucin oligomers bud from the trans-Golgi within membrane bound condensing granules to accumulate in the theca, a centrally located storage zone (476, 501). Further oligomerization through the mucin N-termini (head-to-head) occurs within the condensing granules.

1.5.2. **Goblet cells and mucin secretion**

Goblet cells are found in the lumenal epithelia of the tracheobronchial and gastrointestinal tracts, and are specialized in mucin exocytosis (505, 506, 507). These cells have a highly organized array of microtubules and intermediate filaments known as the theca, which provides a boundary between the mucin granules and the remaining cytoplasm (508). The theca lies below the apical membrane and gives the goblet cells their characteristic shape. It contains no actin filaments, but a network of F-actin filaments overlies the theca providing a physical barrier between the mucin granules and the plasma membrane (509). Depolymerization of these actin filaments using cytochalasin D causes an acceleration of granular secretion (510).

Mucin secretion takes on two forms: (i) unregulated secretion, in which mucins are not stored but are instead secreted constitutively in the absence of a secretagogue, and (ii) regulated secretion, in which mucins are stored prior to their secretagogue-provoked release (505, 507). The various contributions of these processes to overall mucin secretion depends on the cell-type under study. Constitutive release of mucin granules occurs at the apical membrane and is controlled by interactions with microtubules, since the microtubule depolymerizing agent nocodazole disrupts this process. With respect to the process of regulated mucin secretion, a number of diverse mucin secretagogues are known to exist including neurotransmitters (i.e. serotonin, acetylcholine, etc.), inflammatory mediators (i.e. prostaglandins, IL-1, etc.), and chemical agents (i.e. cholera toxin, ionophore, etc.) (505, 507). A variety of second messenger pathways are implicated in the signal transduction of these secretagogues such as intracellular cyclic AMP, intracellular Ca^{2+}, and DAG. Secretagogues normally induce extrusion of the laterally located mucin granules of the theca. However, under extreme circumstances the centrally located mucin granules are released in a process called compound exocytosis (511). This process involves the evacuation of much of the mucin granule mass in addition to a considerable loss of cytoplasm and a sloughing of granule membrane (505). However, goblet cells have been observed to recover quickly with complete refilling of the intestinal goblet cell estimated to take 1-2 hours (509).

1.5.3. **Mucins in gastrointestinal pathophysiology**

The complexity inherent in the mucin family of glycoproteins is a product of the different mucin gene products described earlier and the differential glycosylation of each of these gene products, as facilitated by distinct subpopulations of goblet cells (509). Under conditions of disease the complexity of this mucin heterogeneity is increased, in that both altered glycosylation and aberrant mucin gene expression are observed. For instance, in colonic adenocarcinomas the O-linked oligosaccharide chains tend to be fewer in number and shorter than normal (475, 512). This shortening of oligosaccharides may increase the exposure of the peptide regions of the mucin. This has allowed the targeting of monoclonal antibodies against MUC2 that could be directed specifically to colonic carcinomas (513). Regarding the aberrant expression of mucin genes, gastric metaplasia tissues express the intestinal mucins MUC2 and MUC3 while normal gastric tissues do not (514). These changes may play a role in metastases and/or in assisting the tumour cell to evade destruction by immune effector cells (475).

In contrast, studies of biopsies from ulcerative colitis patients demonstrate that MUC2 is the predominating species expressed and is unchanged from that found in normal tissue (507). The biosynthesis of the MUC2 apoprotein is retarded although the mRNA levels are unchanged. The rate of MUC2 biosynthesis returns to control levels upon remission of the disease (515). However, more recently an alteration in the post-translational modification of MUC2 in both ulcerative colitis and Crohn's disease is reported to allow better detectability by a MUC2 antibody (516). Ulcerative colitis may indeed be multifactorial since a rare TR in the MUC3 is suggested to confer a predisposition to the disease (517).

Another component of mucins in gastrointestinal pathophysiology concerns their interaction with invading microorganisms (475, 507). In this capacity, mucins protect the underlying epithelium by providing a matrix of carbohydrates to which the microbial adhesins can bind and are considered to function as a host defense mechanism. In processes favouring removal of the microbe, organisms are sloughed by peristaltic movements and defecation. However, some microbes might use this attachment to

mucins as a means to colonize and then penetrate the epithelial layer. For instance, the protozoan parasite *Entamoeba histolytica* has a high affinity for rat and human colonic mucins ($K_d = 8.2 \times 10^{-11}$ M) (518, 519). In this process, the microbes would then secrete virulence factors such as cytotoxins, cytolysins, or invasins. They may also release mucin secretagogues. Here, chronic exposure of the epithelium to mucin secretagogues could deplete mucin stores or stimulate the release of immature, poorly glycosylated mucins (520). These compromised mucin forms may be targets for proteases released by the parasites. The parasite could also produce mucinases, such as those released by *E. histolytica* (521). Thus, the fate of the invading microbe is dependent on a number of factors, including mucin composition, mucin quality and quantity, gut motility, and luminal fluid flow.

2. RESEARCH OBJECTIVES

The 2-series prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) evoke a diverse array of biological activities through their preferential interaction with eight individual prostanoid receptors, specifically DP, EP (EP_1, EP_2, EP_3, EP_4), FP, IP, and TP, respectively. Although the endogenous prostanoids demonstrate a degree of receptor selectivity, pharmacological cross-reactivity is well described in the interactions between these ligands and the entire prostanoid receptor family. Thus, PGD_2 preferentially binds to the DP receptor but also has demonstrated affinity for the FP and TP receptors. This cross-reactivity has necessitated the development of synthetic prostanoid analogues with improved receptor selectivity in order to elucidate the biological contributions of the individual prostanoid receptors in heterogeneous systems.

However, the interpretation of the biological consequences of DP receptor activation is further complicated by its highly discrete distribution relative to the broad spectrum of PGD_2-mediated events. Thus, PGD_2 is formed in a variety of tissues including brain, spleen, lung, bone marrow, stomach, skin, and in mast cells. It has been shown to effect physiological events in the central nervous system (such as sleep, body temperature, olfactory function, hormone release, and nociception) and in the peripheral tissues (such as intraocular pressure, platelet aggregation, systemic vasodilation, pulmonary constriction, and bronchoconstriction). However, the DP receptor is the least abundant prostanoid receptor and, consequentially, the least well characterized. Convincing evidence for DP receptor expression has been demonstrated in some tissues (such as in the brain, the eye, and in platelets). However, the expression of the DP receptor and its functional role in many tissues remains unclear.

Thus, the objectives of this thesis were:

A. To investigate the activity of synthetic prostanoid analogues against the recombinant human DP receptor, in an effort to identify novel selective DP-specific ligands that can be used to pharmacologically discern the individual contribution of the DP receptor in heterologous systems.

B. To delineate the cell-specific localization of the DP receptor in the gastrointestinal tract, in an effort to identify a functional correlate that justifies the abundance of DP-specific mRNA in these tissues from various species.

C. To determine whether selective DP-specific ligands can regulate mucin secretion (as the results from Manuscript B would suggest) in an established *in vitro* cell model, in an effort to justify mucin secretion as a functional correlate that reflects the abundance of DP-specific mRNA in the gastrointestinal tissues of various species.

3. STATEMENT OF CONTRIBUTIONS

This thesis is composed of 3 manuscripts. The contributions of the individuals involved are described below.

Manuscript **A**. D. Hamish Wright, Kathleen M. Metters, Mark Abramovitz and Anthony W. Ford-Hutchinson (1998) Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist. *British Journal of Pharmacology* 123: 1317-1324.

All the experiments described in this manuscript were planned and performed by myself, with two exceptions. Verification of the stability of [^{3}H]PGD_2 under the binding assay conditions by r.p.-h.p.l.c. (data not shown) was performed by Nathalie Tremblay prior to my arrival in the laboratory. Additionally, some of the selectivity testing of L-644,698 against the prostanoid receptors other than DP was performed during routine screening in the laboratory, while the remainder was performed by myself. Selectivity testing against the DP receptor was performed by myself. This manuscript was also written by myself. Instruction on various experimental techniques was provided by Drs. M. Abramovitz and K. M. Metters, while supervision was provided by Drs. K. M. Metters and A. W. Ford-Hutchinson.

Manuscript **B**. D. Hamish Wright, François Nantel, Kathleen M. Metters and Anthony W. Ford-Hutchinson (1999) A novel biological role for prostaglandin D_2 is suggested by distribution studies of the rat DP prostanoid receptor. *European Journal of Pharmacology* 377: 101-115.

All the experiments described in this manuscript were planned and performed by myself. This manuscript was also written by myself. Dr. F. Nantel provided instruction on the *in situ* hybridization technique, and the work was supervised by Drs. K. M. Metters and A. W. Ford-Hutchinson.

Manuscript C. D. Hamish Wright, Anthony W. Ford-Hutchinson, Kris Chadee, and Kathleen M. Metters (submitted) The human prostanoid DP receptor stimulates mucin secretion in LS174T cells. *British Journal of Pharmacology*

All the experiments described in this manuscript were planned and performed by myself. This manuscript was also written by myself. Dr. K. Chadee provided instruction on the mucin experiments and access to the LS174T cell line. The work was supervised by Drs. A. W. Ford-Hutchinson and K. M. Metters.

4. MANUSCRIPT A

Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist

British Journal of Pharmacology (1998) 123: 1317-1324

Prostaglandins, prostacyclin and thromboxanes are collectively described as prostanoids. These cyclooxygenase products of arachidonate metabolism mediate various physiological and pathophysiological effects through membrane-bound receptors. The current paper provides a thorough characterization of the recombinant human PGD_2 receptor (DP) stably expressed in a human embryonic kidney cell line, with respect to radioligand binding and signal transduction properties using prostanoids and prostanoid analogues. Because of the diversity of prostanoid-mediated activities, prostanoid receptors are considered potential therapeutic targets and collections of compounds have been established with which to study them further. The current work also describes the use of the characterized receptor, along with a system of other recombinant human prostanoid receptors, to identify a novel specific agonist, L-644,698, for the human DP receptor. At nanomolar concentrations this compound demonstrated both high affinity DP-specific binding and high potency, performing as a full agonist at the human DP receptor. Most importantly, this paper provides evidence that L-644,698 is at least 300-fold more selective for the DP receptor than for any of the seven other recombinant human prostanoid receptors tested, making it one of the most selective DP-specific agonists as yet described. PGJ_2 and Δ^{12}-PGJ_2, the endogenous metabolites of PGD_2, were also tested in this system and PGJ_2 was particularly active, being equipotent to the DP-specific agonists including L-644,698.

Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist

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Summary

1. A human embryonic kidney cell line [HEK 293(EBNA)] stably expressing the human recombinant prostaglandin D_2 (PGD_2) receptor (hDP) has been characterized with respect to radioligand binding and signal transduction properties by use of prostanoids and prostanoid analogues. Radioligand binding studies included saturation analyses, the effects of nucleotide analogues, the initial rate of ligand-receptor association and equilibrium competition assays. In addition, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation in response to ligand challenge was also measured, as this is the predominant hDP signaling pathway.

2. L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate)) was identified as a novel ligand having high affinity for hDP with an inhibitor constant (K_i) of 0.9 nM. This K_i value was comparable to the K_i values obtained in this study for ligands that have previously shown high affinity for DP: PGD_2 (0.6 nM), ZK 110841 (0.3 nM), BW245C (0.4 nM), and BW A868C (2.3 nM).

3. L-644,698 was found to be a full agonist with an EC_{50} value of 0.5 nM in generating cyclic AMP following activation of hDP. L-644,698 is, therefore, comparable to those agonists with known efficacy at the DP receptor (EC_{50}): PGD_2 (0.5 nM), ZK 110841 (0.2 nM), and BW245C (0.3 nM).

4. L-644,698 displayed a high degree of selectivity for hDP when compared to the family of cloned human prostanoid receptors: EP_1 (> 25,400-fold), EP_2 (~300-fold), EP_{3-III} (~4100-fold), EP_4 (~10,000-fold), FP (>25,400-fold), IP (>25,400-fold), and TP (>25,400-fold). L-644,698 is, therefore, one of the most selective DP agonists as yet described.

5. PGJ_2 and Δ^{12}-PGJ_2, two endogenous metabolites of PGD_2, were also tested in this system and shown to be effective agonists with K_i and EC_{50} values in the nanomolar

range for both compounds. In particular, PGJ_2 was equipotent to known DP-specific agonists with a K_i value of 0.9 nM and an EC_{50} value of 1.2 nM.

Introduction

Prostaglandins, prostacyclin (PGI_2), and thromboxane A_2 (TXA_2) are collectively described as prostanoids. Originally it was proposed (Kennedy *et al*, 1982; Coleman *et al*, 1984) that individual prostanoid receptors existed for each of the primary bioactive prostanoids. This classification described distinct receptors for prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 which were denoted DP, EP (EP_1 and EP_2), FP, IP, and TP, respectively. This was followed by a further subdivision of the EP class of receptors into four subtypes: EP_1, EP_2, EP_3, and EP_4 (for review, Coleman *et al*, 1994). The cloning of the human (h) prostanoid receptors currently includes TP (Hirata *et al*, 1991), FP (Abramovitz *et al*, 1994), IP (Boie *et al*, 1994), EP_1 (Funk *et al*, 1993), EP_2 (Regan *et al*, 1994), EP_3 (Adam *et al*, 1994), EP_4 (originally mistakenly described as EP_2)(An *et al*, 1993; Bastien *et al*, 1994), and DP (Boie *et al*, 1995). These receptors form a sub-family within the G protein-coupled receptor (GPCR) superfamily. During cloning, alternatively spliced isoforms of both hTP (Raychowdhury *et al*, 1995) and hEP_3 (Adam *et al*, 1994; Schmid *et al*, 1995; Kotani *et al*, 1995) were identified.

The endogenous prostanoids demonstrate preference towards individual prostanoid receptors but, in general, there is a marked degree of cross-reactivity between these ligands and the entire receptor family (Dong *et al*, 1986; Sheldrick *et al*, 1988; Armstrong *et al*, 1989; Bunce *et al*, 1990). This has driven the development of selective compounds. Several selective and efficacious prostanoid agonists have been identified, including cicaprost (Dong *et al*, 1986; Armstrong *et al*, 1989) at the IP receptor, GR63799X (Bunce *et al*, 1990) at the EP_3 receptor and butaprost (Abramovitz *et al*, unpublished observations) at the EP_2 receptor. To date, the group of synthetic DP agonists includes BW245C, ZK 110841, RS-93520, RS-93427, 572C85, and 192C86. BW245C is the most comprehensively studied of these synthetic DP agonists (Town *et al*, 1983; Whittle *et al*, 1983; Woodward *et al*, 1990; Fernandes & Crankshaw, 1995; Rangachari *et al*, 1995) but although the efficacy of BW245C at DP is well-established (Town *et al*, 1983; Boie *et al*, 1995), the selectivity of this ligand versus the other prostanoid receptors is not completely defined. Data suggest that BW245C has affinity for at least one other prostanoid receptor that couples to stimulation of adenylate cyclase

via the guanine nucleotide binding (G) protein G_s. Some findings suggest that BW245C cross-reacts with EP_2 (Giles *et al*, 1989; Matsugi *et al*, 1995) while other investigators have proposed IP (Trist *et al*, 1989). Another potential limitation associated with BW245C concerns its stability in aqueous solution since it has been shown to produce an epimer which is less biologically active than the parent compound (Brockwell *et al*, 1981).

Studies of the DP receptor have been complicated by its relatively low abundance and narrow scope of distribution. Thus, it was the last known human prostanoid receptor to be cloned (Boie *et al*, 1995). In this study, the radioligand binding and signal transduction properties of recombinant hDP have been more completely studied, both with prostanoids and prostanoid synthetic analogues of varying selectivity. Through these analyses, and by use of the system of cloned recombinant human prostanoid receptors previously described (Abramovitz *et al*, unpublished observations), a novel selective DP agonist, L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate) (Figure 1), has been identified.

Methods

pCEP4-hDP stable expression in HEK 293(EBNA) cells

Stable expression of the hDP receptor was achieved by transfection of the pCEP4-hDP (Abramovitz *et al*, unpublished observations) plasmid into HEK 293(EBNA) cells [maintained under selection with GENETICIN (G418)] by cationic-liposome mediated transfer using LipofectAMINE reagent (Felgner *et al*, 1987). Cells were maintained in culture for 48 h post transfection and then grown in the presence 200 μg ml^{-1} hygromycin B for 2 weeks, to select for resistant colonies expressing the hDP receptor. Resistant colonies were expanded and subsequently tested for hDP expression by radioligand binding. The clone with the highest level of binding activity was then used for signal transduction assays.

Cell culture and membrane preparation

HEK 293(EBNA) cells stably expressing hDP (hDP-HEK) were maintained in culture in Dulbecco's modified Eagle's medium growth medium (Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 20 units ml^{-1} penicillin G, 20 μg ml^{-1} streptomycin sulphate, 250 mg ml^{-1} GENETICIN and 200 mg ml^{-1} hygromycin B). In order to prepare membranes from hDP-HEK cells (all procedures at 4 °C), they were first resuspended by Dounce homogenization (pestle B, 10 strokes) in the presence of 2 mM phenylmethylsulfonylfluoride. Cells were next disrupted by nitrogen-cavitation at 800 psi for 30 min on ice. The resulting cell suspension was subjected to two centrifugation steps: 1000 x g_{max} for 10 min followed by 100 000 x g_{max} for 30 min. The resulting pellet was resuspended to 1/10th the original volume in 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA (tetrasodium salt) by Dounce homogenization (pestle A, 10 strokes), and aliquots were stored at –80 °C at a protein concentration of 8-10 mg ml^{-1}.

[^{3}H]PGD_2 binding to hDP-HEK membranes

Radioligand binding assays were performed in 0.2 ml of 10 mM HEPES-KOH (pH 7.4) 1 mM EDTA containing (unless otherwise noted) 0.8 nM [^{3}H]PGD_2 (115 Ci mmol^{-1}) and 10 mM $MnCl_2$. Compounds were added in dimethylsulfoxide (Me_2SO) at 1% (v/v) of the final incubation volume (vehicle concentration was constant throughout). The reaction was initiated by the addition of 30 µg of hDP-HEK membrane protein to all tubes and the samples were incubated at room temperature for 1 h. The reaction was terminated by rapid filtration at 4 °C in 3-4 ml of 10 mM HEPES/KOH (pH 7.4) through a GF/C filter (Unifilter) which had been presoaked in the same buffer. Each filter was dried for 1-2 h at 55 °C and the residual [^{3}H]PGD_2 bound to the filter (33% efficiency) was determined in 50 µL per well of Ultima Gold scintillation cocktail. Non-specific binding was determined in the presence of 1 µM PGD_2.

Analysis of [^{3}H]PGD_2 binding

Rates of association were calculated through a one-site curve-fit analysis, employing the equation $B_t=B_{eq}-B_{eq}[e^{(-K_{obs}t)}]$; where B_t represents the radioligand bound specifically at time t, B_{eq} represents the radioligand bound specifically at equilibrium and K_{obs} is the observed association rate which is then expressed as K_{obs}/[radioligand].

Specific binding saturation isotherms were deduced by subtracting the non-specific binding from the total binding, both measured experimentally. The saturation isotherms were transformed by use of nonlinear, least-squares, regression analysis adapted from the work of Feldman (1972) where the equation $[(B_{MAX} \times F) / (K_1 + F)] + [(B_{MAX} \times F) / (K_2 + F)]$ represents the radioligand specifically bound, B_{MAX} is the maximal number of binding sites, K is the equilibrium dissociation constant and F is the concentration of free radioligand. The analyses were performed using Accufit Two-Site saturation software (Beckman Instruments).

Sigmoidal curves from equilibrium competition assays were analysed by custom designed software which employs a non-linear least-squares fitting routine based on the four parameter logistic equation: $y=(m1-m2)(1+(m0/m3)e^{m4})^{-1}+m2$; where m1 and m2

represent the maximum and minimum of the curve, m3 represents the inflection point (IP), m4 represents the slope of the curve at the inflection point, m0 represents the concentration of the competing ligand and y represents the % [^{3}H]PGD_2-specific binding. K_i values were calculated from the equation K_i = IP / 1 + [radioligand]/(K_D).

Chemical and metabolic stability of [^{3}H]PGD_2 under the experimental conditions

The stability of [^{3}H]PGD_2 exposed to the incubation conditions for 2 h was verified by reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.). Following incubation of [^{3}H]PGD_2 under standard conditions the reaction was terminated with the addition of 2 x r.p.-h.p.l.c. solvent (r.p.-h.p.l.c. solvent = 66:15:18:1 (v/v) H_2O:CH_3CN:CH_3OH:CH_3COOH, adjusted to pH 5.6 with 10 N NaOH) to the samples. Samples were allowed to incubate for 10 min at 30 °C to dissociate the receptor-bound [^{3}H]PGD_2 and were then subjected to centrifugation at 100 000 x *g* for 15 min at 4 °C. The resulting supernatant fractions, containing both bound and unbound [^{3}H]PGD_2, were then analysed by r.p.-h.p.l.c. using a NovaPak C_{18} column (0.39 x 15 cm; Waters). [^{3}H]PGD_2 was eluted with a linear gradient from 0 to 70% (v/v) acetonitrile developed over 35 min at a flow rate of 1 ml min^{-1} following an initial 3 min elution with r.p.-h.p.l.c. solvent. The 35 min gradient elution was followed by a final 10 min wash with 90% (v/v) acetonitrile. The profile of radioactivity was monitored using an on-line flow-through radioactivity detector (Berthold). Eluant fractions were subsequently mixed with 5 ml of scintillation fluid for the precise quantitation of the radioactivity recovered.

Cyclic AMP assays with hDP-HEK cells

hDP-HEK cells were harvested at 80% confluence by resuspension in 10 ml of enzyme-free cell dissociation buffer (a commercially available, membrane-filtered, isotonic, and enzyme-free aqueous formulation of salts, chelating agents, and cell-conditioning agents) and washed in phosphate-buffered saline by centrifugation (300 x g_{max} for 6 min at room temperature). Cells were then washed in 10 ml of Hanks' balanced salt solution (HBSS) by centrifugation under the same conditions as above and resuspended in HBSS at 4 x

10^6 cells ml^{-1}. Cell viability was determined to be > 95%, by trypan blue exclusion. The generation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) was performed in a final incubation volume of 0.2 ml of HBSS containing 100 μM Ro 20-1724 to abrogate cyclic AMP hydrolysis. Compounds were added in Me_2SO kept constant at 1% (v/v) of the final incubation volume. Assays were initiated with the addition of 2 x 10^5 cells per reaction and samples incubated for 10 min at 37 °C with shaking. Reactions were halted by the incubation of samples in boiling water for 3 min and cyclic AMP was subsequently measured using a [^{125}I] - cyclic AMP scintillation proximity assay.

Sigmoidal concentration-response curves were analysed by custom designed software to determine EC_{50} values. Maximal stimulation was defined as the quantity of cyclic AMP produced by incubation with 1 μM PGD_2. Statistical analysis of the maximal response for each ligand as a percentage of this maximal stimulation was performed with SigmaStat software, version 2.0 (Jandel Scientific). A one-way ANOVA followed by a Tukey test was used to determine full and partial agonists.

Protein assays

Protein concentration was measured by the bicinchoninic acid (BCA) protein assay kit (Pierce) with bovine serum albumin as the standard.

Reagents

PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-$PGF_{2\alpha}$), PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and Ro 20-1724 (4-(3 butoxy-4-methoxybenzyl)-2-imidazolidinone) were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). 13,14-dihydro-15-keto-PGD_2 was from Cayman Chemical (Ann Arbor, MI, U.S.A.). BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)) and BW A868C ((±)-3-benzyl-5-(6-carboxyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) were generous gifts from The Wellcome Foundation Ltd (Beckenham, Kent, U.K.). ZK 110841 ((5Z,13E)-(9R,11R,15S)-9β-chlor-15-cyclohexyl-11,15-dihydroxy-

16,17,18,19,20-pentanor-5,13-prostadienoic acid) was a generous gift from Dr. D. Crankshaw from the Department of Obstetrics & Gynecology (McMaster University, Hamilton, Ontario, Canada). L-644,698 was synthesized at Merck Research Laboratories by Dr. J.B. Bicking. Iloprost and ^{125}I - cyclic AMP scintillation proximity assay kits were from Amersham (Oakville, ON, Canada). [^{3}H]PGD$_2$ was from Dupont NEN (Boston, MA, U.S.A.). GTPγS (Guanosine-5′-O-(3-thiotriphosphate)), ATPγS (Adenosine-5′-O-(3-thiotriphosphate)), GMP-PNP (Guanylyl-imidodiphosphate) and AMP-PNP (Adenylyl-imidodiphosphate) were from Boehringer Mannheim Canada (Laval, QC, Canada). Bicinchoninic Acid (BCA) Protein Assays were from Pierce (Rockford, IL, U.S.A.). GENETICIN (G418) and LipofectAMINE were from GIBCO/BRL (Burlington, ON, Canada). Enzyme-free cell dissociation buffer was from Life Technologies Inc (Gaithersburg, MD, U.S.A.). Hygromycin B was from Calbiochem, (La Jolla, CA, U.S.A.). Unifilters and Ultima Gold were from Packard (Meriden, CT, U.S.A.).

Results

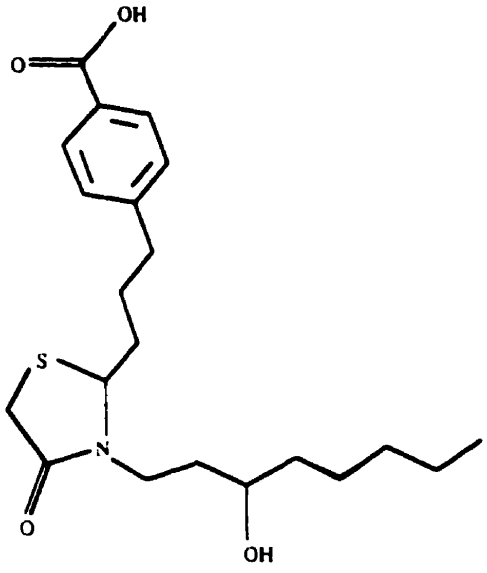

Figure 1. Structure of L-644,698 (racemate).

Rate of association

The rate of association of [^{3}H]PGD_2 with hDP at room temperature is shown in Figure 2. The initial rate of association was 0.15 ± 0.01 min^{-1} nM^{-1}. Equilibrium was reached within 20 min. Once achieved, equilibrium binding was maintained throughout the 2 h time course. The stability of [^{3}H]PGD_2 under the assay conditions was verified by reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.). [^{3}H]PGD_2 was incubated under the assay conditions for 2 h, recovered from the incubation media and resolved by r.p.-h.p.l.c. as a single peak with the same retention time (24.5 min) as a control sample of [^{3}H]PGD_2 analyzed under identical conditions (data not shown). Approximately 87% of the [^{3}H]PGD_2 added to the incubation medium was recovered. [^{3}H]PGD_2 is, therefore, chemically and metabolically stable during a 2h incubation under these experimental conditions as determined by r.p.-h.p.l.c.

The association rate of [^{3}H]PGD_2-specific binding to the hDP receptor was also investigated at 30 °C (data not shown). Equilibrium binding was attained more rapidly

than under conditions of room temperature, at a rate of 0.31 ± 0.03 min^{-1} nM^{-1}. However, it was not maintained as demonstrated by a time-dependent decrease in $[^3H]PGD_2$-specific binding during the 2 h incubation to a value 75% of the maximum level obtained.

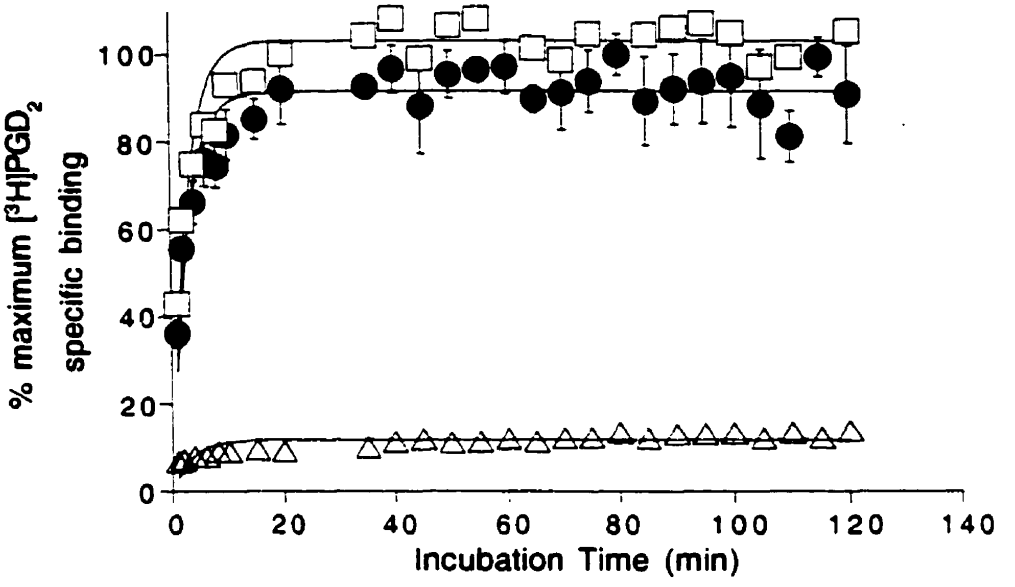

Figure 2. Rates of association of $[^3H]PGD_2$ binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods, with the following modifications: the rates of total (□) and non-specific (Δ) binding were assessed over a 2 h time course by the sequential addition of HEK 293(EBNA) membranes expressing the hDP receptor to separate incubation tubes. Membranes were added initially at the final time point of 2 h and successively at various other intervals until the initial time point of 1 min. Specific binding (●) was calculated as the difference between non-specific binding, measured in the presence of 1 μM PGD_2, and total binding. Data points are the mean from three separate experiments performed in duplicate, vertical lines show s.e.mean.

Effects of nucleotide analogues

The effects of slowly-hydrolyzable nucleotide analogues on $[^3H]PGD_2$-specific binding to the hDP receptor were studied (Figure 3). $[^3H]PGD_2$-specific binding was inhibited in a concentration-dependent manner by GTPγS, ATPγS, GMP-PNP and AMP-PNP. The

IC_{50} values (μM) for these compounds were 0.01, 1.67, 1.51 and 347, corresponding to the following rank order of nucleotide analogue potency at the hDP receptor: GTPγS >> ATPγS = GMP-PNP >> AMP-PNP. The effects of GTPγS were studied further in conjunction with saturation analyses as described below.

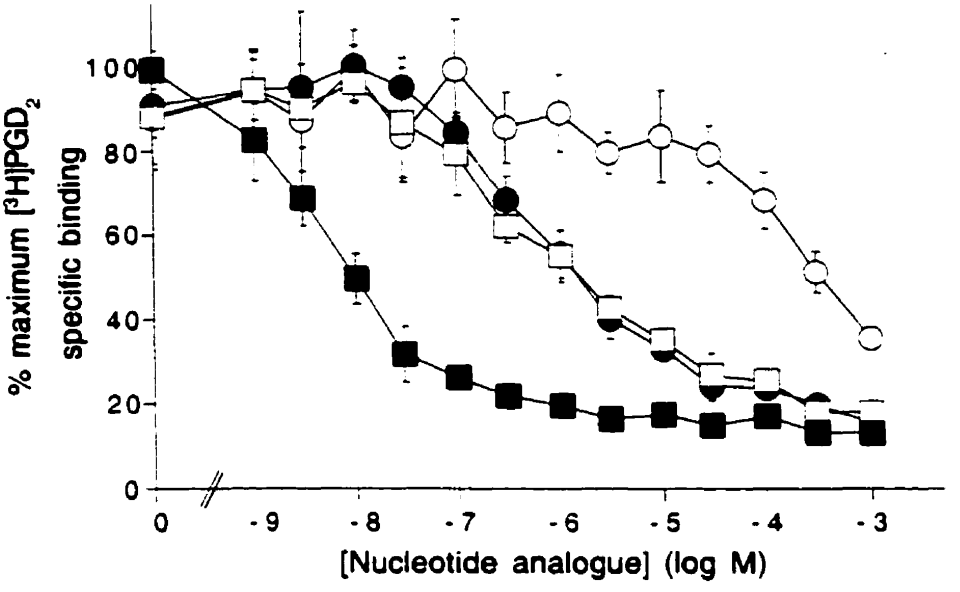

Figure 3. Effects of nucleotide analogues on [^{3}H]PGD_2-specific binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods, in the presence of 0-1000 μM of GTPγS (■), ATPγS (□) GMP-PNP (●), or AMP-PNP (○). The specific binding for each nucleotide analogue concentration was calculated as a percentage of the maximum specific binding obtained in the absence of nucleotide analogue. Data points are the mean from two separate experiments performed in duplicate; vertical lines show s.d.

Saturation analyses

Saturation analysis of [^{3}H]PGD_2-specific binding to the hDP receptor was performed and two populations of specific binding sites were revealed (Figure 4a): a high affinity site of relatively low abundance with an equilibrium dissociation constant (K_D) of 0.3 ± 0.1 nM

and a maximal number of specific binding sites (B_{MAX}) of 0.5 ± 0.1 pmol mg^{-1} of membrane protein and a low affinity site of relatively high abundance with a K_D of 13.4 ± 1.2 nM and a B_{MAX} of 5.9 ± 0.6 pmol mg^{-1} of membrane protein (all values are the mean ± s.e.mean, n=3). The nature of these two populations of [^{3}H]PGD_2-specific binding sites was investigated further with GTPγS. When saturation analysis was conducted in the presence of 100 μM GTPγS (Figure 4b) only the low affinity binding site was identified (K_D of 17.1 ± 1.3 and a B_{MAX} of 5.8 ± 0.3 pmol mg^{-1} of membrane protein).

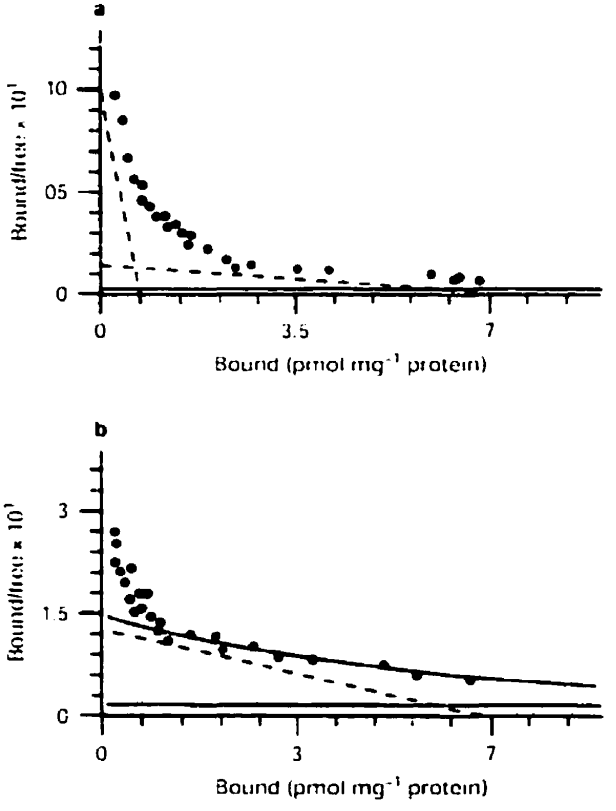

Figure 4. Saturation analysis of [^{3}H]PGD_2 binding to the human DP receptor expressed on membranes from HEK 293(EBNA) cells. Radioligand membrane binding assays were carried out as previously described under Methods. Total and non-specific binding were determined over a concentration range of 0.4 nM to 40 nM [^{3}H]PGD_2 (28.8 Ci $mmol^{-1}$) in the absence (a) and presence (b) of 100 μM GTPγS. Saturation isotherms were analysed as described in Methods. Note the difference in the scales of the ordinates between the two graphs. Non-specific binding was measured with 100-fold (4 μM) PGD_2, as validated in competition assays using [^{3}H]PGD_2. Data are representative of three separate experiments, each done in duplicate, with membrane stocks prepared from different passages of hDP-HEK cells.

Competition for [^{3}H]PGD_2-specific binding

Three groups of compounds were investigated for their ability to compete with [^{3}H]PGD_2-specific binding to hDP in equilibrium competition assays (Table 1): prostanoids, synthetic prostanoid analogues and PGD_2 metabolites. PGD_2 had a K_i of 0.6 ± 0.2 nM. PGE_2, $PGF_{2\alpha}$, and iloprost all had K_i values in the high nanomolar range. Compounds with the highest affinity were the DP-specific synthetic ligands: BW245C, BW A868C, and ZK 110841, all of which were comparable to PGD_2. PGJ_2 had affinity for the DP receptor that was of the same order as the DP-specific synthetic ligands and PGD_2. The other metabolic breakdown products of PGD_2 tested (Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, and 13, 14-dihydro-15-keto-PGD_2) had K_i values ranging from 100 nM to 6000 nM.

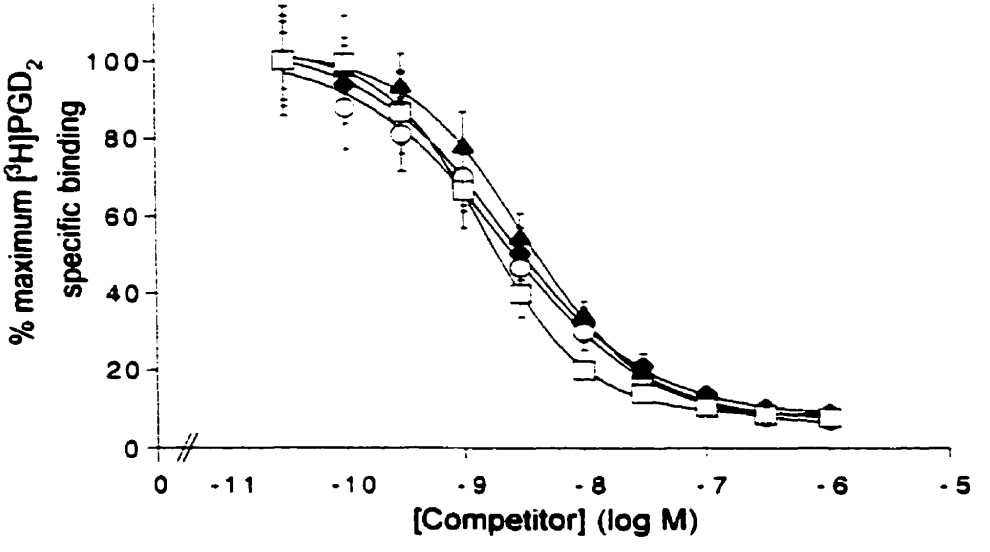

Figure 5. Competition for [^{3}H]PGD_2-specific binding to the human DP receptor expressed on HEK 293(EBNA) membranes by DP selective agonists . Radioligand membrane binding assays were carried out as previously described under Methods. Assays of competition were conducted under conditions of equilibrium at room temperature, with 0.03-1000 nM PGD_2 (◆), L-644,698 (○), BW245C (□), or PGJ_2 (▲). Data points are the mean from four experiments performed in duplicate, vertical lines show s.e.mean.

During this series of assays a novel ligand of high affinity and specificity for hDP, denoted L-644,698 (Figure 1), was identified. L-644,698 had high affinity for the hDP receptor with a K_i of 0.9 ± 0.2 nM, which is equal in magnitude to PGD_2, PGJ_2, BW245C, BW A868C, and ZK 110841 (Figure 5). In addition, L-644,698 demonstrated considerable selectivity for the hDP receptor, as shown in Table 2. Binding of L-644,698 was detectable at hEP_2, hEP_3, and hEP_4, where the respective K_i values were at least 300-fold, 4100-fold, and 10000 fold higher than the K_i value at hDP. L-644,698 did not compete for specific binding to hEP_1, hFP, hIP and hTP at concentrations up to 25.4 μM.

Table 1. Inhibitor constants and Hill coefficients for competing ligands at the human DP receptor.

	Ligand	*K_i (nM)*	*Hill coefficient (n_H)*
	PGD_2 (4)	0.6 ± 0.2	0.6 ± 0.2
I	PGE_1	53 ± 8	0.8 ± 0.1
	PGE_2	107 ± 42	0.6 ± 0.1
	$PGF_{2\alpha}$	367 ± 85	0.7 ± 0.1
	Iloprost	269 ± 82	0.7 ± 0.1
	U-46619	1202 ± 64	0.7 ± 0.1
II	PGJ_2 (4)	0.9 ± 0.1	0.9 ± 0.1
	Δ^{12}-PGJ_2	100 ± 13	0.8 ± 0.1
	15-deoxy-$\Delta^{12,14}$-PGJ_2 (4)	280 ± 30	0.1 ± 0.1
	13,14-dihydro-15-keto-PGD_2	6374 ± 1208	0.9 ± 0.1
III	L-644,698 (4)	0.9 ± 0.2	0.6 ± 0.2
	BW245C (4)	0.4 ± 0.1	0.6 ± 0.2
	BW A868C	2.3 ± 1.4	0.4 ± 0.2
	ZK110841 (4)	0.3 ± 0.1	0.9 ± 0.1

Inhibitor constant (K_i) values ± s.e.mean (nM) and Hill coefficient (n_H) values ± s.e.mean are shown for prostanoids and synthetic prostanoid analogues (I), metabolites of PGD_2 (II) and synthetic prostanoid analogues specific for DP (III). Values are derived from 3 separate experiments unless otherwise noted in parentheses by the ligand name.

Table 2 Selectivity of L-644,698 and reported DP agonists at recombinant human prostanoid receptors stably expressed in HEK 293 (EBNA) cells

Ligand	*K_i (nM)*							
	hEP_1	*hEP_2*	*hEP_3*	*hEP_4*	*hDP*	*hFP*	*hIP*	*hTP*
L-644,698	>25400 (3)	267 ± 39 (3)	3730 ± 738 (3)	*9280 ± 226 (2)	0.9 ± 0.2 (4)	>25400 (3)	>25400 (3)	>25400 (3)
†PGD_2	5820 ± 1801 (3)	2973 ± 100 (3)	421 ± 60 (3)	1483 ± 189 (3)	0.6 ± 0.2 (3)	6.7 ± 0.5 (3)	>25000 (3)	6602 ± 541 (5)
†BW245C	>25000 (4)	219 ± 19 (5)	>25000 (3)	132 ± 26 (4)	0.4 ± 0.1 (4)	>25000 (3)	>25000 (3)	13482 ± 1134 (5)
†ZK 110841	1.8 ± 0.2 (4)	6.0 ± 0.6 (4)	522 ± 81 (2)	41 ± 7 (4)	0.3 ± 0.1 (4)	1670 ± 107 (3)	2147 ± 661 (2)	1121 ± 201 (3)
PGJ_2	15678 ± 3711 (5)	989 ± 311 (4)	319 ± 29 (5)	1065 ± 252 (4)	0.9 ± 0.1 (3)	553 ± 226 (4)	>25000 (2)	6426 ± 1271 (5)

Inhibitor constant (K_i) values ± s.e.mean (where n≥3) or s.d. (where n=2) are derived from radioligand binding assays and are shown with the number of determinations (n) indicated in parentheses. * L-644,698 binding to hEP_4, K_i value ± s.d., data set = 9440 nM, 9120 nM and 43% inhibition at 25.4 µM; ZK 110841 binding to hEP_3, K_i value ± s.d., data set = 442 nM, 604 nM and 50% inhibition at 2.5 µM and to hIP, K_i value ± s.d., data set = 2615 nM, 1680 nM, 64% inhibition at 2.5 µM and 70% inhibition at 2.5 µM. † Data from Metters *et al*, in preparation.

Cyclic AMP generation assays

The ability of prostanoids and synthetic prostanoid analogues to transduce intracellular signals through hDP was explored by measuring stimulation of intracellular cyclic AMP production (Figure 6 & Table 3). PGD_2 had an EC_{50} value of 0.5 ± 0.1 nM for the

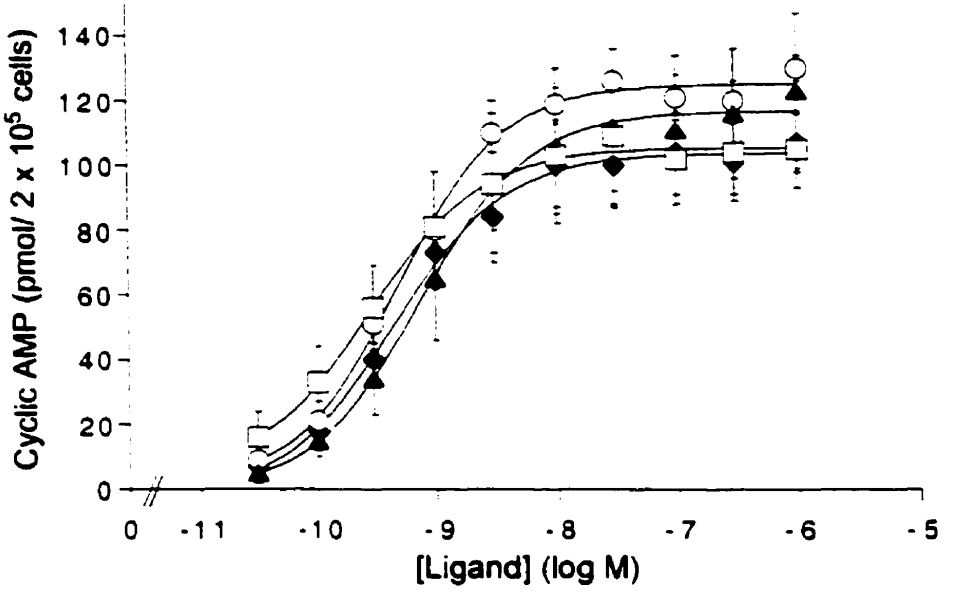

Figure 6. Cyclic AMP production by HEK 293(EBNA) cells expressing the human DP receptor. hDP-HEK cells were incubated in a final volume of 0.2 ml of HBSS containing 100 μM Ro 20-1724, as previously described under Methods. Also, added to the incubation medium was 0.03-1000 nM PGD_2 (◆), L-644,698 (○), BW245C (□), or PGJ_2 (▲). Reactions were initiated with the addition of 2 x 10^5 cells to the incubation volume, samples were incubated for 10 min at 37 °C with shaking and the reaction was terminated by the incubation of the assay plate in boiling water for 3 min. Cyclic-AMP was quantified by use of a commercially available scintillation proximity assay. Data points are the mean of three (L-644,698, BW245C, and PGJ_2) or four (PGD_2) experiments each performed in duplicate; vertical lines show s.e.mean.

production of cyclic AMP. The novel selective ligand L-644,698 was equipotent with an EC_{50} value of 0.5 ± 0.1 nM. BW245C and ZK 110841 had EC_{50} values of 0.3 ± 0.1 nM

and 0.2 ± 0.1 nM, respectively, whereas BW A868C was less potent with an EC_{50} value of 7.5 ± 0.9 nM. The PGD_2 metabolites all showed varying efficacies, the most notable being PGJ_2 with an EC_{50} value of 1.2 ± 0.1 nM.

The maximal response for each ligand as a percentage of the maximal stimulation achieved with 1 μM PGD_2 was also found in an effort to discriminate between full and partial agonists (Table 3). The majority of compounds were not statistically significantly different from PGD_2 and were thus deemed full agonists, including the novel hDP-specific ligand L-644,698. In contrast, BW A868C [documented both as an antagonist (Giles *et al*, 1989) and a partial agonist (Hirata *et al*, 1994; Boie *et al*, 1995] was significantly different from PGD_2 ($p < 0.05$) since it reached only 68 ± 4% of PGD_2-mediated maximal stimulation, which is suggestive of partial agonist activity. The only other partial agonist identified was 15-deoxy-$\Delta^{12, 14}$-PGJ_2 ($p < 0.05$) with a value of 69 ± 5% as compared with the PGD_2 control.

Table 3. Potencies and efficacies for ligands at the human DP receptor.

	Ligand	*EC_{50} (nM)*	*% of maximal stimulation of cyclic AMP production*
	PGD_2 (4)	0.5 ± 0.1	86 ± 2
I	PGE_2	84 ± 1	91 ± 10
	$PGF_{2\alpha}$	264 ± 22	85 ± 10
	Iloprost	377 ± 13	82 ± 2
	U-46619	2903 ± 647	100 ± 12
II	PGJ_2	1.2 ± 0.1	87 ± 1
	Δ^{12}-PGJ_2 (4)	91 ± 18	118 ± 9
	15-deoxy-$\Delta^{12,14}$-PGJ_2	321 ± 15	* 69 ± 5
	13,14-dihydro-15-keto-PGD_2	5283 ± 944	78 ± 3
III	L-644,698	0.5 ± 0.1	86 ± 4
	BW245C	0.3 ± 0.1	90 ± 1
	BW A868C (4)	7.5 ± 0.9	* 68 ± 4
	ZK110841	0.2 ± 0.1	92 ± 1

EC_{50} values ± s.e.mean (nM) and maximal responses (percentage of maximally stimulated control values ± s.e.mean) are shown for prostanoids and synthetic prostanoid analogues (I), metabolites of PGD_2 (II), and synthetic prostanoid analogues specific for the DP receptor (III). Values are derived from 3 separate experiments unless otherwise noted in parentheses by the ligand name. Maximal stimulation was defined as the cyclic-AMP response produced in response to 1 μM PGD_2. An asterisk (*) indicates a value statistically significantly different ($p < 0.05$) from the maximal stimulation (100%).

Discussion

Characterization of a newly-developed hDP-HEK stable cell line has been performed in order to study DP receptor ligands. This characterization includes the radioligand binding properties of hDP determined using cell membrane preparations. In addition, cyclic AMP measurements with hDP-HEK cells have been performed as an index of cell signaling. These features are described below.

Slowly-hydrolyzable nucleotide analogues disrupt GPCR signaling by binding to the G-protein $G\alpha$ subunit (for review: Gilman, 1987; Neer, 1995). This promotes the sustained dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ subunits (Hanski *et al*, 1981; Sternweis *et al*, 1981) thereby conferring a low affinity state for agonist binding on the entire receptor population (Rojas & Birmbaumer, 1985; Gilman, 1987). In this study [^{3}H]PGD_2-specific binding to hDP was inhibited by GTPγS > ATPγS = GMP-PNP > AMP-PNP, indicating a marked difference in agonist affinities between the coupled and uncoupled states of the recombinant hDP receptor. The rank order of potency of the nucleotide analogues probably reflects their structural similarity to guanosine 5′-triphosphate (GTP).

Two populations of hDP-specific binding sites were identified by saturation analysis: a high affinity site of relatively low abundance and a low affinity site of relatively high abundance. Further support is lent to the two-site model with the suppression of the high affinity site in the presence of 100 μM GTPγS which allows detection of only the low affinity site. In addition, the calculated Hill coefficient values (n_H) from equilibrium competition radioligand binding assays ranged from 0.4 to 0.9, except for 15-deoxy-$\Delta^{12,14}$-PGJ_2 where n_H was equal to 0.1. This is indicative of more than one binding site since, classically, a single site of binding is indicated by $n_H = 1$.

Competition for [^{3}H]PGD_2-specific binding to the recombinant hDP receptor identified a novel ligand, L-644,698, with high affinity and selectivity for hDP. The overall rank order of affinities for the ligands tested was: L-644,698 = ZK 110841 = BW245C = PGD_2 = PGJ_2 ≥ BW A868C >> PGE_1 > Δ^{12}-PGJ_2 = PGE_2 > 15-deoxy-$\Delta^{12,14}$-PGJ_2 = Iloprost > $PGF_{2\alpha}$ >> U46619 > 13, 14-dihydro-15-keto-PGD_2. The selectivity of L-644,698 for the hDP receptor distinguishes it from these other compounds of equal

affinity. L-644,698 is comparable to BW245C in terms of its selectivity for hDP, but has a slightly different profile relative to the other prostanoid receptors which couple to stimulation of adenylate cyclase. Thus, L-644,698 and BW245C have equal affinities for hIP and hEP_2, but L-644,698 has a 100-fold lower affinity for hEP_4. The other DP agonists tested demonstrated much less selectivity.

L-644,698 is also a potent agonist in signal transduction assays which assessed cyclic AMP production. The following rank order of potency was observed: L-644,698 = ZK 110841 = BW245C = PGD_2 = PGJ_2 ≥ BW A868C >> Δ^{12}-PGJ_2 = PGE_2 > $PGF_{2\alpha}$ > 15-deoxy-$\Delta^{12,14}$-PGJ_2 = Iloprost >> U46619 > 13, 14-dihydro-15-keto-PGD_2. Due to its demonstrated potency and unique profile of selectivity L-644,698 should, therefore, be valuable in studying DP in heterologous systems.

Prostaglandin J_2 was also potent in its ability to stimulate cyclic AMP production and demonstrated full agonism in the recombinant hDP-HEK system. Previously PGJ_2 has demonstrated only partial agonist activity. The latter study was conducted *in vitro* using the DP receptor in human non-pregnant myometrium (Fernandes & Crankshaw, 1995). This discrepancy is probably attributable to the differences in receptor reserve between the two systems. PGJ_2 can be degraded in the presence of albumin, serum or plasma to its biologically active metabolite Δ^{12}-PGJ_2 (Mahmud *et al*, 1984). Δ^{12}-Prostaglandin J_2 was also shown to be a full agonist in this study and was approximately 100-fold less potent than PGJ_2 and 200-fold less potent than PGD_2. This is consistent with a previous study where Δ^{12}-PGJ_2 was shown to be 300-fold less potent than PGD_2 in mediating inhibition of ADP-induced human platelet aggregation (Narumiya & Toda, 1985). Prostaglandin J_2 and, less probably, Δ^{12}-PGJ_2 have the potential, therefore, to be active *in vivo* at the DP receptor depending on the local concentrations of these ligands.

BW A868C is a DP antagonist described as a hydantoin derivative, having a pK_B value when assayed against BW245C ranging from 8.3 at human myometrium (Senior *et al*, 1992; Senior *et al*, 1993) to 9.3 at human platelets (Giles *et al*, 1989; Trist *et al*, 1989; Barraclough *et al*, 1996). Similarly, at DP in human myometrium BW A868C behaved as an antagonist with a pK_B value of 7.3 against ZK 110841 (Fernandes & Crankshaw, 1995). In contrast, BW A868C behaved as a partial agonist in this recombinant hDP receptor system. Similar activity has been previously described using the cloned hDP

and mouse (m)DP receptors (Boie *et al*, 1995; Hirata *et al*, 1994). A shift from antagonist to partial agonist activity is usually explained by the low level of receptors in systems expressing endogenous receptors relative to the high receptor reserve obtained in recombinant expression systems. However, the stable cell line expressing mDP had a low receptor number (maximum number of specific binding sites of 93 fmol mg^{-1} membrane protein) which is less than many values observed for tissue preparations (Ito *et al*, 1989). Partial agonism associated with BW A868C is, therefore, observed under conditions of low and high expression of specific binding sites.

BW245C and ZK 110841 were 1.5- and 2.5-times as potent as PGD_2, respectively, in the recombinant hDP-HEK system. In independent studies BW245C was determined to be 3-times as potent as PGD_2 (Town *et al*, 1983) and PGD_2 was found to be 1.5-times as potent as ZK 110841 (Thierauch *et al*, 1988; Schulz *et al*, 1990) in inhibiting ADP-induced platelet aggregation. The values obtained in this study are, therefore, in good agreement with the previous data. Variations in reported values do exist, however, for platelet aggregation assays. For example, BW245C has been reported to be 8-fold more potent than PGD_2 (Whittle *et al*, 1983; Narumiya & Toda, 1985). In contrast to other reports in which washed platelets were used, in these, platelet-rich-plasma was employed where the plasma and the accompanying albumin would have the potential to metabolize PGD_2, as has been documented (Fitzpatrick & Wynalda, 1983). Studies addressing hDP-mediated inhibition of neutrophil activation are also in agreement with our data. ZK 110841 was 3-fold more potent than PGD_2 in inhibiting superoxide anion formation and equipotent with PGD_2 in inhibiting β-glucuronidase release in neutrophils (Ney & Schrör, 1991). Finally, investigations of PGD_2-mediated effects in the non-pregnant myometrium have documented a biphasic effect (Senior *et al*, 1992; Fernandes & Crankshaw, 1995), where the initial contractile phase occurs through PGD_2 activation of hFP and the secondary relaxatory phase results from PGD_2 activation of hDP. These results emphasize the importance of selectivity data in the prediction of *in vivo* ligand activity.

In conclusion, a novel DP-specific agonist, L-644,698, has been described. This synthetic prostanoid analogue is one of the most highly selective DP agonists discovered to date. In addition, the PGD_2 metabolites PGJ_2 and Δ^{12}-PGJ_2 have been shown to be

potent at hDP, suggesting that they may have physiological significance *in vivo*. Selectivity data have been presented for some currently accepted DP-specific agonists across the eight human prostanoid receptors cloned to date. Finally, the recombinant hDP receptor has been characterized with available pharmacological tools, both with respect to radioligand binding and signal transduction.

References

ABRAMOVITZ, M., BOIE, Y., NGUYEN, T., RUSHMORE, T.H., BAYNE, M.A., METTERS, K.M., SLIPETZ, D.M. & GRYGORCZYK, R. (1994). Cloning and expression of a cDNA for the human prostanoid FP receptor. *J. Biol. Chem.*, **269(4)**, 2632-2636.

ADAM, M., BOIE, Y., RUSHMORE, T.H., MULLER, G., BASTIEN, L., MCKEE, K.T., METTERS, K.M. & ABRAMOVITZ, M. (1994). Cloning and expression of three isoforms of the human EP_3 prostanoid receptor. *FEBS Lett.*, **338**, 170-174.

AN, S., YANG, J., XIA, M. & GOETZL, E.J. (1993). Cloning and expression of the EP_3-subtype of human receptors for prostaglandin E_2. *Biochem. Biophys. Res. Commun.*, **197**, 263-270.

ARMSTRONG, R.A., LAWRENCE, R.A., JONES, R.L., WILSON, N.H. & COLLIER, A. (1989). Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors. *Br. J. Pharmacol.*, **97**, 657-668.

BARRACLOUGH, P., BOLOFO, M. L., GILES, H., GILLAM, J., HARRIS, C. J., KELLY, M. G., LEFF, P., McNEILL, A., ROBERTSON, A. D., STEPNEY, R. J. & WHITTLE, B. J. R. (1996). Synthesis of hexahydrocyclopentimidazol-2-(1H)-one derivatives displaying selective DP receptor agonist properties. *Bioorg. Med. Chem.*, **4**, 81-90.

BASTIEN, L., SAWYER, N., GRYGORCZYK, R., METTERS, K.M. & ADAM, M. (1994). Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. *J. Biol. Chem.*, **269(16)**, 11873-11877.

BOIE, Y., RUSHMORE, T.H., DARMON-GOODWIN, A., GRYGORCZYK, R., SLIPETZ, D.M., METTERS, K.M. & ABRAMOVITZ, M. (1994). Cloning and expression of a cDNA for the human prostanoid IP receptor. *J. Biol. Chem.*, **269**, 12173-12178.

BOIE, Y., SAWYER, N., SLIPETZ, D.M., METTERS, K.M. & ABRAMOVITZ, M. (1995). Molecular cloning and characterization of the human prostanoid DP receptor. *J. Biol. Chem.*, **270**, 18910-18916.

BROCKWELL, M.A., CALDWELL, A.G., WHITTAKER, N. & BEGLEY, M.J. (1981). Heterocyclic prostaglandin analogues. Part 3. The relationship of configuration to biological activity for some hydantoin prostaglandin analogues. *J. Chem. Soc. Perkin Trans.*, **1**, 706-711.

BUNCE, K.T., CLAYTON, N.M., COLEMAN, R.A., COLLINGTON, E.W., FINCH, H., HUMPHREY, J.M., HUMPHREY, P.P.A., REEVES, J.J., SHELDRICK, R.L.G. & STABLES, R. (1990). GR63799X - a novel prostanoid with selectivity for EP_3 receptors. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, Vol. 21, ed. Samuelsson, B., Ramwell P.W., Paoletti, R., Falco, G. & Granstrom, E. 379-382. New York: Raven Press.

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I., AND LUMLEY, P. (1984). Prostanoid receptors - the development of a working classification. *Trends Pharmacol. Sci.*, **5**, 303-306.

COLEMAN, R.A., SMITH, W.L. & NARUMIYA, S. (1994). VIII. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.*, **46**, 205-229.

DONG, Y.J., JONES, R.L. & WILSON, N.H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br. J. Pharmacol.*, **87**, 97-107.

FELDMAN, H.A. (1972). Mathematical theory of complex ligand binding systems at equilibrium: methods of parameter fitting. *Anal. Biochem.*, **48**, 317-338.

FELGNER, P.L., GADEK, T.R., HOLM, M., ROMAN, R., CHAN, H.W., WENZ, M., NORTHROP, J.P., RINGOLD, G.M. & DANIELSEN, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci.*, **84**, 7413-7417.

FERNANDES, B. & CRANKSHAW, D. (1995). Functional characterization of the prostanoid DP receptor in human myometrium. *Eur. J. Pharmacol.*, **283**, 73-81.

FITZPATRICK, F. A. & WYNALDA, M. A. (1983). Albumin-catalyzed metabolism of prostaglandin D_2, identification of products formed in vitro. *J. Biol. Chem.*, **258**, 11713-11718.

FUNK, C.D., FURCI, L., FITZGERALD, G.A., GRYGORCZYK, R., ROCHETTE, C., BAYNE, M.A., ABRAMOVITZ, M., ADAM, M. & METTERS, K.M. (1993). Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *J. Biol. Chem.*, 268(35), 26767-26772.

GILES, H., LEFF, P., BOLOFO, M.L. KELLY, M.G. & ROBERTSON, A.D. (1989). The classification of prostaglandin DP receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br. J. Pharmacol.*, **96**, 291-300.

GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.*, **56**, 615-649.

HANSKI, E., STERNWEIS, P.C., NORTHUP, J.K., DROMERICK, A.W. & GILMAN, A.G. (1981). The regulatory component of adenylate cyclase. Purification and properties of the turkey erythrocyte protein. *J. Biol. Chem.*, **256**, 12911-12919.

HIRATA, M., HAYAISHI, Y., USHIKUBI, F.L., YOKOTA, Y., KAGEYAMA, R., NAKANISHI, S. & NARUMIYA, S. (1991). Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature*, **349**, 617-620.

HIRATA, M., KAKIZUKA, A., AIZAWA, M., USHIKUBI, F. & NARUMIYA, S. (1994). Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc. Natl. Acad. Sci.*, **91**, 11192-11196.

ITO, S., NARUMIYA, S. & HAYAISHI, O. (1989). Prostaglandin D_2: a biochemical perspective. *Prostaglandins Leukotrienes Essent. Fatty Acids*, **37**, 219-234.

KENNEDY, I., COLEMAN, R.A., HUMPHREY, P.P.A., LEVY, G.P. & LUMLEY, P. (1982). Studies on the characterization of prostanoid receptors: a proposed classification. *Prostaglandins*, **24**, 667-689.

KOTANI, M, TANAKA, I, OGAWA, Y, UNSUI, T. MORI, K, ICHIKAWA, A, NARUMIYA, S, YOSHIMI, T. & NAKAO, K. (1995). Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol.*, **48**, 869-879.

MAHMUD, I., SMITH, D.L., WHYTE, M.A., NELSON, J.T., CHO, D., TOKES, L.G., ALVAREZ, R. & WILLIS, A.L. (1984). On the identification and biological properties of prostaglandin J_2. *Prost. Leuk. Med.*, **16**, 131-146.

MATSUGI, T., KAGEYAMA, M., NISHIMURA, K., GILES, H. & SHIRASAWA, E. (1995). Selective prostaglandin D_2 receptor stimulation elicits ocular hypotensive effects in rabbits and cats. *Eur. J. Pharmacol.*, **275(3)**, 245- 250.

NARUMIYA, S. & TODA, N. (1985). Different responsiveness of prostaglandin D_2-sensitive systems to prostaglandin D_2 and its analogues. *Br. J. Pharmacol.*, **85**, 367-375.

NEER, E.J. (1995). Heterotrimeric G-proteins: organizers of transmembrane signals. *Cell.* **80**, 249-257.

NEY, P. & SHCRÖR, K. (1991). PGD_2 and its mimetic ZK 110.841 are potent inhibitors of receptor-mediated activation of human neutrophils. *Eicosanoids*, **4**, 21-28.

RANGACHARI, P.K., BETTI, P.A., PRIOR, E.T. & ROBERTS II, L.J. (1995). Effects of a selective DP receptor agonist (BW245C) and antagonist (BW A868C) on the canine colonic epithelium: an argument for a different DP receptor. *J. Pharmacol. Exp. Ther.*, **275**, 611-617.

RAYCHOWDHURY, M.K., YUKAWA, M, COLLINS, L.J., MCGRAIL, S.H., KENT, K.C. & WARE, J.C. (1995). Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J. Biol. Chem.*, **270**, 7011-7014.

REGAN, J.W., BAILY, T.J., PEPPERL, D.J., PIERCE, K.L., BOGARDUS, A.M., DONELLO, J.E., FAIRBAIRN, C.E., KEDZIE, K.M., WOODWARD, D.F. & GIL, D.W. (1994). Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. *Mol. Pharmacol.*, **46(2)**, 213-20.

ROJAS, F.J. & BIRMBAUMER, L. (1985). Regulation of glucagon receptor binding. Lack of effect of Mg and preferential role for GDP. *J. Biol. Chem.*, **260**, 7829-7835.

SCHMID, A, THIERAUCH, K.H., SCHLEUNING, W.D. & DINTER, H. (1995). Splice variants of the human EP_3 receptor for prostaglandin E_2. *Eur. J. Biochem.*, **228**, 23-30.

SCHULZ, B. G., BECKMAN, R., MÜLLER, B., SCHRÖDER, G., MAASS, B., THIERAUCH, K. -H., BUCHMANN, B., VERHALLEN, P. F. J. & FRÖHLICH, W. (1990). Cardio- and hemodynamic profile of selective PGD_2 analogues. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, Vol. 21, ed. Samuelsson, B., Ramwell P.W., Paoletti, R., Falco, G. & Granstrom, E. 591-594. New York: Raven Press.

SENIOR, J., SANGHA, R., BAXTER, G. S., MARSHALL, K. & CLAYTON, J. K. (1992). In vitro characterization of prostanoid FP-, DP-, IP- and TP-receptors on the non-pregnant human myometrium. *Br. J. Pharmacol.*, **107**, 215-221.

SENIOR, J., MARSHALL, K., SANGHA, R. & CLAYTON, J.K. (1993). In vitro characterization of prostanoid receptors on human myometrium at term pregnancy. *Br. J. Pharmacol.*, **108**, 501-506.

SHELDRICK, R.L.G., COLEMAN, R.A. & LUMLEY, P. (1988). Iloprost - a potent EP_1 and IP receptor agonist. *Br. J. Pharmacol.*, **94**, 334P.

STERNWEIS, P.C., NORTHUP, J.K., SMIGEL, M.D. & GILMAN, A.G. (1981). The regulatory component of adenylate cyclase. Purification and properties. *J. Biol. Chem.*, **256**, 11517-11526.

THIERAUCH, K.-H., STÜRZEBECHER, C.-St. & SCHILLINGER, E. (1988). Stable 9β- or 11α-halogen-15-cyclohexyl-prostaglandins with high affinity to the PGD_2 receptor. *Prostaglandins*, **35**, 855-868.

TOWN, M.-H., CASALS-STENZEL, J. & SCHILLENGER, E. (1983). Pharmacological and cardiovascular properties of a hydantoin derivative, BW245C, with high affinity and selectivity for PGD_2 receptor. *Prostaglandins*, **25**, 13-28.

TRIST, D.G., COLLINS, B.A., WOOD, J., KELLY, M.G. & ROBERTSON, A.D. (1989). The antagonism by BW A868C of PGD_2 and BW245C activation of human platelet adenylate cyclase. *Br. J. Pharmacol.*, **96(2)**, 301-306.

WHITTLE, B.J.R., MONCADA, S., MULLANE, K. & VANE, J.R. (1983). Platelet and cardiovascular activity of the hydantoin BW245C, a potent prostaglandin analogue. *Prostaglandins*, **25**, 205-223.

WOODWARD, D.F., HAWLEY, S.B., WILLIAMS, L.S., RALSTON, T.R., PROTZMAN, C.E., SPADA, C.S. & NIEVES, A.L. (1990). Studies on the ocular pharmacology of prostaglandin D_2. *Inv. Opthamol. Vis. Sci.*, **31**, 138-146.

5. MANUSCRIPT B

A novel biological role for prostaglandin D_2 is suggested by distribution studies of the rat DP prostanoid receptor

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DP, the receptor which preferentially interacts with PGD_2, is the least studied and understood of the prostanoid receptors. Thus, the limited distribution of the DP receptor has made it hard to characterize and it was the last known homologue of the human prostanoid receptors to be cloned. During cloning of both the murine and human DP receptors, DP-specific mRNA was observed in the small intestine and other GI tract tissues by Northern blot analyses of their mRNA distribution. A corresponding functional role for DP in these tissues has not yet been postulated. However, the cross-species similarity of this mRNA distribution suggests a similar role for DP in these tissues. The current work has extended this premise by investigating the cell-specific localization of mRNA corresponding to the rat homologue of rDP. These studies necessitated the cloning and functional expression of the rDP receptor. Prostanoids and prostanoid analogues, including L-644,698, demonstrated the expected rank orders of affinity and potency during pharmacological characterization of the functionally expressed protein, confirming its identity as the rat homologue of the DP receptor. This validated the use of the cDNA corresponding to rDP as a template from which to make rDP-specific riboprobes for *in situ* hybridization studies. rDP-specific mRNA was localized to the CNS (brain and spinal cord) and GI tract by the *in situ* hybridization technique. Within the stomach, duodenum, ileum, and colon of the GI tract, rDP-specific signals were observed repeatedly in the mucous-secreting goblet cells and, less often, in the adjacent epithelium. These findings suggest a novel biological role for the DP receptor, namely the regulation of mucin secretion.

A novel biological role for prostaglandin D_2 is suggested by distribution studies of the rat DP prostanoid receptor

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Summary

We report the cloning, functional expression and cell-specific localization of the rat homologue of the prostaglandin D_2 receptor (DP). *In situ* hybridization, utilizing multiple digoxigenin-labelled riboprobes and their complementary sense controls, was performed to determine the detailed distribution of DP receptor mRNA in the central nervous system and the gastrointestinal tract. Within the brain, the leptomeninges and choroid plexus expressed DP receptor mRNA. Transcripts detected in the spinal cord were localized to the sensory and motor neurons of the dorsal and ventral horns, respectively, suggesting a role for the DP receptor in the modulation of central nervous system processes, including pain transmission. Within the gastrointestinal tract (stomach, duodenum, ileum and colon) signals were highly localized to the mucous-secreting goblet cells and the columnar epithelium. These findings suggest a novel biological role for prostaglandin D_2-mediated activity at the DP receptor, namely mucous secretion. In addition, radioligand binding assays (saturation analyses and equilibrium competition assays) and functional assays (measuring cyclic AMP accumulation) were performed to characterize the recombinant rat DP receptor expressed in human embryonic kidney (HEK) 293(EBNA) cells. A single site of binding (K_D=14 nM, B_{max}=115 fmol/mg protein) was measured for prostaglandin D_2-specific binding to the rat DP receptor. Prostaglandin D_2 proved to be a potent agonist at the rat DP receptor (EC_{50}=5 nM). The rank order of efficacy for DP-specific agonists [prostaglandin D_2 = prostaglandin J_2 = BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)) > L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate)] reflected the affinity with which the ligands bound to the receptor.

Introduction

The eicosanoids referred to as the primary bioactive prostanoids encompass prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostacyclin (prostaglandin I_2) and thromboxane A_2. They act through eight individual prostanoid receptors DP, EP (EP_1, EP_2, EP_3, EP_4), FP, IP and TP (for review, Coleman et al., 1994) which form a distinct sub-family within the rhodopsin-type G-protein coupled receptor superfamily. To date, the rat prostanoid receptors EP_1 (Boie *et al.*, 1997), EP_2 (Boie *et al.*, 1997), $EP_{3\alpha}$ (Boie *et al.*, 1997), EP_4 (Boie *et al.*, 1997), FP (Lake *et al.*, 1994), IP (Sasaki *et al.*, 1994), and TP (Kitanaka *et al.*, 1995) have been cloned. In this report we describe the cloning, functional expression and cell-specific localization of the rat DP prostanoid receptor.

Prostaglandin D_2 is the prostanoid interacting preferentially with the DP receptor. It is generated from arachidonic acid in a number of tissues by the sequential actions of prostaglandin H synthase and prostaglandin D synthase, and is associated with both central and peripheral physiological effects. Within the central nervous system, prostaglandin D_2 has been associated with sleep induction, modulation of body temperature, olfactory function, hormone release, nociception and neuromodulation. Peripherally, prostaglandin D_2 has been shown to mediate vasodilation, inhibition of platelet aggregation, glycogenolysis, bronchoconstriction and vasoconstriction (for review Hayaishi, 1988; Ito *et al.*, 1989). As well, prostaglandin D_2 is the major prostanoid generated by mast cells upon immunological challenge (Roberts et al., 1980). However, several of these effects may be due to interaction of prostaglandin D_2 with prostanoid receptors other than the DP receptor (e.g. bronchoconstriction potentially occurs via the FP receptor) (Liston and Roberts, 1985).

The cloning and characterization of the human (Boie et al., 1995) and mouse (Hirata et al., 1994) DP receptor homologues has previously been reported and limited distribution data described. In both cases, the detection of DP receptor mRNA transcripts in various tissues by northern blot analysis was presented. For the human DP receptor, nineteen tissues were investigated (brain, retina, heart, aorta, lung, liver, stomach, small intestine, pancreas, kidney, adrenal, spleen, thymus, mammary gland, placenta, uterus, ovary, testis and skeletal muscle) and only the retina and small intestine were positive for

human DP receptor mRNA. In the case of the mouse DP receptor, ten tissues were probed (brain, thymus, lung, heart, spleen, stomach, liver, ileum, kidney and uterus) and positive signals were identified in ileum, lung, stomach and uterus. Although species variation with respect to DP receptor-mediated activity is well-established (Giles and Leff, 1988, and references within), much of the observed biological activity of prostaglandin D_2 cannot be accounted for by the reported distribution of the DP receptor. In order to address the link between prostaglandin D_2-stimulated and DP receptor-mediated activities, the rat homologue of the DP receptor has been used to study the cell-specific localization of rat DP receptor-specific mRNA transcripts as identified by *in situ* hybridization in selected tissues.

Materials and methods

Cloning of the rat DP prostanoid receptor

A DNA probe (rDPTMD2-5) of size 416 bp was amplified from a rat genomic DNA library by polymerase chain reaction (PCR) using the primers xDP-fTMD2 (5′ ACG GTC ACC GAC TTG CTG GGC AAG N 3′) and xDP-rTMD5 (5′ CAT GAG GCT GGA GTA GAG CAC AGA N 3′). These DP receptor-specific primers, based on sequences conserved between the mouse (Hirata et al., 1994) and human (Boie et al., 1995) homologues, correspond to regions within the second and fifth transmembrane domains, respectively. The PCR conditions were: initial denaturation at 95 °C, 2 min; followed by 35 cycles of denaturation at 95 °C, 15 s; then annealing and extension at 60 °C, 30 s; and then a final extension at 72 °C, 10 min (GeneAmp 9600 system; Perkin-Elmer/Applied Biosystems, Foster City, CA).

A rat spinal cord cDNA library (1.0 - 1.2 x 10^6 plaques) (Stratagene, La Jolla, CA) was screened with the rDPTMD2-5 cDNA probe which was random-labelled with [α-^{32}P]cytidine 5′-triphosphate (NEN, Mississauga, ON.). Three positive clones were obtained and their cDNA inserts were subcloned into bluescript pKS (Stratagene, La Jolla, CA) at the *Eco*R I site and sequenced (ABI-373 Stretch automated sequencer; Perkin-Elmer/Applied Biosystems). Two independent clones were used to construct the open reading frame (ORF) of the rat DP receptor: 10-2-I which spanned from the 5' untranslated region to a position 10 residues 3' of the first in-frame stop codon, and 24-1-II which extended from the cDNA corresponding to the first transmembrane domain to the 3' untranslated region. The complete cDNA for the receptor (pKS-rDP) was constructed by ligation of the 5′ end of 10-2-I and the 3′ end of 24-1-II at a unique *Vgo*M I site which occurs at a position 150 bp from the start codon.

Construction of the FLAG-epitope tagged rat DP and human DP receptors

The 5′-FLAG-epitope tagged rat DP receptor was generated by first amplifying by PCR the 10-2-I clone using the primers FLAGrDPf72 (5′ ACT AAG CTT ACC ATG GAC

TAC AAG GAC GAC GAT GAC AAG TGG CAA GGA CTA GCT GCG AAT GAG TCC TAT CGC 3′) and xDP-rTMD5, to generate DNA corresponding to the FLAG-epitope tagged 5′ terminus of the rat DP receptor. The PCR protocol used was as described above. The amplified DNA was digested using *Hin*d III and *Ngo*M I. The digested fragment was then ligated into the 24-1-II clone, which had previously been digested with *Hin*d III and *Ngo*M I, to produce the full length 5′-FLAG-epitope tagged rat DP receptor in bluescript pKS (pKS-FLAG-rDP). The 5′-FLAG-epitope tagged human DP receptor was generated by first amplifying by PCR human DP receptor cDNA in pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) (pcDNA3-hDP; described previously by Boie et al., 1995) using the primers FLAGhDPf72 (5′ AAG CTT ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG CTG CTC CCG CAC GCC GCG AAG TCG CCG TTC TAC 3′) and hDP-m22 (5′ CTT GGC ACA ACG AGT TGT CCA A 3′), as described above. The amplified DNA was digested at a *Hin*d III site which was engineered into the FLAGhDPf72 primer, as well as at the *Bst*E II site 207 bp from the start codon. The digested fragment was then ligated into pcDNA3-hDP, digested with *Hin*d III and *Bst*E II, to produce the full length 5′-FLAG-epitope tagged human DP receptor in pcDNA3 (pcDNA3-FLAG-hDP).

Construction of pCEP4-rDP, pCEP4-FLAG-rDP and pCEP4-FLAG-hDP mammalian expression vectors

Digestion of both pKS-rDP and pKS-FLAG-rDP with *Hin*d III and *Not* I released their respective fragments from pKS, which were subsequently subcloned into pCEP4 (Invitrogen) at the same restriction sites employed for construction of the pCEP4-rDP and pCEP4-FLAG-rDP expression vectors. Digestion of pcDNA3-FLAG-hDP with *Hin*d III and *Xho* I released FLAG-human DP from pcDNA3, which was subsequently subcloned in pCEP4 at the same restriction sites for construction of the pCEP4-FLAG-hDP expression vector.

pCEP4-rDP, pCEP4-FLAG-rDP and pCEP4-FLAG-hDP bulk stable expression in HEK 293(EBNA) cells, cell culture and membrane preparation

Transfection of pCEP4-rDP, pCEP4-FLAG-rDP and pCEP4-FLAG-hDP plasmids into HEK 293(EBNA) for bulk stable expression of the rat DP, FLAG-rat DP and FLAG-human DP receptor constructs was achieved as previously described (Wright *et al.*, 1998). Cells were maintained in culture for 48 h post transfection and then grown in the presence of 200 μg/ml hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) for two weeks, to select for resistant colonies expressing the rat DP, FLAG-rat DP or the FLAG-human DP receptors. Maintenance of HEK 293(EBNA) cells in culture was as previously described (Wright *et al.*, 1998) for subsequent analyses by radioligand binding assays, immunoblot and cyclic AMP accumulation assays.

In order to prepare membranes from HEK 293(EBNA) cells (all procedures at 4 °C), they were first resuspended in the presence of protease inhibitors (2mM phenylmethylsulfonylfluoride, 10 μM E-64, 100 μM leupeptin and 0.05 mg/ml pepstatin) by Dounce homogenization (pestle B, 10 strokes). Cells were next disrupted by nitrogen-cavitation at 800 psi for 30 min on ice. The resulting cell suspension was subjected to two centrifugation steps: 1000 x g_{max} for 10 min followed by 100 000 x g_{max} for 30 min. The resulting pellet was resuspended to 1/10th the original volume in 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA (tetrasodium salt) by Dounce homogenization (pestle A, 10 strokes), and aliquots were quickly frozen in liquid nitrogen and then stored at –80 °C at a protein concentration of 8-10 mg/ml.

[^{3}H]prostaglandin D_2 binding to FLAG-rat DP and FLAG-human DP receptors expressed in HEK 293(EBNA) cell membranes

Radioligand binding assays were carried out as previously described (Wright et al., 1998). Briefly, assays were performed in 0.2 ml of 10 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 8 nM [^{3}H]prostaglandin D_2 (115 Ci/mmol) and 10 mM $MnCl_2$. Compounds were added in dimethylsulphoxide (Me_2SO) at 1% (v/v) of the final incubation volume (vehicle concentration was constant throughout). The reaction was initiated by the

addition of 200 µg (FLAG-rat DP receptor) or 50 µg (FLAG-human DP receptor) cell membrane protein to all tubes and the samples were incubated at room temperature for 1 h. The reaction was terminated by rapid filtration at 4 °C in 4 ml of 10 mM HEPES-KOH (pH 7.4) through GF/C filters (Brandel) which had been presoaked in the same buffer. The residual [^{3}H]prostaglandin D_2 bound to the filter was determined in 5 ml per well of Ultima Gold (Packard, Meriden, CT, U.S.A.) scintillation cocktail (59% efficiency). Non-specific binding was determined in the presence 1 µM prostaglandin D_2. Analysis of [^{3}H]prostaglandin D_2 binding was performed as previously described (Wright et al., 1998). In particular, sigmoidal curves from equilibrium competition assays were analyzed by proprietary software custom designed, based on the work described in Kenakin (1997), which employs a non-linear least-squares fitting routine based on the four parameter logistic equation: $y=(m1-m2)(1+(m0/m3)e^{m4})^{-1}+m2$; where m1 and m2 represent the maximum and minimum of the curve, m3 represents the inflection point (IP), m4 represents the slope of the curve at the IP, m0 represents the concentration of the competing ligand and y represents the % [^{3}H]prostaglandin D_2-specific binding. K_i values were calculated from the equation $K_i=IP/1+[radioligand]/K_D$ (Cheng and Prusoff, 1973). The integrity of the radioligand was maintained throughout the incubation in the presence of HEK 293(EBNA) membranes.

Cyclic AMP assays with FLAG-rat DP and FLAG-human DP receptor-expressing HEK 293(EBNA) cells

Assays of cyclic AMP accumulation were carried out as previously described (Wright et al., 1998). Briefly, FLAG-rat DP or FLAG-human DP receptor-expressing HEK 293(EBNA) cells were harvested at 80% confluence by resuspension in 10 ml of enzyme-free cell dissociation buffer and washed in phosphate-buffered saline (PBS) by centrifugation (300 x g_{max}, 6 min, room temperature). Cells were then washed in 10 ml of Hank's balanced salt solution (HBSS) by centrifugation under the same conditions as above and resuspended in HBSS at 4 x 10^6 cells/ml. Cell viability was determined to be >95% by trypan blue exclusion. The generation of cyclic AMP was performed in a final incubation volume of 0.2 ml of HBSS containing 100 µM Ro 20-1724 to abrogate cyclic

AMP hydrolysis. Compounds were added in Me_2SO, kept constant at 1% (v/v) of the final incubation volume. The reaction was initiated by the addition of 2×10^5 cells per incubation and the samples were incubated for 10 min at 37 °C with shaking. Reactions were halted by the incubation of samples in boiling water for 3 min and cyclic AMP was subsequently measured by use of a [^{125}I]-cyclic AMP scintillation proximity assay (Albano *et al.*, 1974). Sigmoidal concentration-response curves were analyzed by custom designed software to determine EC_{50} values. Maximal stimulation was defined as the quantity of cyclic AMP produced by incubation of a given receptor construct (either the FLAG-rat DP or FLAG-human DP receptor) with 1 μM prostaglandin D_2.

Protein assays

Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.) using bovine serum albumin as the standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Samples (66 μg of protein) from the 100 000 x g_{max} membrane fraction were solubilized in 10 μl of 2% (w/v) SDS with shaking for 1 h at room temperature. Samples were subsequently diluted 10-fold in 10 mM $NaPO_4$ (pH 7.4) containing 10 mM EDTA, 1% (v/v) β-mercaptoethanol, 1% (w/v) n-octylglucoside and 2 mM phenylmethylsulfonylfluoride, and treated with N-glycosidase F (0.01 U/μg protein) (Boehringer Mannheim) with shaking for 1 h at room temperature. Samples (33 μg of protein equivalent) were diluted 3:1 (v/v) in SDS-PAGE sample buffer (Laemmli, 1970) and subjected to SDS-PAGE on a 10% gel (Novel Experimental Technology, San Diego, CA, U.S.A.), and transferred onto nitrocellulose by electroblotting. Samples were not boiled prior to SDS-PAGE because previous experiments in this laboratory and others (Herberg *et al.*, 1984) have shown that heating samples results in subsequent aggregation and retention in the stacking gel during electrophoresis. Immunoreactive proteins were detected using a primary anti-FLAG m2 monoclonal antibody (IBI) and a secondary

sheep anti-mouse immunoglobulin linked to horseradish peroxidase (Kodak). FLAG-bacterial alkaline phosphatase (IBI) was used as a positive control. Immunoreactivity was detected with the Renaissance chemiluminescence kit (NEN).

In situ hybridization: tissue preparation and storage

All procedures were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research in accordance with the guidelines established by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 200-250 g were administered a lethal injection of sodium pentobarbital (85-100 mg/kg, intraperitoneally). Transcardial infusion of 0.9%-sodium chloride was followed by perfusion with 4.0% paraformaldehyde in cold 0.1 M PBS (pH 7.3). Tissues were removed immediately and processed using standard procedures for paraffin-embedding. Tissue blocks were cut into 4 μM thick sections and mounted on aminoalkylsilane-coated microscope slides. All solutions and glassware were pre-treated to destroy nuclease activity.

In situ hybridization: preparation of cRNA probes

PCR was used to amplify three non-overlapping sequences of 300–400 bp found within the rat DP receptor using the following primer pairs: Probe 1: rDP-ATG (5′ ATG AAT GAG TCC TAT CGC TGT CAG GCA 3′) and rDP-ri2 (5′ CAG AGA CAG CCA GCA CTC TAG TG 3′); Probe 2: rDP-fi2 (5′ CAC TAG AGT GCT GGC TGT CTC TG 3′) and rDP-re3 (5′ CAT GCC TGT AGT CTG AGC CTG ACT G 3′); and Probe 3: rDP-fe3 (5′ AGT CAG GCT CAG ACT ACA GGC ATG 3′) and rDP-TGA (5′ AGC CCT CAC AAA GTG GAT TCC ATG T 3′). Probes 1-3 were cloned into pCR2.0 (Invitrogen) which contains a SP6 polymerase binding site and a T7 polymerase binding site flanking the multiple cloning site at the 5′ and 3′ termini, respectively. pCR2.0-rDP1, pCR2.0-rDP2, and pCR2.0-rDP3 were generated using the TA cloning kit according to the manufacturers instructions. Probes 1-3 in pCR2.0 were digested at the multiple cloning site at either the *Xho* I site (5′ of the cloned insert) or the *Spe* I site (3′ of the cloned insert). The linearized probes were then treated with proteinase K (0.2 μg/μg

DNA) at 37 °C for 45 min, followed by phenol/chloroform extraction and ethanol precipitation. The DNA was subsequently resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O and the concentration determined by UV spectrophotometry. Digoxigenin-labelled cRNA riboprobes were prepared by transcription of 1 µg of linearized probe template DNA with SP6 or T7 polymerases in the presence of digoxigenin-UTP using an RNA labelling kit (Boehringer Mannheim). Following the labelling reaction the samples were treated with 20 units of Dnase I to remove the DNA template prior to storage of the digoxigenin-labelled riboprobes in hybridization buffer [75% (v/v) formamide, 15% (v/v) 20x NaCl, sodium citrate (SSC) buffer, 2% (v/v) 50x Denhardt's, 4% yeast tRNA (5 mg/ml), 5% 1 M $NaPO_4$ (pH 7.4), 10% (w/v) dextran sulfate] at –20 °C.

In situ hybridization: reaction

All procedures were performed at room temperature unless otherwise noted. Glass slides with tissue slices were treated as follows: xylene [3 x 15 min], graded ethanol (100% ethanol: 2 x 10 min; 95%, 80%, 70%, 50% ethanol in DEPC-H_2O, each 5 min) and 10 mM PBS, pH 7.4 (2 x 10 min). The tissues were then treated with 0.1 µg/ml proteinase K in 100 mM Tris-HCl pH 8 (10 min, 37 °C), washed sequentially in DEPC-H_2O (5 min), 0.1 M triethanolamine pH 8 (5 min), 0.25% (v/v) fresh acetic anhydride in triethanolamine (10 min), 2x SSC (5 min), then briefly dipped in graded ethanol (50%, 70%, 80%, 90%, 100%, 100% ethanol) and air dried. The tissues were then incubated with the digoxigenin-labelled probes in hybridization buffer (30 µl per slide) covered with Parafilm in a chamber humidified with 75% (v/v) formamide solution in H_2O at 56-57 °C for 16 h. The concentrations of the digoxigenin-labelled antisense and sense probes added to tissues were standardized by comparison on a dot blot prior to each experiment. Parafilm coverslips were removed by dipping slides in 2x SSC. The tissues were then treated with 40 µg/ml RNase A (Boehringer Mannheim) (45 min), and washed as follows: 2x SSC (10 min), 1x SSC (10 min), 0.5x SSC (10 min), 0.1x SSC (60 °C, 45 min). Digoxigenin-labelling was then detected by immunochemistry at room temperature in a H_2O-humified chamber. Non-specific antibody sites were blocked by incubation

with Buffer 1 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin for 30 min. The tissues were incubated with Immunopure peroxidase suppressor (Pierce) for 30 min and washed in Buffer 1 (3 x 10 min). The slides were incubated with horseradish peroxidase-linked anti-digoxigenin antibody (Boehringer Mannheim) (1:75 in Buffer 1 containing 3% BSA) for 2 h and then washed in Buffer 1 (3 x 5 min), and positive labelling was visualized by incubation with diaminobenzidine (DAB) in substrate buffer (Pierce) for 15-45 min. The substrate reaction was stopped by overnight incubation in 10 mM Tris-HCl, 1 mM Na_2EDTA buffer, pH 8. Tissues were counterstained with Gill's hematoxylin (Fisher Scientific) and mounted using aqueous mounting media (Immunon).

Reagents

Prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$), prostaglandin J_2 and Ro 20-1724 (4-(3 butoxy-4-methoxybenzyl)-2-imidaxlidinone) were from Biomol Research Laboratories (Plymouth Meeting, PA, USA). BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)) was a generous gift from The Wellcome Foundation Ltd (Beckenham, Kent, U.K.). L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate) was synthesized at Merck Research Laboratories by Dr. J.B. Bicking. Iloprost (5-(hexahydro-5-hydroxy-4-(3-hydroxy-4-methyl-1-octen-6-ynyl)-2(1H)-pentalenylidene) pentanoic acid) and [^{125}I]-cyclic AMP scintillation proximity assay kits were from Amersham (Oakville, ON, Canada). [^{3}H]prostaglandin D_2 was from Dupont NEN (Boston, MA, U.S.A.).

Results

Cloning of the rat DP receptor

The rat DP prostanoid receptor open reading frame (ORF) was constructed as described in the Methods. The ORF consists of 1074 nucleotides which encode a polypeptide of 357 amino acids with a predicted molecular mass of 39 800. There are two potential N-glycosylation sites at positions 2 and 89, four potential protein kinase C phosphorylation sites at residues 47, 144, 239 and 325, and five protein kinase A consensus phosphorylation sites at positions 144, 239, 245, 253 and 325. The rat DP receptor is 92% and 78% identical at the nucleic acid level, falling to 90% and 73% at the amino acid level, to the mouse and human homologues, respectively (Figure 1).

Immunodetection of FLAG-rat DP and FLAG-human DP receptors expressed in HEK 293(EBNA) cell membranes

Membranes from HEK 293(EBNA) cells expressing rat DP, FLAG-rat DP, and FLAG-human DP receptors were solubilized, deglycosylated, and then analysed by SDS-PAGE followed by immunoblotting as described in the Methods (Figure 2). For both the FLAG-rat DP and FLAG-human DP receptors (lanes 1 and 3, respectively), a major immunoreactive species was detected at a molecular mass of approximately 36 000, close to that predicted for the DP receptor. This probably represents the DP receptor because this band was not detected in lane 2 containing the recombinant rat DP receptor without the FLAG epitope. In addition, several less prominent higher molecular weight forms of immunoreactive material were detected, including species migrating at approximately 72 kDa, double the molecular weight of the DP receptor. These type of SDS-resistant receptor dimers have been observed for a number of G-protein coupled receptors including D2 dopamine receptors (Ng et al., 1996), β_2-adrenoceptors (Hebert et al., 1996)

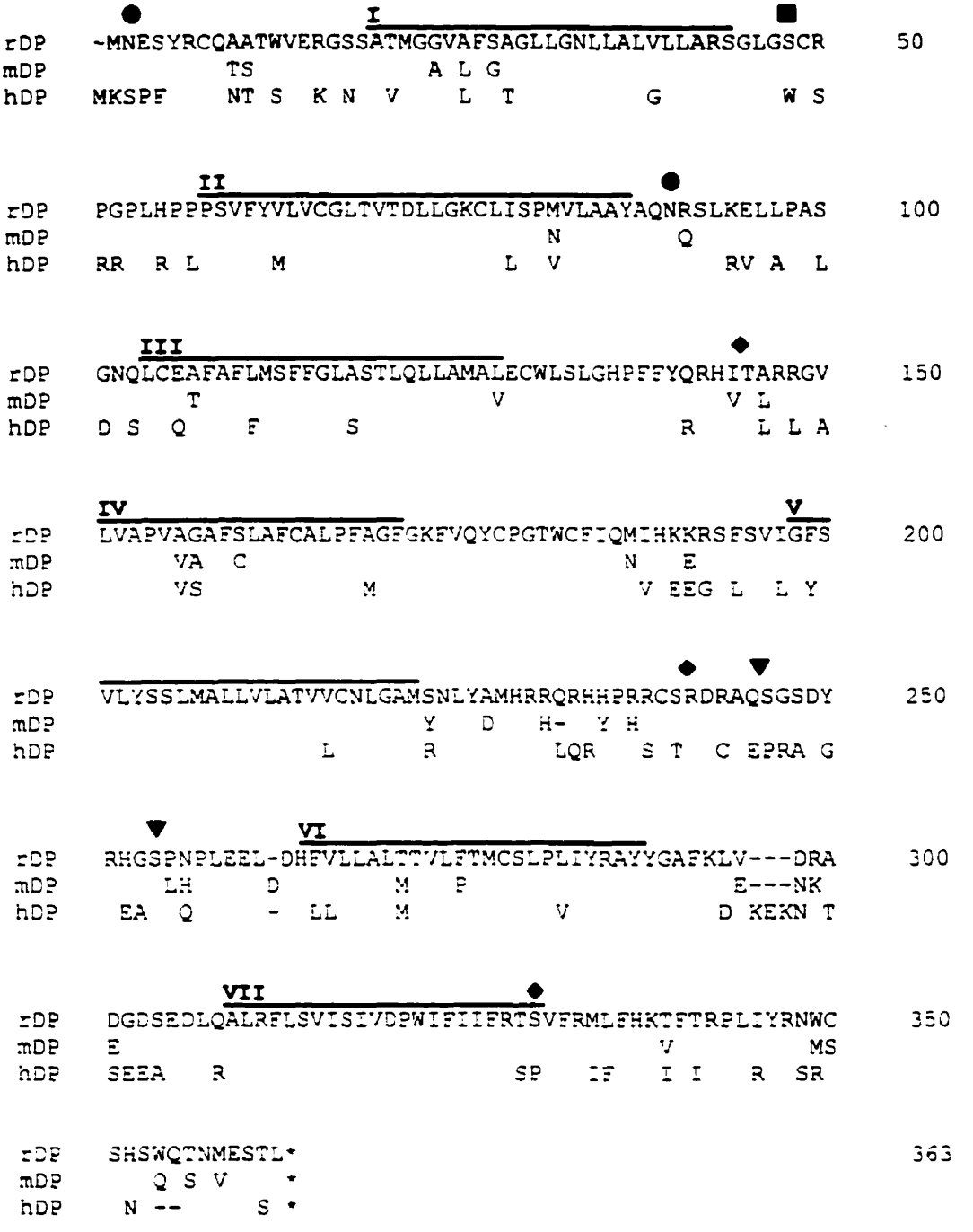

Figure 1. Amino acid sequence alignments of the rat, mouse and human DP receptors. Alignments were performed using GCG Wisconsin DNA software. Shown are the amino acids which are not conserved in the human DP and mouse DP receptors compared to the rat DP receptor. DNA sequences corresponding to transmembrane domains I-VII are indicated by overlines. Gaps in the sequences to facilitate alignment are indicated by dashes, and the termination codon by an asterisk. Consensus sites within the rat DP receptor are indicated above the alignment for N-glycosylation (●), protein kinase C (■), protein kinase

A (▼), and both protein kinase C and protein kinase A (◆).Two residues varied between our predicted amino acid sequence and a previously published sequence submitted to GenBank under accession number U92289. The current study identified aspartate instead of histidine at position 71, and threonine instead of alanine at position 338. The nucleotide and peptide sequences identified for the rat DP receptor have been submitted to the GenBank™/EMBL Data Bank with accession number AF120101.

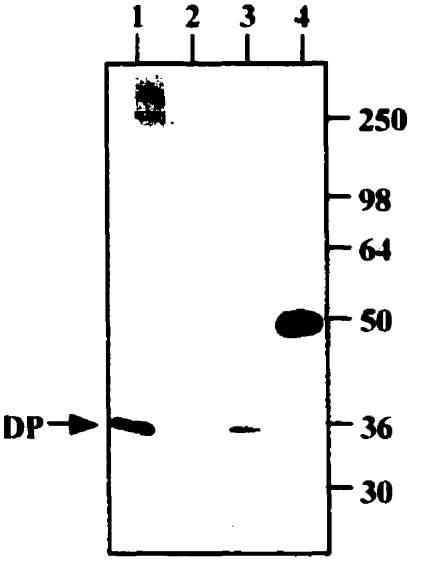

Figure 2. Immunoblot of FLAG epitope-tagged rat and human homologues of the DP receptor expressed in HEK 293(EBNA) cell membranes using the anti-FLAG m2 antibody. Lane 1: FLAG-rat DP receptor; Lane 2: rat DP receptor; Lane 3: FLAG-human DP receptor; Lane 4: FLAG-bacterial alkaline phosphatase (FLAG-BAP) (Mr = 55 000). Cell membranes were solubilized, treated with N-glycosidase F, analyzed by SDS-PAGE, transferred to nitrocellulose and immunoblotted as described under Methods. Lanes were loaded with 33 μg of original membrane protein except for lane 4 where 25 μg of FLAG-BAP was used.

and δ-opioid receptors (Cvejic et al., 1997). Although there are reports that these receptor dimers are physiologically relevant, this remains to be definitely established

(Hebert et al., 1996; Cvejic et al., 1997). FLAG-bacterial alkaline phosphatase (molecular weight of 55 kDa) (lane 4, positive control) was also detected by the anti-FLAG m2 antibody. Membranes prepared from pCEP4-transfected HEK 293(EBNA) cells have also been analyzed by Western blot and did not demonstrate any immunoreactive species (Deborah Slipetz, personal communication).

Expression of the FLAG-rat DP receptor in HEK 293(EBNA) cells: receptor binding studies

The affinity (K_D) of prostaglandin D_2 for the expressed DP receptors and the maximum number of detectable prostaglandin D_2-specific binding sites (B_{max}) were identified by saturation analysis (data not shown). Specific binding of [^{3}H]prostaglandin D_2 to the FLAG-rat DP receptor was best described by a single site model with a K_D value of 14 nM and a B_{max} value of 115 fmol/mg protein. Similarly, [^{3}H]prostaglandin D_2-specific binding to the FLAG-human DP receptor was also fitted most appropriately by a one-site model with a K_D value of 3.8 nM and a B_{max} value of 84 fmol/mg protein.

Equilibrium competition binding assays employing [^{3}H]prostaglandin D_2 were performed to determine the affinities of prostanoids and related analogues at both rat and human DP receptors (Table 1). Prostaglandin D_2 and BW245C had K_i values of 25 nM and 15 nM, respectively, at the FLAG-rat DP receptor. The FLAG-rat DP receptor had lower affinity for L-644,698, however, with a K_i value of 173 nM, while prostaglandin E_2, prostaglandin $F_{2\alpha}$, iloprost and U46619 all had K_i values greater than 1 μM. Comparable results were obtained upon challenge of the non-epitope tagged rat DP receptor (data not shown). In contrast, prostaglandin D_2 and its analogue BW245C bound to the FLAG-human DP receptor with K_i values of 2.8 nM and 3.8 nM, respectively. In particular, the DP receptor-specific agonist L-644,698 had a 350-fold higher affinity for the FLAG-human DP receptor as compared with the FLAG-rat DP receptor. Again, prostaglandin E_2, prostaglandin $F_{2\alpha}$, iloprost and U46619 all demonstrated K_i values greater than 1 μM at the FLAG-human DP receptor. There was no binding of [^{3}H]prostaglandin D_2 to membranes generated from non-transfected HEK 293(EBNA) wild type cells (data not shown).

Table 1. Inhibitor constants for competing ligands at HEK 293(EBNA) cell membranes expressing either FLAG-rat DP or FLAG-human DP receptors.

Ligand	K_i (nM)	
	FLAG-rat DP receptor	FLAG-human DP receptor
prostaglandin D_2	25	2.8
L-644,698	173	0.5
BW245C	15	3.8
prostaglandin E_2	>1000	>1000
prostaglandin $F_{2\alpha}$	>1000	>1000
Iloprost	>1000	>1000
U46619	>1000	>1000

Inhibitor constant (K_i) values are shown for prostanoids and synthetic prostanoid analogues. Values were derived from a single experiment.

Expression of the FLAG-rat DP receptor in HEK 293(EBNA) cells: functional assays - cyclic AMP generation

Second messenger assays monitoring the predominant signaling pathway for the DP receptor, the stimulation of cyclic AMP production by adenylate cyclase, were performed on both the FLAG-rat DP and FLAG-human DP receptors (Figure 3 and Table 2). In general, the rank order of efficacies followed the rank order of affinities determined in the competition radioligand binding assays. Prostaglandin J_2, a metabolite of prostaglandin D_2, was also tested since it has previously demonstrated affinity and efficacy equal to the parent compound at the human DP receptor (Wright et al., 1998). The DP receptor-specific ligand BW245C had the highest efficacy at the FLAG-rat DP receptor with an EC_{50} value of 1.9 nM. Prostaglandin D_2 and prostaglandin J_2 were equipotent with EC_{50} values of 4.8 and 6.9 nM. Once again, L-644,698 had reduced efficacy compared to the other DP receptor-specific agonists at this receptor, with an EC_{50} value of 32 nM. Of the other ligands tested, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were markedly less potent while iloprost and U46619 were silent at this receptor (Figure 3A,B and Table 2). Similar results were obtained upon challenge of cells expressing the non-epitope tagged rat DP receptor (data not shown). In comparison, the profile of efficacy at the FLAG-human DP receptor was as expected, with the DP receptor-specific ligands prostaglandin D_2, prostaglandin J_2, BW245C and, in particular L-644,698, all demonstrating marked efficacies with EC_{50} values below 10 nM. Again, the other ligands tested (prostaglandin E_2, prostaglandin $F_{2\alpha}$, iloprost and U46619) were less efficient at this receptor (Figure 3C, D and Table 2). There was no cyclic AMP accumulation above the level obtained with vehicle in non-transfected HEK 293(EBNA) wild type cells or vector-transfected HEK 293(EBNA) cells (data not shown).

Maximal responses for each ligand (the cyclic AMP produced at a concentration of 1 μM) at the FLAG-rat DP and FLAG-human DP receptors were normalized to those elicited by 1 μM prostaglandin D_2 (Table 2). The DP receptor-specific ligands (prostaglandin D_2, prostaglandin J_2, L-644,698 and BW245C) responded as full agonists at the rat DP receptor, with normalized maximal responses all above 80%. In fact BW245C was a very efficient agonist with a maximal response of 130% compared to

prostaglandin D_2. Similar results were obtained for the FLAG-human DP receptor except that the maximal stimulation with BW245C was comparable to prostaglandin D_2. The maximal production of cyclic AMP produced with 1μM of prostaglandin D_2 was 30-40 pmol/well for the FLAG-rat DP receptor and 80 pmol/well for the FLAG-human DP receptor.

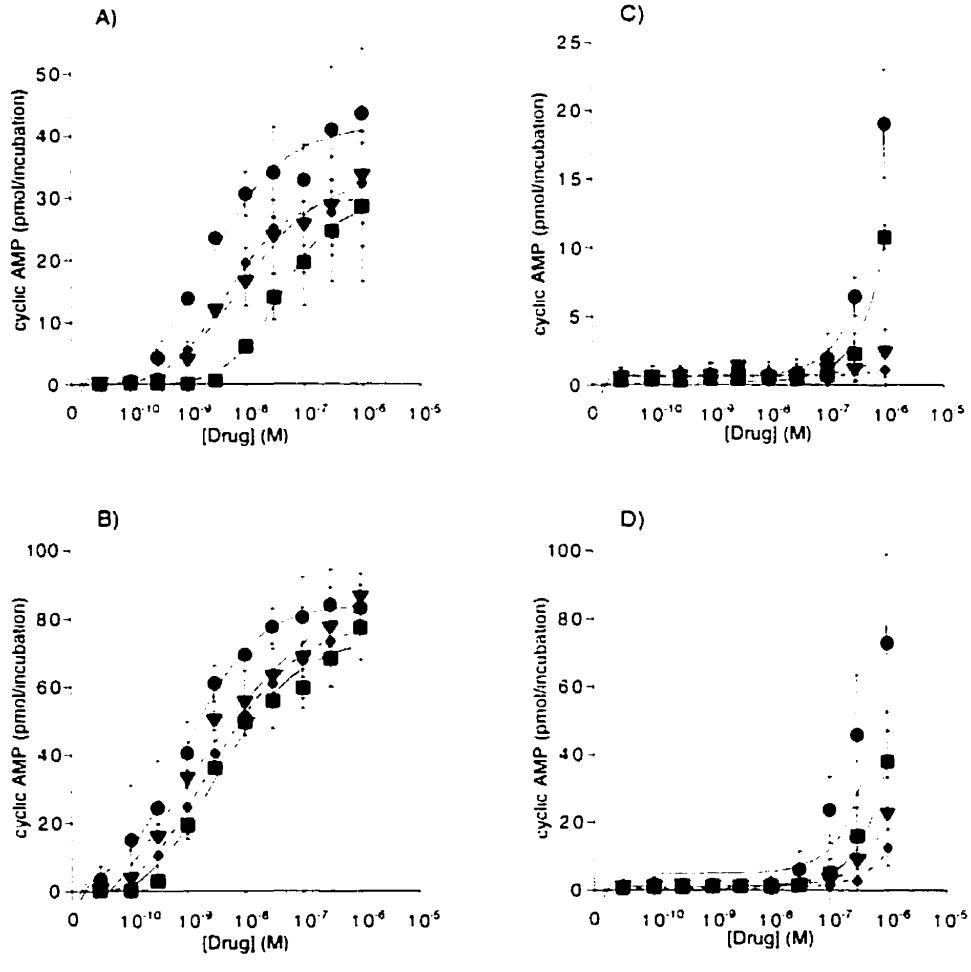

Figure 3. Cyclic AMP production by HEK 293(EBNA) cells expressing FLAG epitope-tagged rat or human homologues of the DP receptor. FLAG-rat DP (Panels A,C) or FLAG-human DP (Panels B,D) receptor-expressing cells were challenged with either DP receptor-specific prostanoids or prostanoid analogues, or prostanoids and prostanoid analogues of other specificity's. The incubation medium contained 0.03-1000 nM of (in Panels A,B): prostaglandin D_2 (◆), prostaglandin J_2 (▼), L-644,698 (■), or BW245C (●); or (in Panels C,D): prostaglandin E_2 (●), prostaglandin $F_{2\alpha}$ (■), iloprost (▼), or U46619 (◆). Data points are the mean of two experiments each performed in duplicate.

Table 2. Potencies and efficacies for ligands at HEK 293(EBNA) cells expressing either FLAG-rat DP or FLAG-human DP receptors.

Ligand	FLAG-rat DP receptor		FLAG-human DP receptor	
	EC_{50} (nM)	[a] % of maximal stimulation of cyclic AMP production	EC_{50} (nM)	[a] % of maximal stimulation of cyclic AMP production
prostaglandin D_2	4.8 ± 2.6	100	1.4 ± 0.1	100 ± 6
prostaglandin J_2	6.9 ± 3.2	103 ± 12	0.4 ± 0.1	118 ± 27
L-644,698	32 ± 16	90 ± 21	4.1 ± 2.7	105 ± 11
BW245C	1.9 ± 1.2	130 ± 8	0.8 ± 1.0	114 ± 2
[b]prostaglandin E_2	2120 ± 360	-	169 ± 22	-
[b]prostaglandin $F_{2\alpha}$	296	-	580 ± 20	-
[b]Iloprost	-	-	665 ± 313	-
[b]U46619	-	-	>1000	-

EC_{50} values ± S.D. and maximal responses (percentage of maximally stimulated control values ± S.D.) are shown for prostanoids and synthetic prostanoid analogues. Values were derived from 2 separate experiments. [a]Maximal stimulation was defined as the cyclic AMP response produced with 1 μM prostaglandin D_2. [b]Maximal responses could not be calculated for prostaglandin E_2, prostaglandin $F_{2\alpha}$, Iloprost and U46619 because the concentration-response curves were not complete.

In situ hybridization of rat tissues with rat DP receptor-specific probes

In situ hybridization reactions were carried out to determine the cell-specific localization of rat DP receptor mRNA transcripts using labelled rat DP receptor-specific cRNA riboprobes (Figure 4 and 5). Each positive result was corroborated by analogous experiments performed using at least one other non-overlapping rat DP receptor-specific antisense riboprobe. Negative controls were performed employing exact complementary sense riboprobes. In order to validate the *in situ* hybridization conditions, initial experiments used brain tissue (Figure 4A-C), which has previously been reported to contain DP receptor mRNA (Oida *et al.*, 1997). A positive signal was detected discontinuously around the periphery of the brain, in the area defined as the leptomeninges (Figure 4C). A specific signal also appeared in the choroid plexus (Figure 4A, B).

The presence of rat DP receptor-specific transcripts was also investigated in spinal cord tissue sections (Figure 4D-G). Cervical, thoracic and lumbar spinal cord sections were evaluated and only the latter was found to contain a positive signal. The signal was most abundantly detected in neurons located in the ventral horn of the transversely-sectioned lumbar spinal cord (Figure 4D,E). The relatively large size of these cell bodies and their ventral location suggests that these cells are motor neurons (Figure 4E). In addition, less abundant staining was detected in the dorsal horn of the lumbar spinal cord (Figure 4G) suggesting the presence of the rat DP receptor transcripts in the interneurons.

The existence of rat DP receptor-specific transcripts in the stomach, duodenum, ileum, and colon of the rat gastrointestinal tract was also investigated (Figure 5). Positive staining identified with the anti-sense probe appeared in all tissues (Figure 5A,C,E,G). Staining was judged specific because it was not detected in tissues probed with sense control probes (Figure 5B,D,F,H). In the stomach (Figure 5A,B), the mucous-secreting goblet cells which lie within the epithelium of the rugae were stained very intensely. Limited staining of the columnar epithelium which lies next to the lumen was also apparent. The duodenum (Figure 5C,D) also demonstrated staining of the goblet cells. Less intense staining was also present in some of the connective tissue cells of the villi.

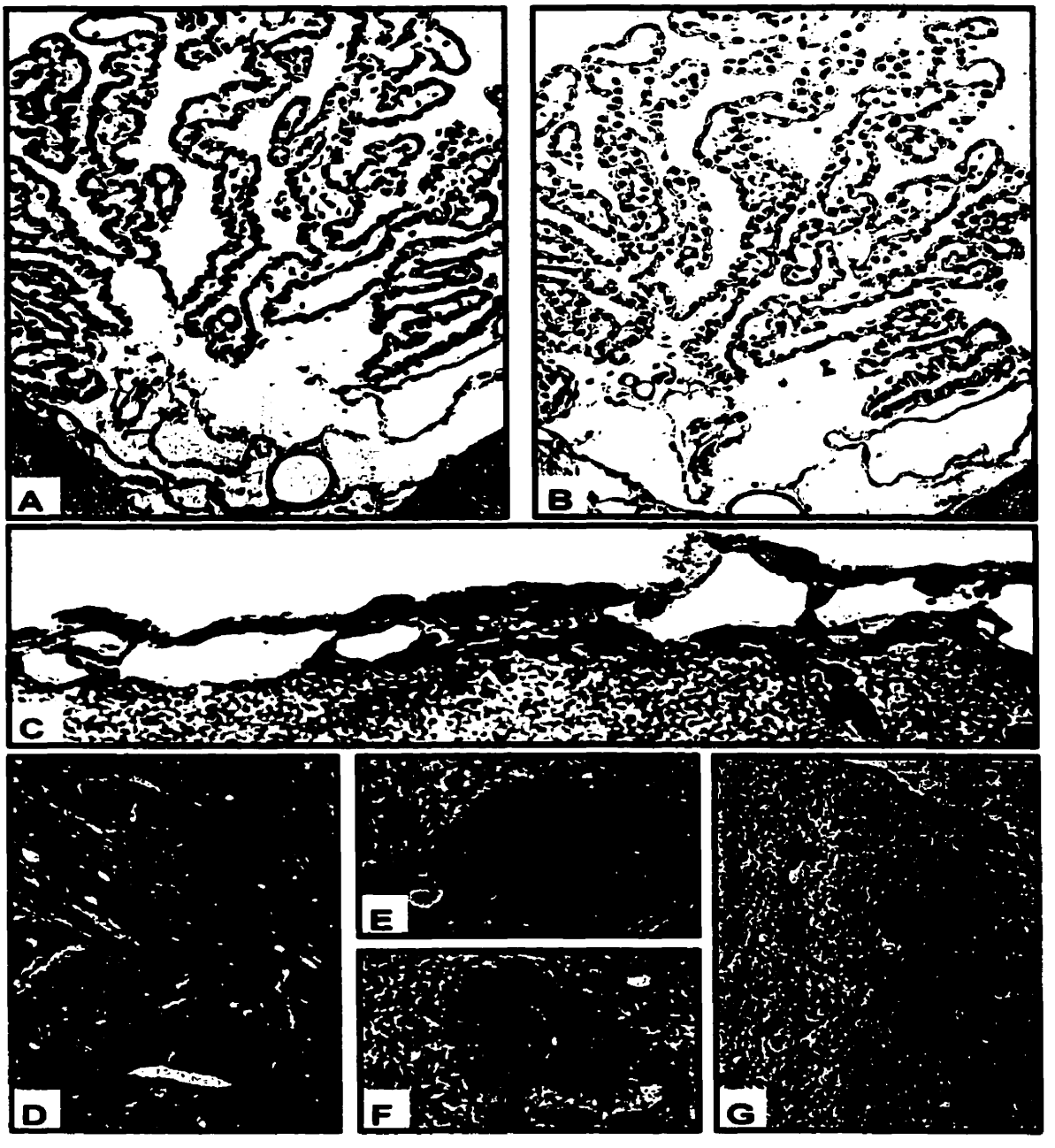

Figure 4. Histochemical localization of DP receptor mRNA in the central nervous system of the rat by in situ hybridization. Reactions within the choroid plexus (200x mag) of the rat brain demonstrate a signal with application of the antisense probe (Panel A) which was absent with application of the exact complementary sense probe (Panel B), used as a negative control. A positive signal was also obtained in the leptomeninges (600x mag) of the rat brain (Panel C). In the rat spinal cord, abundant staining was detected within the ventral horn (Panels D and E; 200x mag and 600x mag, respectively) which was completely absent following application of the sense control probes (Panel F, 600x mag). Staining of lesser intensity was present in the superficial dorsal horn (Panel G, 200x mag), which was also completely absent following application of the sense control probes. Data were confirmed by at least two independent non-overlapping anti-sense riboprobes with respective complementary sense riboprobes used as negative controls.

The ileum (Figure 5E,F) demonstrated intense, positive staining within both the goblet cells and the columnar epithelium. Some of the connective tissue cells within the body of the villi also stained positively. No staining of the goblet cells was evident in the colon (Figure 5G,H), however, a positive signal was observed within the cuboidal epithelium. Some of the connective tissue cells also stained positive, though this staining was much less pronounced than observed within other portions of the gastrointestinal tract. Confirmation of the presence and localization of goblet cells within all gastrointestinal tract tissue preparations was confirmed by staining with Alcian Blue (data not shown) which is known to stain acidic mucopolysaccharides in tissue sections (Steedman, 1950).

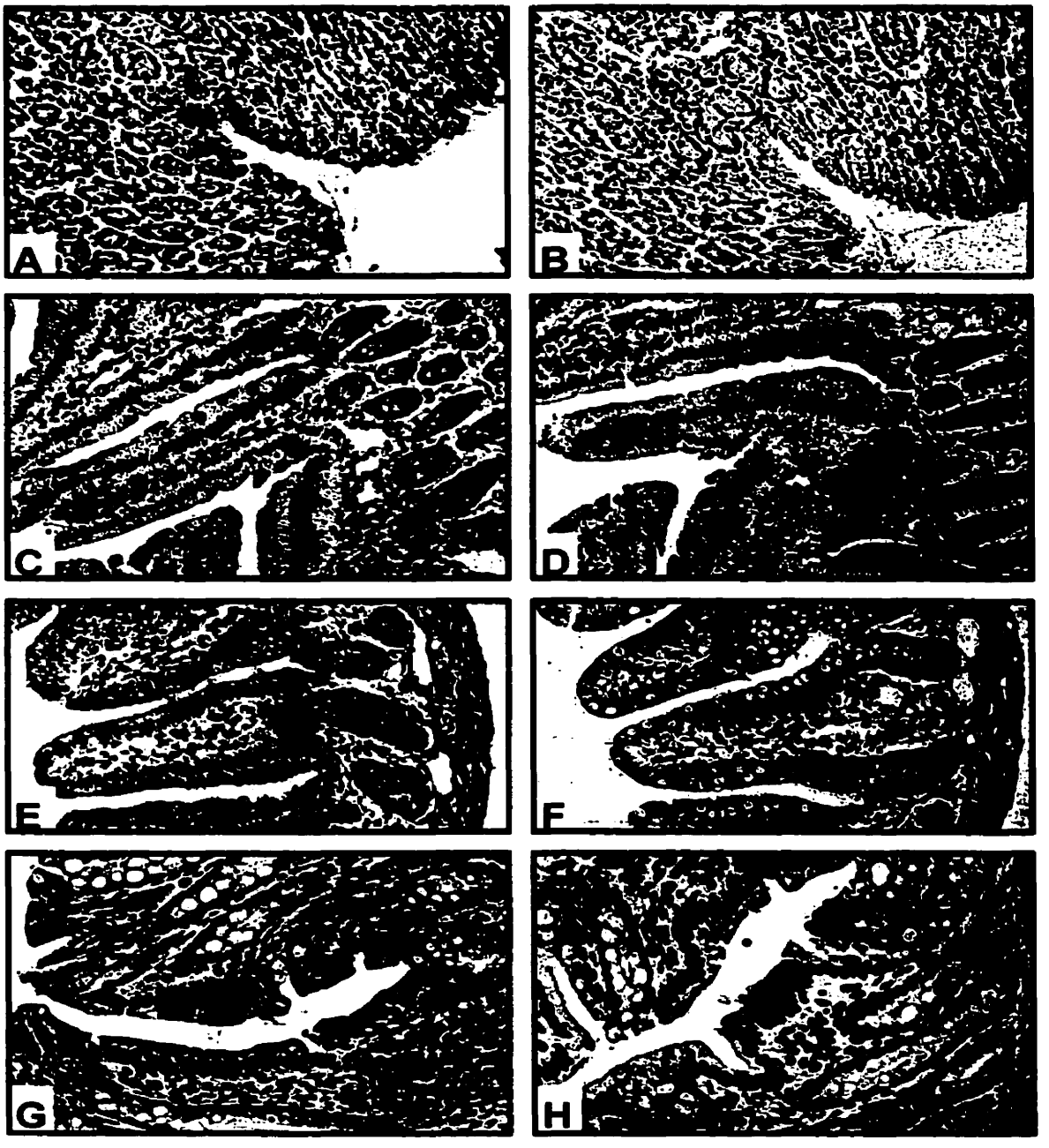

Figure 5. Histochemical localization of DP receptor mRNA in the gastrointestinal tract of the rat by in situ hybridization. Reactions results (200x mag) are presented from rat stomach (Panels A,B), duodenum (Panels C,D), ileum (Panels E,F), and colon (Panels G,H). Panels A, C and E illustrate, in each case, a strong, specific, positive signal obtained within the mucous-producing goblet cells upon application of the anti-sense probe which was not present with application of the exact complementary sense probe (Panels B, D and F), used as a negative control. In the colon, a specific signal was detected instead within the cuboidal epithelium (Panel G) which lies adjacent to the goblet cells. No signal was present in the sense-probed negative control tissues (Panel H). Data were confirmed by at least two independent non-overlapping anti-sense riboprobes with respective complementary sense riboprobes used as negative controls.

Discussion

The rat homologue of the DP receptor is the latest member of the prostanoid receptor family to be cloned for this species. In addition to the cloning and characterization of the rat DP receptor, we present *in situ* hybridization results which provide evidence for the cell-specific localization of rat DP receptor mRNA transcripts in the central nervous system and the gastrointestinal tract. The latter data suggests a novel physiological mechanism for prostaglandin D_2.

In this study, the ORF differs at two positions from a previously published sequence (GenBank™ accession U92289) identified as the rat DP receptor (Gerashchenko *et al.*, 1998). The current study identifies D instead of H at position 71 and T instead of A at position 338. The D for H substitution is of particular importance since D is conserved at this position in virtually all G-protein coupled receptors (Savarese and Fraser, 1992). This D appears to be vital for both the stimulation and attenuation of adenylate cyclase, as well as the activation of phospholipase C. Furthermore, this H for D substitution in the angiotensin II type I receptor impairs both G-protein coupling and downstream signal transduction (Hunyady *et al.*, 1994). Gerashchenko *et al.* did not report functional expression of their receptor so we are unable to address this substitution directly. The sequence described here was, however, successfully functionally expressed.

Saturation analyses performed on the FLAG-rat DP receptor identified a single site of specific binding which, though equal in abundance, had ~4-fold lesser affinity for prostaglandin D_2 relative to the FLAG-human DP receptor. The expresssion of rat and human receptors in a rat cell line was not attempted. Therefore, a distinction between the effects of cellular milieu and inherent receptor affinity cannot be made. The B_{max} value for the FLAG-rat DP receptor expressed in HEK 293(EBNA) cells is comparable to that found previously for the mouse DP receptor (Hirata *et al.*, 1994) expressed in Chinese Hamster Ovary (CHO) cells. However, the affinity of prostaglandin D_2 for the mouse DP receptor in CHO cells is lower than for the FLAG-rat DP receptor (K_D values of 40 nM and 14 nM, respectively).

Competition for [^{3}H]prostaglandin D_2 binding identified a rank order of affinities of prostaglandin D_2 = BW245C > L-644,698 for the FLAG-rat DP receptor and L-

644,698 > prostaglandin D_2 = BW245C for the FLAG-human DP receptor. Similar values were identified for the compounds at the recombinant human DP receptor without the epitope tag expressed on HEK 293(EBNA) cells (Wright *et al.*, 1998), suggesting that the FLAG-epitope tag does not interfere with the receptor's ability to bind ligand. The prostanoids and related analogues demonstrated lower affinities for the FLAG-rat DP receptor relative to the FLAG-human DP receptor. The decrease in affinity is ligand-dependent, ranging from 4-fold for BW245C to over 180-fold for L-644,698. Although inhibitor constants (K_i) are not directly calculated in the characterization of the mouse DP receptor, it appears from Figure 3A within Hirata *et al.* (1994) that the K_i value for BW245C is slightly less than 1 μM. The more recent values for the mouse DP receptor of Kiriyama *et al.* (1997) are in better agreement with the current data, where K_i values of 21 nM and 250 nM for prostaglandin D_2 and BW245C, respectively, were determined.

The rank order of ligand potencies for receptor-mediated increases in intracellular cyclic AMP at the FLAG-rat DP receptor expressed in HEK 293(EBNA) cells was BW245C = prostaglandin D_2 = prostaglandin J_2 > L-644,698, whereas for the FLAG-human DP receptor expressed in HEK 293(EBNA) cells the rank order was BW245C = prostaglandin D_2 = prostaglandin J_2 = L-644,698. EC_{50} values for these DP receptor ligands were from 2-fold to 20-fold lower when measured at the FLAG-rat DP receptor relative to the FLAG-human DP receptor. All DP receptor-specific ligands behaved as full agonists at both receptors. Although EC_{50} values are not presented for the mouse DP receptor (Hirata *et al.*, 1994), it appears from Figure 4B therein that compounds exhibited similar effects at the mouse DP receptor and the FLAG-rat DP receptor in the current study. In both cases, BW245C was slightly more potent than prostaglandin D_2 and EC_{50} values were in the very low nanomolar range for both compounds.

This report also describes the distribution of the rat DP receptor in the central nervous system (CNS) and gastrointestinal tract by *in situ* hybridization. Within the CNS a positive signal was observed in the leptomeninges. A similar distribution was recently reported for both the mouse DP receptor (Oida *et al.*, 1997) and the rat DP receptor (Geraschenko *et al.*, 1998). As mentioned previously (Oida *et al.*, 1997), the discontinuous nature of the signal observed around the leptomeninges may be an artifact introduced during tissue preparation rather than a genuine characteristic of rat DP

receptor distribution, although the lack of signal observed with the sense probes (negative controls) in this study argues against this possibility.

Positive signals were also located within the choroid plexus. Previous *in situ* studies have not reported the DP receptor within the choroid plexus, probably due to the differing sensitivities of the techniques used. Earlier reports addressing the distribution within rat brain of the DP receptor by ligand binding (Yamashita *et al.*, 1983; Watanabe *et al.*, 1986) and prostaglandin D synthase by *in situ* hybridization (Urade *et al.*, 1993) support the present findings. The co-localization of DP receptors and prostaglandin D synthase in the leptomeninges and choroid plexus suggests an autocrine/paracrine relationship, a common aspect of prostanoid-mediated bioactivity (Pierce *et al.*, 1995). This includes the third ventricle that lies directly adjacent to a prostaglandin D_2-sensitive zone within the rostral forebrain shown to modulate sleep (Matsumura *et al.*, 1994) and brain temperature (Hayaishi, 1988). The question remains as to what function is served by the DP receptors localized within the subarachnoid space but outside the sleep-sensitive / brain temperature zone.

In situ hybridization was also performed using rat spinal cord sections since there are reports describing prostaglandin D_2- mediated inhibition of allodynia (discomfort and pain evoked by innocuous tactile stimuli) invoked by prostaglandin E_2 (Minami *et al.*, 1996) and nociceptin (Minami *et al.*, 1997), as well as prostaglandin D_2-mediated hypoalgesia (lowered sensitivity to painful stimuli) (Horiguchi *et al.*, 1986). Within the dorsal horn, signals are confined to the substantia gelatinosa, a spinal cord region involved in the processing of nociceptive stimuli. This is consistent with previous experiments in porcine (Watanabe *et al.*, 1986) and chick (Vesin *et al.*, 1995) spinal cord where prostaglandin D_2-binding proteins and prostaglandin D synthase, respectively, were localized. Recently, murine prostaglandin D synthase has been similarly localized and prostaglandin E_2-mediated allodynia has been shown to be facilitated by prostaglandin D_2 in femtogram to picogram amounts and inhibited by prostaglandin D_2 in picogram to nanogram amounts (Eguchi *et al.*, 1999). Hyperalgesia (increased sensitivity to painful stimuli) has also been demonstrated following the administration of prostaglandin D_2 into mouse spinal cord (Uda *et al.*, 1990) and Substance P antagonists can block the hyperalgesia induced by prostaglandin D_2. Thus, within the substantia

gelatinosa of the dorsal horn. prostaglandin D_2 may modulate hyperalgesia and allodynia through different populations of DP receptor-expressing cells.

The localization of DP receptor mRNA transcripts to the ventral horn of the lumbar spinal cord is consistent with the localization of prostaglandin D synthase in chick spinal cord (Vesin *et al.*, 1995). Previously, prostaglandin E_2-sensitive EP_2 receptors were identified in the same regions of both the ventral and dorsal horn of the rat spinal cord (Kawamura *et al.*, 1997) where a role for EP_2 in monosynaptic reflexes was suggested (Vesin *et al.*, 1991). Co-localization of prostaglandin D synthase and EP_2 suggests an efferent role for prostaglandin D_2 which may include modulation of monosynaptic reflexes. Prostaglandin D_2 and prostaglandin E_2 have opposing functions on the regulation of sleep, where the former promotes sleep and the latter wakefulness (Hayaishi, 1988), and in the regulation of body temperature, where prostaglandin D_2 lowers it and prostaglandin E_2 raises it (Ito *et al.*, 1989). It is possible, therefore, that prostaglandin D_2 and prostaglandin E_2 also demonstrate opposing functions in the modulation of monosynaptic reflexes. The activity opposing prostaglandin D_2 may be mediated by EP_1 in the spinal cord. A prostaglandin E_2-mediated calcium-dependent mechanism of allodynia has recently been reported (Sakai *et al.*, 1998) which is sensitive to EP_1 (but not EP_3) agonists and can be blocked by prostaglandin D_2.

The absence of analogous signals from the dorsal and ventral horns of both the cervical and thoracic spinal cord is suggestive of a discrete contribution to pain transmission by the DP receptor in the neuromodulation of a particular subset of neurons, rather than a more general neuromodulatory role.

DP receptor mRNA was also identified in the stomach, duodenum, ileum and colon. Signals were localized to the mucous-secreting goblet cells, and within the columnar epithelium in some cases. Components of the mucosal connective tissue were also stained, though much less intensely. These results confirm previous reports of DP receptor mRNA expression in human small intestine (Boie *et al.*, 1995) and mouse ileum (Hirata *et al.*, 1994). Previously, both contractile and relaxant roles for prostaglandin D_2 had been reported in the gastrointestinal tract, but other reports failed to identify prostaglandin D_2 effects in the gastrointestinal tract (Giles and Leff, 1988). Prostaglandin D_2 administration has been correlated with contraction in the rat fundus, rabbit jejunum

(Horton *et al.*, 1974) and guinea pig (longitudinal) ileum (Bennett *et al.*, 1980), as well as relaxation of guinea pig ileum (Bennett *et al.*, 1978) and rabbit stomach (Whittle *et al.*, 1979). In the human colon and stomach, however, prostaglandin D_2 was without effect (Sanger *et al.*, 1982). The current report is the first indication that the DP receptor may mediate mucous secretion and cytoprotection.

Interestingly, prostaglandin E_1 has previously been shown to increase both cyclic AMP levels and mucin and water secretion in human intestine (Neutra and Forstner, 1987, and references within; McCool *et al.*, 1990). In addition, 16,16-dimethyl prostaglandin E_2 was shown to increase cell-proliferation rates and mucous granule secretion from the gastrointestinal mucosa of a human colon-derived (HT29) cell line (Phillips *et al.*, 1993). More recently, in an *ex vivo* isolated vascularly perfused rat colon preparation, 16,16-dimethyl prostaglandin E_2 was further demonstrated to be a potent mucin secretagogue (Plaisancie *et al.*, 1997). Stimulation of the DP receptor on epithelial cells could induce mucin secretion directly through increases in intracellular cyclic AMP or indirectly by facilitating the vectorial transport of chloride ions which has been shown to stimulate mucous secretion from goblet cells (Cozens *et al.*, 1992 and references within). Experiments addressing the possibility of DP receptor-mediated mucous secretion and gastrointestinal cytoprotection are currently being undertaken.

In conclusion, the rat homologue of the DP receptor has been cloned. The current sequence differs from that previously presented by Gerashchenko *et al.* (1998) at two residues, one of them (D71) being highly conserved amongst the family of G-protein coupled receptors where, as other studies have suggested, it probably plays a vital role in G-protein coupling and signal transduction. The fidelity of the current sequence allowed for successful functional expression of the rat DP receptor, as assessed by radioligand binding and cyclic AMP accumulation studies. In addition, the cell-specific localization of DP receptor mRNA transcripts has been studied in the CNS and the gastrointestinal tract. DP receptor mRNA was confirmed within the leptomeninges and the choroid plexus of the brain, as well as in the motor neurons and interneurons of the lumbar spinal cord. Within the stomach, duodenum and ileum of the gastrointestinal tract, DP receptor mRNA was repeatedly detected in the mucous-secreting goblet cells and, to a lesser extent, within the columnar epithelium. In the colon, DP receptor mRNA was absent

from the goblet cells but was present in the cuboidal epithelium. These data suggest the intriguing possibility of a role for the DP receptor in mucous regulation and cytoprotection.

References

Albano, J.D., Barnes, G.D., Maudsley, D.V., Brown, B.L., Etkins, R.P., 1974. Factors affecting the saturation assay of cyclic AMP in biological systems. Anal. Biochem. 60: 130-141.

Bennett, A., Sanger, G.J., 1978. The effects of prostaglandin D_2 on the circular muscle of guinea-pig isolated ileum and colon [Proceedings]. Br. J. Pharmacol. 63, 357P-358P.

Bennett, A., Pratt, D., Sanger, G.J., 1980. Antagonism by fenamates of prostaglandin action in guinea-pig and human alimentary muscle. Br. J. Pharmacol. 68, 357-362.

Boie, Y., Sawyer, N., Slipetz, D.M., Metters, K.M., Abramovitz, M., 1995. Molecular cloning and characterization of the human prostanoid DP receptor. J. Biol. Chem. 270, 18910-18916.

Boie, Y., Stocco, R., Sawyer, N., Slipetez, D.M., Ungrin, M.D., Neuschafer-Rube, F., P , schel, G.P., Metters, K.M., Abramovitz, M., 1997. Molecular cloning and characterization of the four rat prostaglandin E_2 prostanoid receptor subtypes. Eur. J. Pharmacol. 340, 227-241.

Cheng, Y.C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099-3108.

Coleman, R.A., Smith, W.L., Narumiya, S., 1994. VIII. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol. Rev. 46, 205-229.

Cozens, A.L., Yezzi, M.J., Yamaya, M., Steiger, D., Wagner, J.A., Garber, S.S., Chin, L., Simon, E.M., Cutting, G.R., Gardner, P., et al., 1992. A transformed human epithelial cell line that retains tight junctions post crisis. In Vitro Cell. Dev. Biol. 28A, 735-744.

Cvejic, S., Devi, L.A., 1997. Dimerization of the δ opioid receptor: implication for a role in receptor internalization. J. Biol. Chem. 272, 26959-26964.

Eguchi, N., Minami, T., Shirafuji, N., Kanaoka, Y., Tanaka., T., Nagata, A., Yoshida., N., Urade, Y., Ito, S., Hayaishi, O., 1999. Lack of tactile pain (allodynia) in lipocalin-type prostaglandin D synthase-deficient mice. Proc. Natl. Acad. Sci. USA 96, 726-730.

Gerashchenko, D., Beuckmann, C.T., Kanaoka, Y., Eguchi, N., Gordon, W.C., Urade, Y., Bazan, N.G., Hayaishi, O., 1998. Dominant expression of rat prostanoid DP receptor mRNA in leptomeninges, inner segments of photoreceptor cells, iris epithelium, and ciliary processes. J. Neurochem. 71, 937-945.

Giles, H., Leff, P., 1988. The biology and pharmacology of PGD_2. Prostaglandins 35(2), 277-300.

Hayaishi, O., 1988. Sleep-wake regulation by prostaglandins D_2 and E_2. J. Biol. Chem. 263, 14593-14596.

Herberg, J.T., Codina, J., Rich, K.A., Rojas, F.J., Iyengar, R., 1984. The hepatic glucagon receptor: solubilization, characterization, and development of an affinity adsorption assay for the soluble receptor. J. Biol. Chem. 259, 9285-9294.

Hebert, T.E., Moffett, S., Morello, J.P., Loisel, T.P., Bichet, D.G., Barret, C., Bouvier, M., 1996. A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J. Biol. Chem. 271, 16384-16392.

Hirata, M., Kakizuka, A, Aizawa, M., Ushikubi, F., Narumiya, S., 1994. Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. Proc. Natl. Acad. Sci. USA 91, 11192-11196.

Horiguchi, S., Ueno, R., Hyodo, M., Hayaishi, O., 1986. Alterations in nociception after intracisternal administration of prostaglandin D_2, E_2, or $F_{2\alpha}$ to conscious mice. Eur. J. Pharmacol. 122, 173-179.

Horton, E.W., Jones, R.L., 1974. Proceedings: biological activity of prostaglandin D_2 on smooth muscle. Br. J. Pharmacol. 52, 110P-111P.

Hunyady, L., Baukal, A.J., Balla, T., Catt, K.J., 1994. Independence of type I Angiotensin II receptor endocytosis from G protein coupling and signal transduction. J. Biol. Chem. 269, 24798-24804.

Ito, S., Narumiya, S., Hayaishi, O., 1989. Prostaglandin D_2: a biochemical perspective. Prostaglandins Leukot. Essent. Fatty Acids. 37(4), 219-234.

Kawamura, T., Yamauchi, T., Koyama, M., Maruyama, T., Akira, T., Nakamura, N., 1997. Expression of prostaglandin EP_2 receptor mRNA in the rat spinal cord. Life Science 61, 2111-2116.

Kenakin, T.P. 1997. Pharmacologic analysis of drug-receptor interaction, 3rd edition. Lippincott-Raven Publishers, Philadelphia, PA, pp 231-241.

Kiriyama, M., Ushikubi, F., Kobayashi, T., Hirata, M., Sugimoto, Y., Narumiya, S., 1997. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br. J. Pharmacol. 122, 217-224.

Kitanaka, J., Hashimoto, H., Sugimoto, Y., Sawada, M., Negishi, M., Suzumura, A., Marunouchi, T., Ichikawa, A., Baba, A., 1995. cDNA cloning of a thromboxane A_2 receptor from rat astrocytes. Biochim. Biophys. Acta 1265, 220-223.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lake, S., Gullberg, H., Wahlqvist, J., Sjogren, Kinhult, A., Lind, K.P., Hellstrom-Lindahl, E., Stjernschantz, J., 1994. Cloning of the rat and human prostaglandin $F_{2\alpha}$ receptors and the expression of the rat prostaglandin $F_{2\alpha}$ receptor. FEBS Lett. 355, 317-325.

Liston, T.E., Roberts II, L.J., 1985. Metabolic fate of radiolabeled prostaglandin D_2 in a normal human male volunteer. J. Biol. Chem. 260, 13172-13180.

Matsumura, H., Nakajima, T., Osaka, T., Satoh, S., Kawase, K., Kubo, E., Sri Kantha, S., Kasahara, K., Hayaishi, O., 1994. Prostaglandin D_2-sensitive, sleep-promoting zone defined in the ventral surface of the rostral basal forebrain. Proc. Natl. Acad. Sci. USA 91, 11998-12002.

McCool, D.J., Marcon, M.A., Forstner, J.F., Forstner, G.G., 1990. The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. Biochem. J. 267, 491-500.

Minami, T., Okuda-Ashitaka, E., Mori, H., Ito, S., Hayaishi, O., 1996. Prostaglandin D_2 inhibits prostaglandin E_2-induced allodynia in conscious mice. J. Pharmacol. Exp. Ther. 278, 1146-1152.

Minami, T., Okuda-Ashitaka, E., Nishizawa, M., Mori, H., Ito, S., 1997. Inhibition of nociception-induced allodynia in conscious mice by prostaglandin D_2. Br. J. Pharmacol. 122, 605-610.

Neutra, M.R., Forstner, J.F., 1987. Gastrointestinal Mucus. In: Johnson, L.R. (Ed.), Physiology of the Gastrointestinal Tract, vol. 2. Raven Press, New York, NY, pp. 975-1009.

Ng, G.Y.K., O'Dowd, B.F., Lee, S.P., Chung, H.T., Brann, M.R., Seeman, P., George, S.R., 1996. Dopamine D2 receptor dimers and receptor-blocking peptides. Biochem. Biophys. Res. Comm. 227, 200-204.

Oida, H., Hirata, M., Sugimoto, Y., Ushikubi, F., Ohishi., H., Mizuno, N., Ichikawa, A., Narumiya, S., 1997. Expression of messenger RNA for the prostaglandin D receptor in the leptomeninges of the mouse brain. FEBS Lett. 417, 53-56.

Phillips, T.E., Stanley, C.M., Wilson, J., 1993. The effect of 16,16-dimethyl prostaglandin E_2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoproteins. Prostaglandins Leukotrienes Essential Fatty Acids 48, 423-428.

Pierce, K.L., Gil, D.W., Woodward, D.F., Regan, D.F., 1995. Cloning of human prostanoid receptors. Trends Pharmacol. Sci. 16, 253-256.

Plaisancie, P., Bosshard, A., Meslin, J.-C., Cuber, J.-C., 1997. Colonic mucin discharge by a cholinergic agonist, prostaglandins, and peptide YY in the isolated vascularly perfused rat colon. Digestion 58, 168-175.

Roberts II, L.J., Sweetman, B.J., Lewis, R.A., Austin, K.F., Oates, J.A., 1980. Increased production of prostaglandin D_2 in patients with systemic mastocytosis. New Eng. J. Med. 303(24), 1400-1404.

Sakai, M., Minami, T., Hara, N., Nishihara, I., Kitade, H., Kamiyama, Y., Okuda, K., Takahashi., H., Mori, H., Ito, S., 1998. Stimulation of nitric oxide release from rat spinal cord by prostaglandin E_2. Br. J. Pharmacol. 123(5), 890-894.

Sanger, G.J., Jackson, A., Bennett, A., 1982. A prostaglandin analogue which potently relaxes human uterus but not gut muscle. Eur. J. Pharmacol. 81, 141-143.

Sasaki, Y., Usui, T., Tanaka., I., Nakagawa, O., Sando, T., Takahashi, T., Namba, T., Narumiya, S., Nakao, K., 1994. Cloning and expression of a cDNA for rat prostaglandin receptor. Biochim. Biophys. Acta 1224, 601-605.

Savarese, T.M., Fraser, C.M., 1992. In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. Biochem. J. 283, 1-19.

Steedman, H.F., 1950. Alcian Blue 8GS: A new stain for mucin. Q. J. Microsc. Sci. 91, 477-479.

Uda, R., Horiguchi, S., Ito, S., Hyodo, M., Hayaishi, O., 1990. Nociceptive effects induced by intrathecal administration of prostaglandin D_2, E_2 or F_2 alpha to conscious mice. Brain Research. 510, 26-32.

Urade, Y., Kitahama, K., Ohishi, H., Kaneko, T., Mizuno, N., Hayaishi, O., 1993. Dominant expression of mRNA for prostaglandin D synthase in leptomeninges, choroid plexus, and oligodendrocytes of the adult rat brain. Proc. Natl. Acad. Sci. USA 90, 9070-9074.

Vesin, M.-F., Pralong, E., Droz, B., 1991. PGE_2 synthesis in chick spinal cord and high affinity PGE_2 receptors on motoneurons. Adv. Prostaglandin Thromboxane Leukot. Res. 21B, 735-738.

Vesin, M.-F., Billotte, C., Droz, B., 1995. Biosynthesis of prostaglandin D_2 by motoneurons and dorsal horn microneurons: a biochemical and high resolution immunocytochemical study in chick spinal cord. Neuroscience 69, 967-975.

Watanabe, YU., Watanabe, Y., Hayaishi, O., 1986. Prostaglandin bindings in the central nervous system. In: Hayaishi, O., Torizuka, K. (Eds.), Biomedical Imaging. Academic Press, Tokyo, pp. 227-238.

Whittle, B.J.R., Mugridge, K.G., Moncada, S., 1979. Use of rabbit transverse stomach-strip to identify and assay prostacyclin, PGA_2, PGD_2 and other prostaglandins. Eur. J. Pharmacol. 53, 167-172.

Wright, D.H., Metters, K.M., Abramovitz, M., Ford-Hutchinson, A.W., 1998. Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist. Br. J. Pharmacol. 123, 1317-1324.

Yamashita, A., Watanabe, Y., Hayaishi, O., 1983. Autoradiographic localization of a binding protein(s) specific for prostaglandin D_2 in rat brain. Proc. Natl. Acad. Sci. USA. 80, 6114-6118.

6. MANUSCRIPT C

The human prostanoid DP receptor stimulates mucin secretion in LS174T cells

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From Manuscript B it is apparent that mRNA corresponding to the rat DP receptor is specifically localized to the mucous-secreting goblet cells and/or adjacent epithelium of the stomach, duodenum, ileum, and colon of the GI tract. These observations suggest a novel biological role for the DP receptor, namely the regulation of mucin secretion. In light of both these results and the previously discussed data regarding the abundance of both hDP- and mDP-specific mRNA in GI tract tissues, especially in small intestine, it is plausible that DP might play a general role in the secretion of mucous which is not species-specific. The current study first demonstrates that, indeed, DP-specific mRNA is localized to the mucous-secreting goblet cells of the human colon. These observations validated the use of an established *in vitro* cell model for the study of mucin secretion, the LS174T human colonic adenocarcinoma cell line. Mucins are high molecular weight glycoproteins that are the chief determinates of the physical and functional properties of mucous. Because of the inherent cross-reactivity among the prostanoid receptors, it was important to determine which of the prostanoid receptors were expressed in LS174T. RT-PCR analyses of mRNA demonstrate that LS174T is a heterologous cell system which endogenously expresses many of the human prostanoid receptors. In addition to DP, all of the other prostanoid receptors were present in LS174T by RT-PCR with the exception of the IP receptor. These findings necessitated the use of highly selective DP receptor ligands, such as L-644,698, to discriminate DP-specific activity from that of other prostanoid receptors. Thus, the current study demonstrates the endogenous expression of a functional receptor that exhibits the pharmacological profile expected for hDP, within LS174T cells. Furthermore, this work confirms that hDP stimulates mucin secretion in this cell line, using DP-specific agonists (PGD_2, L-644,698) and a DP-specific antagonist (BW A868C).

The human prostanoid DP receptor stimulates mucin secretion in LS174T cells

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Summary

1. This study demonstrates both the cell-specific localization of the prostaglandin (PG)D_2 receptor (DP) within the human colon and a potential role for endogenously expressed human (h)DP in regulating mucin secretion from LS174T human colonic adenocarcinoma cells.
2. *In situ* hybridization of DP receptor mRNA in human colon tissue demonstrated a positive signal that was highly localized to the mucous-secreting goblet cells. Because these results are suggestive of a role in mucous secretion, the ability of endogenous DP to regulate mucous secretion was studied in LS174T cells through the use of selective high-affinity ligands.
3. The expression of hDP in LS174T cells was confirmed at the mRNA level by reverse transcriptase-polymerase chain reaction, and at the protein level by radioligand binding assays and signal transduction assays (measuring cyclic AMP accumulation). PGD_2 and the highly selective DP-specific agonist L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate)), but not PGE_2, competed for [^{3}H]PGD_2-specific binding to LS174T cell membranes with K_i values of 0.4 nM and 7 nM, respectively. Several DP-specific agonists (PGD_2, PGJ_2, BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)), and L-644,698) showed similar potencies in stimulating cyclic AMP accumulation through hDP (EC_{50} values: 45-90 nM) and demonstrated the expected rank order of potency based on previous studies. PGE_2 also elicited cyclic AMP production in this cell line (EC_{50} value: 162 nM).
4. The activation of cyclic AMP production by PGD_2 and L-644,698, but not PGE_2, was inhibited by the selective DP antagonist BW A868C showing that PGD_2 and L-644,698 act through hDP in LS174T cells. PGD_2, L-644,698, and PGE_2 (an established mucin secretagogue) were all able to potently stimulate mucin secretion in LS174T cells in a concentration-dependent manner ($EC_{50} < 50$ nM). However, BW A868C effectively antagonized only the mucin secretion mediated by PGD_2 and L-644,698 and not PGE_2. These data are supportive of a role for the DP receptor in the regulation of mucous secretion.

Introduction

The primary bioactive prostanoids encompass prostaglandin (PG)D_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A_2. These products of arachidonic acid metabolism mediate biological activities through eight individual prostanoid receptors [DP, EP (EP_1, EP_2, EP_3, EP_4), FP, IP and TP], some of which have associated spliced variants due to differential mRNA alternative splicing. The prostanoid receptors form a distinct sub-family within the rhodopsin-type G protein-coupled receptor superfamily (for review Coleman *et al.*, 1994).

PGD_2 is the bioactive prostanoid interacting preferentially with the DP receptor, and its synthesis is governed by the sequential actions of PGH synthase and PGD synthase. PGD_2 is associated with both central and peripheral physiological effects. Centrally, PGD_2 has been associated with sleep induction, modulation of body temperature, olfactory function, hormone release, nociception and neuromodulation. Within peripheral tissues, PGD_2 has been shown to mediate vasodilation, inhibition of platelet aggregation, glycogenolysis, bronchoconstriction and vasoconstriction (for review: Giles & Leff, 1988; Ito *et al.*, 1989). In addition, PGD_2 is the major prostanoid synthesized by immunologically challenged mast cells (Lewis *et al.*, 1982) and its release within this system is well characterized (Kawata *et al.*, 1995; Murakami *et al.*, 1995).

Comparatively little is known about the DP receptor. The cloning and characterization of the human (Boie *et al.*, 1995), mouse (Hirata *et al.*, 1994) and rat (Wright *et al.*, 1999) species homologues have previously been reported and in each case the recombinant receptor demonstrated the ability to increase intracellular cyclic AMP in response to PGD_2 and its various analogues. Various studies utilizing mouse and rat tissue slices to investigate the cell-specific localization of DP mRNA by *in situ* hybridization support the roles for the DP receptor in the brain and spinal cord (Oida *et al.*, 1997; Urade *et al.*, 1993; Wright *et al.*, 1999). In addition, strong positive signals were identified for mouse (Hirata *et al.*, 1994) and human (Boie *et al.*, 1995) DP receptor mRNA in the small intestine and ileum, respectively, by northern blot analysis. A much weaker signal was also present in the stomach of the mouse. However, a defined role for

DP in the gastrointestinal tract does not exist. Recently, we localized DP-specific mRNA transcripts to the mucous-secreting goblet cells and/or adjacent epithelium of the stomach, small intestine (duodenum, ileum) and colon whilst performing *in situ* hybridization in selected rat tissues (Wright *et al.*, 1999). These results suggested that the regulation of mucin secretion may be a novel physiological role for the DP receptor. In support of this hypothesis, this report describes the DP-specific regulation of mucin secretion in a human colonic epithelial cell line.

Methods

In situ hybridization

Human colon tissue was removed during surgical biopsy and immediately processed using standard procedures for paraffin embedding (Martinez *et al.*, 1995). Tissue blocks were cut into 4 μM sections and mounted on aminoalkylsilane-coated microscope slides. Solutions were made using water pretreated with diethyl pyrocarbonate (DEPC) and new glassware was used to minimize nuclease activity.

Polymerase chain reaction (PCR) was used to amplify two non-overlapping sequences of 300-400 bp found within the human DP receptor using the following primer pairs: Probe 1: hDP-ATG (5′ G CTC CCG CAC GCC ATG AAG TCG CCG 3′) and hDP-NcoA (5′ CCA GCA CTC CAG TGC CAT GGC CAG G 3′) and Probe 2: hDP-NcoSv2 (5' TC CTG GCC ATG GCA CTG GAG TGC 3') and hDP-PstAv2 (5' CCG GTG CAT CGC ATA GAG GTT GC 3'). Probes 1and 2 were cloned into pCR2.0 (Invitrogen).

Cloning into the pCR2.0 vector, preparation of pCR2.0 clones for use as templates for cRNA riboprobe synthesis (including DNA linearization by restriction digest, proteinase K treatment and phenol/chloroform extraction) and synthesis of digoxigenin-labelled cRNA riboprobes have previously been described (Wright *et al.*, 1999). I*n situ* hybridization including tissue preparation and probe hybridization, was also conducted as previously described (Wright *et al.*, 1999).

Identification of prostanoid receptor transcripts

Gene expression of the eight prostanoid receptors within LS174T cells was analyzed by reverse transcriptase (RT)-PCR. Total RNA was isolated from LS174T cells using Trizol reagent (Gibco BRL Life Technologies, Burlington, Canada) according to the manufacturer's protocol. RT-PCR was performed on LS174T total RNA using a RT-PCR Core Kit (Perkin Elmer), with slight amendments to the manufacturer's protocol as follows: following the reverse transcriptase reaction, sense (S) and anti-sense (AS)

primers were used to amplify cDNAs for human (h)EP_1 (hEP_1S: 5' CCT TGG GTG TAC ATC CTA CTG C 3' and hEP_1AS: 5' AGA ATG GCT TTT TAT TCC CAA AG 3'), hEP_2 (hEP_2S: 5' GAA AGC CCA GCC ATC AGC TC 3' and hEP_2AS: 5' GCG AAG AGC ATG AGC ATC G 3'), hEP_3 (hEP_3S: 5' AGA CGG CCA TTC AGC TTA TGG GGA 3' and hEP_3AS: 5' GAA GAA GGA TCT TTC TTA ACA G 3'), hEP_4 (hEP_4S: 5' ATC TTC GGG GTG GTG GGC AAC 3' and hEP_4AS: 5' CGC TTG TCC ACG TAG TGG C 3'), hDP (hDPS: 5' GGA GCA TTT AAG GAT GTC AAG G 3' and hDPAS: 5' TTC CAT GTT AGT GGA ATT GCT G 3'), hFP (hFPS: 5' TTT CAA CTT GTT TTT GCC AAT G 3' and hFPAS: 5' CAT GCA TGT GTT AAT TGA GGC T 3'), hIP (hIPS: 5' CTG CTC CCT CTG CTG ACA TT 3' and hIPAS: 5' TCC TCT GTC CCT CAC TCT CTT C 3'), hTP (hTPS: 5' CCC TGG GGA TCC ATC CTG TTC CGC CGC 3' and hTPAS: 5' GAG AAG GAA TTC CTA CTG CAG CCC GGA 3'), and β-actin (hBACS: 5' ACA TTA AGG AGA AGC TGT GCT ATG T 3' and hBACAS: 5' CTT CAT GAT GGA GTT GAA GGT AGT T 3'). PCR was performed in 2 mM Mg^{2+} for all primer pairs except those for hTP as follows: initial denaturation at 99 °C, 2 min; followed by 40 cycles of denaturation at 95 °C, 15 s, then annealing and extension at 57 °C, 30 s; and a final extension at 72 °C, 10 min (GeneAmp 9600 system; Perkin-Elmer/Applied Biosystems, Foster City, CA). In the case of hTP, PCR was performed in 3.5 mM Mg^{2+} as follows : initial denaturation at 99 °C, 2 min; followed by 40 cycles of denaturation at 95 °C, 15 s; then annealing and extension at 68 °C, 30 s; and then a final extension at 72 °C, 10 min. RT-PCR reaction products were resolved by gel electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining. The molecular sizes of the amplified products were determined by comparison with the molecular weight markers run in parallel.

LS174T cell culture and membrane preparation

Maintenance of LS174T human colonic adenocarcinoma cells (ATCC No. CL-188; Rockville, MD) in culture was as previously described (Belley *et al.*, 1996; Tse & Chadee, 1992).

Briefly, a high mucin expressing variant was obtained by serially passing LS174T cells through nude mice (*nu/nu* BALB/c). Cells from xenograph tumors were cultured at 37 °C and 5% CO_2 in complete minimal essential medium (MEM) [MEM (Gibco BRL Life Technologies, Burlington, Canada) containing 10% fetal bovine serum, 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulfate, and 20 mM HEPES]. LS174T cell membranes were prepared by cell disruption in the presence of protease inhibitors using nitrogen cavitation as previously described (Wright *et al.*, 1999).

[^{3}H]PGD_2 binding to LS174T cell membranes

Radioligand binding assays were carried out as previously described (Wright *et al.*, 1998). Briefly, assays were performed in 0.2 ml of 10 mM HEPES-KOH (pH 7.4) containing 1 mM EDTA, 8 nM [^{3}H]PGD_2 (115 Ci $mmol^{-1}$) and 10 mM $MnCl_2$. Compounds were added in dimethylsulphoxide (Me_2SO) at 1% (v/v) of the final incubation volume (vehicle concentration was constant throughout). The reaction was initiated by the addition of 350 μg cell membrane protein to all tubes and the samples were incubated at room temperature for 1 h. Termination of the reaction and analysis of [^{3}H]PGD_2-specific binding were performed as previously described (Wright *et al.*, 1999).

Protein assays

Protein concentrations were measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.) using the manufacturer's protocol and bovine serum albumin as the standard.

Cyclic AMP accumulation assays in LS174T cells

Cyclic AMP accumulation assays were conducted using LS174T cells in suspension as previously described (Wright *et al.*, 1998). LS174T cells were harvested at 80% confluence by rinsing the cells in prewarmed Versene 1:5000 (Gibco BRL Life Technologies, Burlington, Canada) followed by enzyme-free cell dissociation buffer to

facilitate their dissociation from the culture dish. Cells were thoroughly washed twice in phosphate-buffered saline pH 7.4 (PBS) by resuspension and centrifugation (300 x g_{max}, 6 min, room temperature) and finally resuspended in HBSS for functional assays. Cell viability (> 60%) was determined by trypan blue exclusion. Generation of cyclic AMP was performed in a final incubation volume of 0.2 ml of HBSS containing 100 µM Ro 20-1724 to abrogate cyclic AMP hydrolysis. Compounds were added in Me_2SO and the vehicle was 1% (v/v) of the final incubation volume throughout. The reaction was initiated by the addition of 2 x 10^5 cells per incubation for both agonist and antagonist assays and the samples were incubated for 10 min at 37 °C. Reactions were terminated and agonist concentration-response curves were analyzed as previously described (Wright *et al.*, 1999).

Antagonist potencies were determined by Schild analysis as follows: agonist concentration-response curves were constructed in the absence and presence of a fixed and increasing concentration of antagonist. Concentration-response curves were analysed and EC_{50} values determined. The equilibrium dissociation constant for the antagonist-receptor complex (K_b) was calculated from the following equation: $dr = [B] / K_b + 1$, where dr is the equiactive dose ratio of the agonist (determined in the presence and absence of the antagonist) and [B] is the antagonist concentration used. Schild regression involves the following logarithmic manipulation of dr on [B]: $\log (dr-1) = \log [B] - \log K_b$.

Analyses of statistical significance employed the statistical modeling software SuperANOVA (Abacus Concepts, Inc.; Berkley, CA, U.S.A.) to perform an analysis of variance (ANOVA). A two-way ANOVA was used to establish differences in the maximal efficacy of DP-specific agonists and a one-way ANOVA was performed to identify differences in agonism in the presence and absence of the DP-specific antagonist BW A868C. All statistically significant differences were analyzed further by way of a Bonferroni/Dunn test of all means.

Mucin secretion assays

LS174T cells were grown in complete MEM to 80% confluence (approximately 4 x 10^5 cells per well) in 6-well plastic culture dishes (well diameter: 34.6 mm). Mucins were subjected to metabolic labeling by incubating cells for 48 h in complete MEM containing 1 μCi ml^{-1} [^{3}H]glucosamine (specific activity: 40 Ci $mmol^{-1}$; ICN Biomedicals Inc., Irvine, CA). Cells were washed three times with warm MEM and incubated in 3 ml complete MEM. The cell media were collected at the required time points for analysis of secreted radiolabelled glycoproteins as follows: samples were centrifuged to remove cell debris (2500 x *g*, 5 min, room temperature) and then an equal volume of 10% (w/v) trichloroacetic acid (TCA), 1% (w/v) phosphotungstic acid (PTA) (Sigma Chemical Co.) was added to precipitate the secreted radiolabelled glycoproteins overnight. Precipitated material was collected by centrifugation (1800 x *g*, 5 min, 4 °C), solubilized in PBS (pH 7.2), and neutralized to pH 7 with 0.1 M NaOH. Radiolabelled mucins were separated from non-mucin radiolabelled glycoproteins by Sepharose 4B column chromatography as described previously (Belley *et al.*, 1996).

High molecular weight mucins elute in the void volume by Sepharose 4B column chromatography (Chadee *et al.*, 1987). It was determined that 98 ± 2 % of the TCA/PTA-precipitable [^{3}H]glycoproteins were recovered in the void volume. Accordingly, subsequent analyses were performed directly on the solubilized, neutralized, TCA/PTA protein precipitates. Data were calculated as the percentage change in secreted [^{3}H]glucosamine relative to vehicle-challenged control wells.

Analyses of statistical significance employed the statistical modeling software SuperANOVA (Abacus Concepts, Inc.) to perform a two-way ANOVA to analyze differences in mucin secretagogue activity mediated by increasing ligand concentrations. A one-way ANOVA was performed to identify differences in secretagogue activity in the presence and absence of the DP-specific antagonist BW A868C. All statistically significant differences were further analyzed by way of a Bonferroni/Dunn test of all means.

Reagents

PGD_2, PGE_2, $PGF_{2\alpha}$, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-$PGF_{2\alpha}$), PGJ_2 and Ro 20-1724 (4-(3 butoxy-4-methoxybenzyl)-2-imidaxlidinone) were from Biomol Research Laboratories (Plymouth Meeting, PA, USA). BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin)) and BW A868C ((±)-3-benzyl-5-(6-carboxyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) were generous gifts from The Wellcome Foundation Ltd (Beckenham, Kent, U.K.). L-644,698 ((4-(3-(3-(3-hydroxyoctyl)-4-oxo-2-thiazolidinyl) propyl) benzoic acid) (racemate) was synthesized at Merck Research Laboratories by Dr. J.B. Bicking. Iloprost (5-(hexahydro-5-hydroxy-4-(3-hydroxy-4-methyl-1-octen-6-ynyl)-2(1H)-pentalenylidene) pentanoic acid) and [^{125}I]-cyclic AMP scintillation proximity assay kits were from Amersham (Oakville, ON, Canada). [^{3}H]PGD_2 was from Dupont NEN (Boston, MA, U.S.A.).

Results

In situ hybridization of DP in human colon

In situ hybridization reactions were carried out to determine the cell-specific localization of DP receptor mRNA transcripts within human colon tissue using digoxigenin-labelled human DP receptor-specific cRNA riboprobes (Figure 1). Experiments using two distinct, non-overlapping, anti-sense riboprobes specific for the hDP receptor corroborated positive results. Negative control experiments were performed employing exact complementary sense riboprobes. Positive staining was identified using the anti-sense riboprobes within the mucous-secreting goblet cells of human colon tissue (Figure 1*A*). This staining was absent in the presence of the negative sense control riboprobes (Figure 1*B*). The localization of goblet cells within the human colon biopsy sections under study was confirmed by staining with Alcian Blue (Figure 1*C*), an established stain for acidic mucopolysaccharides (Steedman, 1950).

Figure 1. Histochemical localization of DP receptor mRNA in human colon tissue by *in situ* hybridization. Results (400x mag) illustrate a strong, specific, positive signal obtained within the mucous-producing goblet cells upon application of the anti-sense probe (*A*) that was not present with application of the exact complementary sense probe (*B*) used as a negative control. Data were confirmed using two independent non-overlapping anti-sense riboprobes with the respective complementary sense riboprobes. Goblet cells were identified using Alcian Blue (*C*).

Identification of prostanoid receptor transcripts in LS174T cells

Total RNA was subjected to RT-PCR to survey the gene expression of mRNA transcripts corresponding to the eight human prostanoid receptors within LS174T cells (Figure 2). Expression of transcripts corresponding to hEP_1, hEP_2, hEP_3, hEP_4, hDP, hFP and hTP in LS174T cells was confirmed by the migration of each receptor-specific amplified cDNA fragment (lanes 1, 4, 7, 10, 13, 16 and 22, respectively) to the predicted position. Results were confirmed by the migration pattern of receptor-specific positive control reactions (lanes 3, 6, 9, 12, 15, 18 and 24, respectively). Receptor-specific negative control reactions (lanes 2, 5, 8, 11, 14, 17, 20 and 23, respectively) performed without reverse transcriptase confirmed in each case that the template for amplification was RNA. In

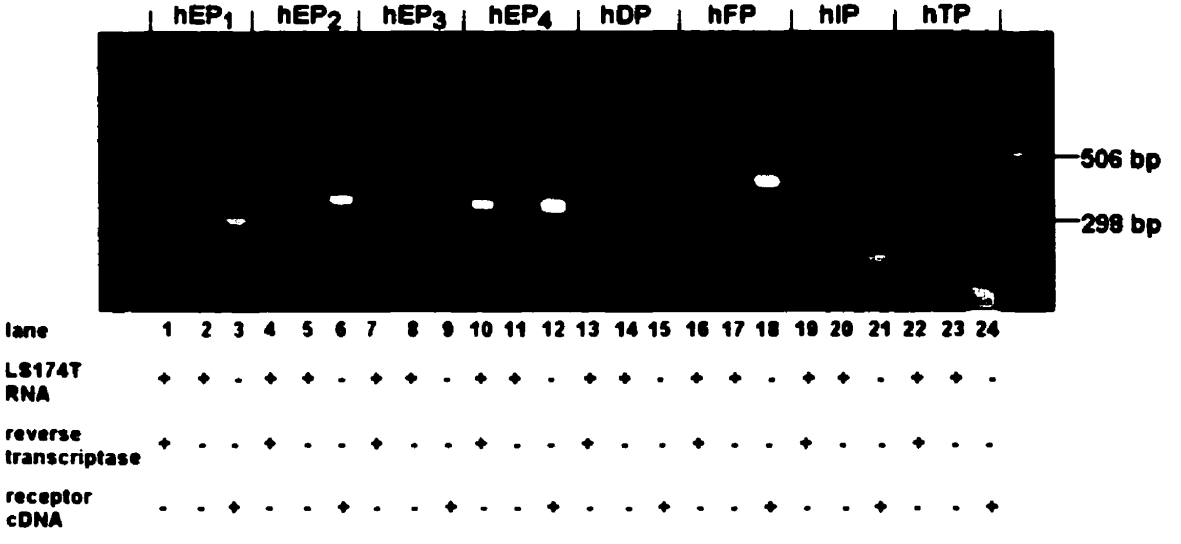

Figure 2. Expression of human prostanoid receptor mRNA in LS174T cells. As described under Methods, RT-PCR was performed using Poly(A)$^+$ RNA isolated from cultured LS174T cells, in the presence of reverse transcriptase and employing primers specific for each of the human prostanoid receptors: EP_1, EP_2, EP_3, EP_4, DP, FP, IP and TP (lanes 1, 4, 7, 10, 13, 16, 19 and 22, respectively). Analogous negative control reactions for each primer pair were performed under the same conditions but in the absence of reverse transcriptase (lanes 2, 5, 8, 11, 14, 17, 20 and 23). Positive control PCR reactions were performed for each primer pair using the corresponding receptor cDNA as the template, in order to generate PCR products of the expected size as markers (lanes 3, 6, 9, 12, 15, 18, 21 and 24). PCR products were resolved on a 1.2% agarose gel and visualized by ethidium bromide staining. Results are representative of three independent experiments.

contrast to these results, there was no detectable cDNA fragment synthesized corresponding to the hIP receptor (lane 19) that co-migrated with the amplified product generated in the positive control reaction for hIP (lane 21). RT-PCR reactions employing primers designed to human β-actin were used as a positive control (data not shown). cDNA fragments corresponding to human β-actin were amplified in a reverse transcriptase-dependent manner. The β-actin fragments co-migrated with fragments generated from an analogous positive control PCR reaction employing human β-actin cDNA as a template.

Radioligand binding to LS174T cell membranes

Equilibrium competition binding experiments were performed employing [^{3}H]PGD_2 to determine if DP-specific binding to LS174T cell membranes could be measured (Figure 3). Both PGD_2 and the selective DP-agonist L-644,698 competed in a concentration-

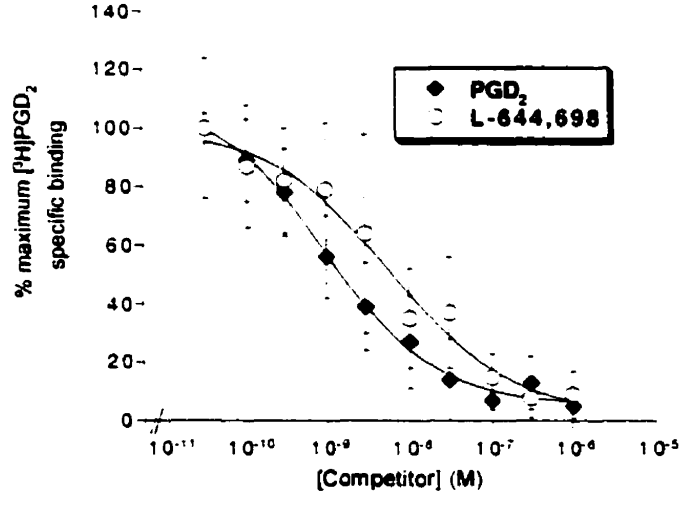

Figure 3. Competition for [^{3}H]PGD_2-specific binding to LS174T membranes by DP-specific agonists. Radioligand membrane binding assays were carried out as previously described under Methods. Equilibrium competition binding assays were conducted using 0.03-1000 nM PGD_2 or L-644,698. Data points are the mean and s.e. mean of values from three independent experiments each performed in duplicate.

dependent manner for [^{3}H]PGD_2-specific binding to LS174T membranes, with inhibitor constant (K_i) values of 0.4 ± 0.1 nM and 7.0 ± 7.5 nM, respectively (n=3). However, PGE_2 did not compete for [^{3}H]PGD_2-specific binding to LS174T membranes at concentrations up to 1 μM (data not shown). These results support the hypothesis that radioligand binding is to DP.

Cyclic AMP accumulation in LS174T cells

The stimulation of cyclic AMP production by adenylate cyclase is the predominating signaling pathway for the DP receptor. Second messenger assays were performed to investigate the capacity of LS174T cells to generate cyclic AMP in response to DP-specific agonists and agonists selective for other receptor specificity's (Figure 4*A* and Table 1). Several DP agonists were potent in this regard with EC_{50} values of approximately 50 nM, resulting in a rank order of BW245C ≥ PGD_2 ≥ L-644,698 ≥ PGJ_2. Generally, prostanoids and prostanoid analogues were without measurable response with the exception of PGE_2, which demonstrated an EC_{50} value of 162 nM (Table 1).

The maximal response for each ligand (the amount of cyclic AMP produced at a concentration of 10 μM) was normalized to that elicited by 10 μM PGD_2 (Table 1). The DP-specific ligand BW245C responded as a full agonist in LS174T cells, with a normalized maximal response of approximately 100%. In contrast, the DP-specific ligands PGJ_2 and L-644,698 were significantly less efficacious than the maximal stimulation defined by PGD_2 ($p < 0.01$), with normalized maximal responses of 67% and 65%, respectively and therefore demonstrated partial agonism. PGE_2 mediated a maximal response greater than that mediated by 10 μM PGD_2.

The ability of the DP antagonist BW A868C to abrogate the cyclic AMP production stimulated in LS174T cells by the DP-specific agonists PGD_2 (Figure 4*B*) and L-644,698 (Figure 4*C*), as well as PGE_2 (Figure 4*D*), was then investigated. BW A868C functioned as a potent, insurmountable, DP-specific antagonist. This was demonstrated by the ability of 3-300 nM BW A868C to depress the maximal responses of PGD_2 and L-644,698. The insurmountable nature of the antagonism produced by BW

Table 1. Potencies and efficacies for (I) DP-specific ligands and (II) ligands for other prostanoid receptors, in the production of cyclic AMP in LS174T cells.

		EC_{50} (nM)	[c] Percentage of maximal stimulation of cyclic AMP production
I	PGD_2	54 ± 8	98 ± 3
	PGJ_2	89 ± 6	*67 ± 3
	L-644,698	62 ± 10	*65 ± 9
	BW245C	45 ± 11	96 ± 15
II	[a] PGE_2	162 ± 66	> 100
	[b] $PGF_{2\alpha}$	-	-
	[b] iloprost	-	-
	[b] U46619	-	-

EC_{50} values ± s.e. mean and maximal responses (percentage of maximally stimulated control values ± s.e. mean) are shown for prostanoids and synthetic prostanoid analogues. Values are the mean and s.e. mean of values from three independent experiments performed in duplicate.

[a] For PGE_2, EC_{50} values ± s.d. and maximal responses (percentage of maximally stimulated control values ± s.d.) are shown, values are derived from two independent experiments each performed in duplicate.

[b] $PGF_{2\alpha}$, iloprost, and U46619 did not generate measurable responses.

[c] Maximal stimulation is defined as the cyclic AMP response produced with 10 μM PGD_2.

* Value is statistically significantly ($p < 0.01$) different from the maximal stimulation (100%) defined with 10 μM PGD_2.

A868C in this cell line precluded calculation of pK_b values or Schild plot ratios. The antagonism mediated at all concentrations of BW A868C against the DP-specific agonists was statistically significant ($p < 0.01$). However, the published pK_b value for BW A868C is approximately 3 nM (Giles *et al.*, 1989). In marked contrast, BW A868C was inefficient at antagonizing the PGE_2-mediated cyclic AMP response.

Mucin secretion in LS174T cells

The LS174T cell line was used to investigate the mucin secretagogue activity of DP receptor agonists because it is an established model for the *in vitro* study of mucin secretion (Belley & Chadee, 1999; Kuan et al., 1987). In preliminary experiments, the time course of PGD_2-mediated mucin secretion was established (data not shown). Forskolin was used as a positive control, since it is a known mucin secretagogue (McCool et al, 1990). Over a 12 h time course forskolin (50 μM) stimulated mucin secretion to a level 87% above vehicle-stimulated control values. In comparison, PGD_2 (250 nM) attained a maximal amount of mucin secretion of 52% above vehicle control over the same period of time. Forskolin stimulated mucin secretion in a linear fashion over the 12 h time course, while PGD_2-mediated mucin secretion reached a plateau after 3 to 6 h of incubation. These experiments confirmed that PGD_2 has the ability to stimulate mucin secretion and experiments were routinely conducted over a 6 h incubation period.

To establish further that the DP receptor was specifically mediating mucin secretion, increasing concentrations of DP-specific agonists were investigated to ascertain if they could stimulate a concentration-dependent response (Figure 5*A*). PGE_2 was included as a positive control, because it is an established mucin secretagogue in LS174T cells (Belley & Chadee, 1999). Incubation with increasing concentrations (1, 50, and 500 nM) of PGD_2, L-644,698 or PGE_2 resulted in a concentration-dependent increase in mucin secretion that was statistically significant ($p<0.01$). The EC_{50} values for mucin secretion were comparable for all three agonists. The rank order for maximal mucin secretion was $PGE_2 > PGD_2 =$ L-644,698.

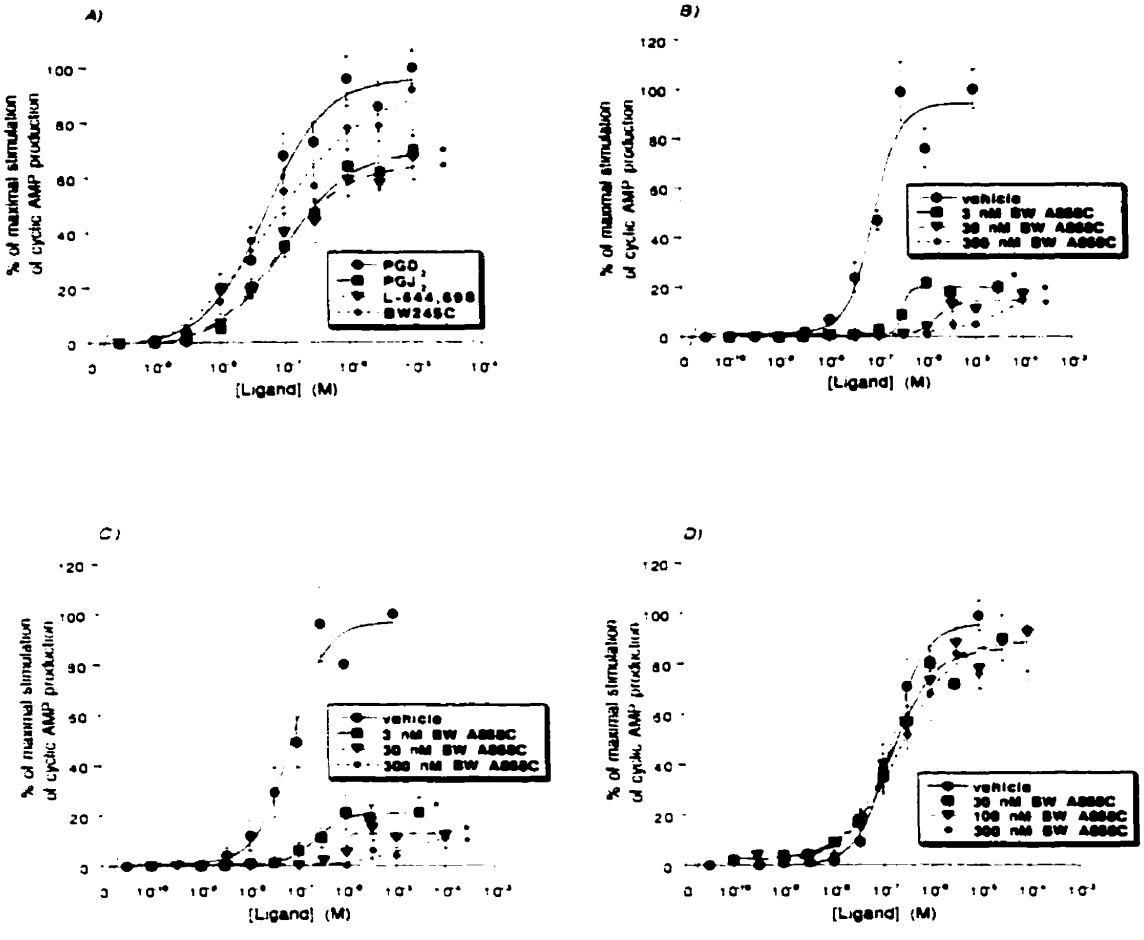

Figure 4. Cyclic AMP production in LS174T cells. (*A*) LS174T cells were challenged with 0.3 nM -10 μM of PGD_2, PGJ_2, L-644,698, or BW245C. Maximal (100%) cyclic AMP production is defined as the amount of cyclic AMP generated in response to 10 μM PGD_2. Data points are the mean and s.e. mean of values from three independent experiments performed in duplicate. The asterisk (*) denotes a ligand demonstrating a statistically significant difference ($p < 0.01$) in maximal efficacy relative to the maximal stimulation (100%) defined with 10 μM PGD_2. (*B*),(*C*) LS174T cells were pre-incubated with vehicle, 3 nM BW A868C, 30 nM BW A868C or 300 nM BW A868C and then challenged with 0.03 nM-100 μM (*B*) PGD_2, or (*C*) L-644,698. (*D*) LS174T cells were pre-incubated with vehicle, 30 nM BW A868C, 100 nM BW A868C or 300 nM BW A868C and then challenged with 0.03 nM-100 μM PGE_2. In Panels (*B*)-(*D*) the maximal (100%) cyclic AMP production for a given agonist is defined as the amount of cyclic AMP generated in response to 10 μM of that agonist in the absence of BW A868C. Data points are the mean and s.d. of values from two independent experiments performed in duplicate. The asterisk (*) denotes a ligand demonstrating a statistically significant ($p < 0.01$) difference in maximal efficacy in the presence, relative to the absence, of BW A868C.

The DP-specific antagonist BW A868C was employed to demonstrate that mucin secretion was DP receptor-specific (Figure 5*B*). LS174T cells were first pre-incubated for 30 min with 30 nM BW A868C or ethanol (vehicle) and were then incubated for 6 h in the presence or absence of PGD_2, L-644,698, or PGE_2 (100 nM). Cells incubated with the antagonist alone responded with a slight increase in secreted mucins of 8%. The mucin secretion observed when cells were stimulated with 100 nM PGD_2 or L-644,698 was significantly inhibited ($p < 0.01$) by 53% and 59%, respectively, when cells were pre-incubated with 30 nM BW A868C prior to agonist addition. However, pre-incubation of cells with 30 nM BW A868C had no effect on the PGE_2-mediated mucin secretion, demonstrating that DP agonist induced mucin secretion in LS174T occurs via an independent pathway to that provoked by PGE_2. These data suggest that DP receptor activation stimulates mucin secretion.

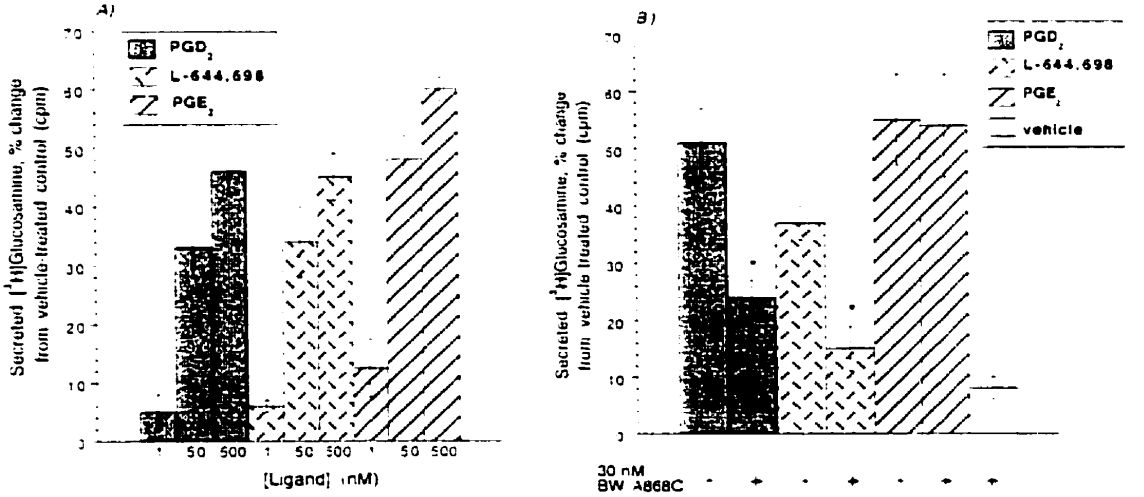

Figure 5. Mucin secretion from LS174T cells. (*A*) LS174T cells were challenged with 1, 50, or 500 nM PGD_2, L-644,698, or PGE_2 for 6 h. A statistically significant ($p < 0.01$) difference was observed for each ligand in the amount of [^{3}H]glucosamine secreted at 50 nM relative to 1 nM and at 500 nM relative to 50 nM. (*B*) Following pre-incubation with vehicle or 30 nM BW A868C for 30 min, LS174T cells were challenged with 100 nM PGD_2, L-644,698, PGE_2, or vehicle for 6 h. Data points are the mean and s.d. of values from two independent experiments performed in duplicate. The asterisk (*) denotes a ligand demonstrating a statistically significant ($p < 0.01$) difference in the presence relative to the absence of the antagonist.

Discussion

This paper provides evidence for a novel physiological role for the DP receptor, namely the regulation of mucin secretion. This role was first suggested by work from this laboratory showing the presence of the DP receptor in mucous-secreting goblet cells of the rat by *in situ* hybridization. In this report we confirm this is not a species-specific observation, by demonstrating that DP receptor mRNA is also localized in mucous-secreting goblet cells of the human colon. This result validated the use of the human LS174T colonic adenocarcinoma cell line, an established *in vitro* model of mucin secretion that endogenously expresses the DP receptor, as a system to study the role of DP in mucous production. The presence of DP within LS174T cells was confirmed at the level of transcription, specific radioligand binding, second messenger coupling and mucin secretion, utilizing DP-specific agonists (PGD_2, L-644,698) and a DP-specific antagonist (BW A868C).

The endogenous DP receptor expressed in LS174T cells shows similar properties to recombinant hDP expressed in the human embryonic kidney cell line, HEK 293(EBNA) (Wright *et al.*, 1998). The K_i values for PGD_2 and the selective DP agonist L-644,698 determined in radioligand binding assays were comparable between endogenous and recombinant hDP (0.5 - 7 nM). As expected, PGE_2 had reduced affinity compared with PGD_2 and L-644,698. These results support $[^3H]PGD_2$ binding specifically to hDP in LS174T cells. Activation of DP stimulates intracellular cyclic AMP production. The EC_{50} values for cyclic AMP production stimulated by DP agonists in LS174T cells (54 - 89 nM) were all 100-fold less potent than those previously identified for recombinant hDP in HEK 293(EBNA) cells (Wright *et al.*, 1998), though a similar rank order of potency was observed. In the current study, PGD_2 and BW245C both demonstrated full agonism while PGJ_2 and L-644,698 were both partial agonists, in contrast to results with recombinant hDP where all four DP compounds functioned as full agonists. This disparity is probably due to the high number of hDP receptors in the recombinant cell line compared with that of endogenous hDP in LS174T, as demonstrated by the level of radioligand binding.

Cyclic AMP accumulation was also measured in LS174T cells challenged with the preferred ligands for prostanoid receptors other than DP, since RT-PCR suggested the presence of multiple types of prostanoid receptors (hDP, hEP_1, hEP_2, hEP_3, hEP_4, hFP, and hTP) in these cells. PGE_2 induced increases in intracellular cyclic AMP (EC_{50} = 162 nM). This represents a balance of contributions from different EP receptors: the stimulation of cyclic AMP production mediated by hEP_2 and hEP_4 and the inhibition of cyclic AMP production mediated by hEP_3. The IP receptor agonist iloprost was functionally silent, corroborating the RT-PCR experiments that did not detect hIP mRNA. This data supports the functional expression of hDP in the LS174T cell line. Furthermore, it verifies the presence of multiple prostanoid receptors within LS174T cells that signal through the cyclic AMP pathway. In this report, therefore, we employ DP selective ligands to investigate the contribution of DP to mucous secretion in this cell line.

BW A868C clearly functions as an insurmountable antagonist at the DP receptor in LS174T cells in the current study. Previous characterization of BW A868C, however, suggested that it functions as a DP-specific competitive, surmountable antagonist (Giles *et al.*, 1989). The differences observed between these two studies may be attributable to the kinetics of formation of the antagonist-receptor complex, as has previously been suggested for the angiotensin II AT_1 receptor (Vanderheyden *et al.*, 1999). Although no kinetic data is available for BW A868C, the time interval of pre-incubation of the antagonist with cells prior to agonist addition differs between the two studies, 2 min previously and 10 min here. If BW A868C has a slow K_{on}/K_{off}, then its competitive antagonism could be surmountable under conditions of short pre-incubation time and insurmountable under conditions of longer pre-incubation time.

There are no previous reports describing a role for PGD_2 in the regulation of DP-mediated mucin secretion. The current study is the first to identify that mucin secretion can occur in response to DP-specific ligands, i.e. using PGD_2 and L-644,698 at concentrations ranging from 1–500 nM with an EC_{50} of less than 50 nM for both ligands. The concentrations used of the DP agonists might be considered close to those relevant *in vivo*, and agree well in this regard with a report identifying a significant increase in mucin secretion upon challenge of rabbit gastric mucosal explants with 10 nM PGE_2 (Seidler *et*

al., 1988). The ability of PGE_2 and $PGF_{2\beta}$ to regulate mucin secretion is well established (Belley & Chadee, 1999; Enss *et al.*, 1995; Lamont *et al.*, 1983; McCool *et al.*, 1990; Phillips *et al.*, 1993; Plaisancie *et al.*, 1997; Seidler & Sewing, 1989) in several cell lines and tissue explants from various species. In contrast to the results observed with the DP agonists, many of these studies have used supra-physiological concentrations of ligands, typically in the micromolar range. For instance, concentrations of ligand (PGE_1, PGE_2 and 16,16-dimethyl PGE_2, respectively) greater than or equal to 1 μM were employed in studies with the cultured human mucous-secreting cell lines T84 (McCool *et al.*, 1990) and HT29-18N2 (Phillips *et al.*, 1993), and cultured pig gastric mucous cells (Enss *et al.*, 1995). Similarly, in a study employing carbachol, A23187 and histamine, the lowest concentration used of these three compounds was 10 μM (McCool *et al.*, 1990).

In this report we have used the selective DP agonist L-644,698 to show that mucin secretion occurs through the DP receptor. L-644,698 was employed because it is one of the most selective DP agonists reported to date with at least 300-fold higher affinity for hDP over any of the other cloned human prostanoid receptors (Wright *et al.*, 1998). In a recent report, Belley & Chadee (1999) show that mucin secretion in LS174T cells is regulated by hEP_4 which acts by increasing cyclic AMP like PGD_2. It was particularly important, therefore, to use selective DP-specific tools to delineate clearly the contribution of the hDP receptor in the mucin response. In comparison to BW245C, L-644,698 has a 100-fold reduced affinity for hEP_4 versus DP (130 nM and 9.3 μM)(Wright *et al.*, 1998). In addition, using the antagonist BW A868C in combination with both PGD_2 and PGE_2 provides further support for the DP-specific regulation of mucin secretion. PGD_2-and L-644,698-mediated increases in cyclic AMP production and stimulation of mucin secretion in LS174T cells were both effectively antagonized by BW A868C at 3 nM. In contrast, BW A868C had no effect on PGE_2-mediated increases in cyclic AMP and mucin release at concentrations up to 300 nM. These data provide compelling evidence that mucin secretion in LS174T cells can be induced by the DP receptor.

A definitive role for the DP receptor in gastrointestinal (GI) physiology has not been demonstrated. Studies of PGD_2-mediated effects within the GI tract have focused on smooth muscle contraction and ion secretion. Both contractile and relaxant roles for

PGD_2 have been reported in the GI tract, but several studies were also negative (Giles & Leff, 1988). PGD_2 administration has been correlated with contraction in the rat fundus, rabbit jejunum (Horton & Jones, 1974) and guinea pig (longitudinal) ileum (Bennett *et al.*, 1980), as well as relaxation of guinea pig ileum (Bennett & Sanger, 1978) and rabbit stomach (Whittle *et al.*, 1979). PGD_2 was without effect in the human colon and stomach (Sanger *et al.*, 1982). PGD_2 has also been reported to both facilitate (Frieling *et al.*, 1994) and inhibit (Goerg *et al.*, 1991) chloride secretion in guinea pig colon and rat colon, respectively. In addition, the T84 human intestinal cell line was reported to stimulate chloride secretion with low potency when challenged with PGD_2 (Barrett, 1992). The current report is the first indication that the DP receptor mediates mucin secretion. PGD_2-mediated mucin secretion may occur through a mechanism originally described by Powell (1991) and modified by Eberhart and Dubois (1995) for electrolyte transport, whereby inflammatory cells, particularly mast cells, release mediators upon activation of the inflammatory response. PGD_2 is the major prostanoid released by mast cells (Lewis *et al.*, 1982) and mast cell degranulation is a prominent feature of certain conditions such as Crohn's disease (Dvorak *et al.*, 1978).

In conclusion, we report the identification of DP mRNA in the mucous-secreting goblet cells of the human colon. We have used the LS174T human colonic adenocarcinoma cell line, an established *in vitro* model of mucin secretion, to investigate a potential role for DP. The expression of endogenous functional DP in LS174T cells has been demonstrated and its ability to activate mucin secretion has been confirmed through the use of selective DP ligands.

References

BARRETT, K.E. (1992). Effect of histamine and other mast cell mediators on T84 epithelial cells. *Ann N Y Acad Sci*, **664**, 222-31.

BELLEY, A. & CHADEE, K. (1999). Prostaglandin E(2) Stimulates Rat and Human Colonic Mucin Exocytosis Via the EP(4) Receptor. *Gastroenterology*, **117**, 1352-1362.

BELLEY, A., KELLER, K., GROVE, J. & CHADEE, K. (1996). Interaction of LS174T human colon cancer cell mucins with Entamoeba histolytica: an in vitro model for colonic disease. *Gastroenterology*, **111**, 1484-92.

BENNETT, A., PRATT, D. & SANGER, G.J. (1980). Antagonism by fenamates of prostaglandin action in guinea-pig and human alimentary muscle. *Br J Pharmacol*, **68**, 357-62.

BENNETT, A. & SANGER, G.J. (1978). The effects of prostaglandin D2 on the circular muscle of guinea-pig isolated ileum and colon [proceedings]. *Br J Pharmacol*, **63**, 357P-358P.

BOIE, Y., SAWYER, N., SLIPETZ, D.M., METTERS, K.M. & ABRAMOVITZ, M. (1995). Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem*, **270**, 18910-6.

CHADEE, K., PETRI, W.A., Jr., INNES, D.J. & RAVDIN, J.I. (1987). Rat and human colonic mucins bind to and inhibit adherence lectin of Entamoeba histolytica. *J Clin Invest*, **80**, 1245-54.

COLEMAN, R.A., SMITH, W.L. & NARUMIYA, S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev*, **46,** 205-29.

DVORAK, A.M., MONAHAN, R.A., OSAGE, J.E. & DICKERSIN, G.R. (1978). Mast-cell degranulation in Crohn's disease [letter]. *Lancet*, **1,** 498.

EBERHART, C.E. & DUBOIS, R.N. (1995). Eicosanoids and the gastrointestinal tract. *Gastroenterology*, **109,** 285-301.

EGUCHI, N., MINAMI, T., SHIRAFUJI, N., KANAOKA, Y., TANAKA, T., NAGATA, A., YOSHIDA, N., URADE, Y., ITO, S. & HAYAISHI, O. (1999). Lack of tactile pain (allodynia) in lipocalin-type prostaglandin D synthase-deficient mice. *Proc Natl Acad Sci U S A*, **96,** 726-30.

ENSS, M.L., SCHMIDT-WITTIG, U., HEIM, H.K. & SEWING, K.F. (1995). Prostaglandin E2 alters terminal glycosylation of high molecular weight glycoproteins, released by pig gastric mucous cells in vitro. *Prostaglandins Leukot Essent Fatty Acids*, **52,** 333-40.

FRIELING, T., RUPPRECHT, C., KROESE, A.B. & SCHEMANN, M. (1994). Effects of the inflammatory mediator prostaglandin D2 on submucosal neurons and secretion in guinea pig colon. *Am J Physiol*, **266,** G132-9.

GILES, H. & LEFF, P. (1988). The biology and pharmacology of PGD2. *Prostaglandins*, **35,** 277-300.

GILES, H., LEFF, P., BOLOFO, M.L., KELLY, M.G. & ROBERTSON, A.D. (1989). The classification of prostaglandin DP receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br J Pharmacol*, **96,** 291-300.

GOERG, K.J., DIENER, C., DIENER, M. & RUMMEL, W. (1991). Antisecretory effect of prostaglandin D2 in rat colon in vitro: action sites. *Am J Physiol*, **260,** G904-10.

HIRATA, M., KAKIZUKA, A., AIZAWA, M., USHIKUBI, F. & NARUMIYA, S. (1994). Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc Natl Acad Sci U S A*, **91,** 11192-6.

HORTON, E.W. & JONES, R.L. (1974). Proceedings: Biological activity of prostaglandin D2 on smooth muscle. *Br J Pharmacol*, **52,** 110P-111P.

ITO, S., NARUMIYA, S. & HAYAISHI, O. (1989). Prostaglandin D2: a biochemical perspective. *Prostaglandins Leukot Essent Fatty Acids*, **37,** 219-34.

KAWATA, R., REDDY, S.T., WOLNER, B. & HERSCHMAN, H.R. (1995). Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced prostaglandin D2 production in mast cells. *J Immunol*, **155,** 818-25.

KUAN, S.F., BYRD, J.C., BASBAUM, C.B. & KIM, Y.S. (1987). Characterization of quantitative mucin variants from a human colon cancer cell line. *Cancer Res*, **47,** 5715-24.

LAMONT, J.T., VENTOLA, A.S., MAULL, E.A. & SZABO, S. (1983). Cysteamine and prostaglandin F2 beta stimulate rat gastric mucin release. *Gastroenterology*, **84,** 306-13.

LEWIS, R.A., SOTER, N.A., DIAMOND, P.T., AUSTEN, K.F., OATES, J.A. & ROBERTS, L.J.D. (1982). Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. *J Immunol*, **129,** 1627-31.

MARTINEZ, A., MILLER, M.J., QUINN, K., UNSWORTH, E.J., EBINA, M. & CUTTITTA, F. (1995). Non-radioactive localization of nucleic acids by direct in situ PCR and in situ RT-PCR in paraffin-embedded sections. *J Histochem Cytochem*, **43**, 739-47.

McCOOL, D.J., MARCON, M.A., FORSTNER, J.F. & FORSTNER, G.G. (1990). The T84 human colonic adenocarcinoma cell line produces mucin in culture and releases it in response to various secretagogues. *Biochem J.* **267**, 491-500.

MURAKAMI, M., BINGHAM, C.O., 3RD, MATSUMOTO, R., AUSTEN, K.F. & ARM, J.P. (1995). IgE-dependent activation of cytokine-primed mouse cultured mast cells induces a delayed phase of prostaglandin D2 generation via prostaglandin endoperoxide synthase-2. *J Immunol*, **155**, 4445-53.

OIDA, H., HIRATA, M., SUGIMOTO, Y., USHIKUBI, F., OHISHI, H., MIZUNO, N., ICHIKAWA, A. & NARUMIYA, S. (1997). Expression of messenger RNA for the prostaglandin D receptor in the leptomeninges of the mouse brain. *FEBS Lett.* **417**, 53-6.

PHILLIPS, T.E., STANLEY, C.M. & WILSON, J. (1993). The effect of 16,16-dimethyl prostaglandin E2 on proliferation of an intestinal goblet cell line and its synthesis and secretion of mucin glycoproteins. *Prostaglandins Leukot Essent Fatty Acids*, **48**, 423-8.

PLAISANCIE, P., BOSSHARD, A., MESLIN, J.C. & CUBER, J.C. (1997). Colonic mucin discharge by a cholinergic agonist, prostaglandins, and peptide YY in the isolated vascularly perfused rat colon. *Digestion*, **58**, 168-75.

POWELL, D.W. (1991). Immunophysiology of intestinal electrolyte transport. In *Handbook of physiology*. ed. Schultz, S.G., Field, M., Frizzell, R.A. & Rauner, B.B. Bethesda, MD: American Physiological Society.

SANGER, G.J., JACKSON, A. & BENNETT, A. (1982). A prostaglandin analogue which potently relaxes human uterus but not gut muscle. *Eur J Pharmacol*, **81,** 141-3.

SEIDLER, U., KNAFLA, K., KOWNATZKI, R. & SEWING, K.F. (1988). Effects of endogenous and exogenous prostaglandins on glycoprotein synthesis and secretion in isolated rabbit gastric mucosa. *Gastroenterology*, **95,** 945-51.

SEIDLER, U. & SEWING, K.F. (1989). Ca2+-dependent and -independent secretagogue action on gastric mucus secretion in rabbit mucosal explants. *Am J Physiol*, **256,** G739-46.

TSE, S.K. & CHADEE, K. (1992). Biochemical characterization of rat colonic mucins secreted in response to Entamoeba histolytica. *Infect Immun*, **60,** 1603-12.

VANDERHEYDEN, P.M., FIERENS, F.L., DE BACKER, J.P., FRAEYMAN, N. & VAUQUELIN, G. (1999). Distinction between surmountable and insurmountable selective AT1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT1 receptors. *Br J Pharmacol*, **126,** 1057-65.

WHITTLE, B.J., MUGRIDGE, K.G. & MONCADA, S. (1979). Use of the rabbit transverse stomach-strip to identify and assay prostacyclin, PGA2, PGD2 and other prostaglandins. *Eur J Pharmacol*, **53,** 167-72.

WRIGHT, D.H., METTERS, K.M., ABRAMOVITZ, M. & FORD-HUTCHINSON, A.W. (1998). Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist. *Br J Pharmacol*, **123,** 1317-24.

WRIGHT, D.H., NANTEL, F., METTERS, K.M. & FORD-HUTCHINSON, A.W. (1999). A novel biological role for prostaglandin D2 is suggested by distribution studies of the rat DP prostanoid receptor. *Eur J Pharmacol*, **377**, 101-15.

7. DISCUSSION

7.1. Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist

Modulation of hDP binding activity by divalent cations and nucleotide analogues was studied in the characterization of this receptor as a GPCR. Divalent cations, especially Mg^{2+}, increase the affinity of an agonist for its receptor by binding to the $G\alpha$ subunit and stabilizing its interaction with GTP, thus influencing such downstream events as $G\alpha\beta\gamma$ subunit dissociation and effector signaling (522, 523). Of the divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}) investigated for their modulatory effects on [^{3}H]PGD_2 specific binding, Mn^{2+} preferentially facilitated agonist specific binding (maximal response at 10 mM, EC_{50} of 2 mM). In light of Mn^{2+} being the optimal cation (instead of Mg^{2+}), this cation may have mediated general effects of stabilization in these studies rather than the site-specific effects of Mg^{2+}. This claim is supported by studies that have found Mg^{2+} to be catalytically essential for GTP binding to the $G\alpha$ subunit of the agonist-receptor- G-protein complex and, subsequently, for the dissociation of the heterotrimeric G-protein into its $G\alpha$ and $G\beta\gamma$ subunits (524, 525).

Slowly-hydrolyzable nucleotide analogues disrupt the G protein cycle by binding to the $G\alpha$ subunit and inducing its sustained dissociation from the $G\beta\gamma$ subunit (526, 527), thereby conferring a low affinity state on the entire receptor population (528, 529, 530). This was demonstrated at the hDP receptor by the inhibition of [^{3}H]PGD_2 specific binding using various nucleotide analogues (GTPγS, ATPγS, GMP-PNP, and AMP-PNP). More recently, all 8 recombinant human prostanoid receptors have been shown to be modulated by nucleotide analogues in their ability to specifically bind ligand (423). Like hDP, most receptors demonstrated a decreased affinity for their cognate ligand following treatment with GTPγS. However, the $hEP_{3\text{-III}}$ receptor has an increased affinity for its ligand under these conditions. Similar results were observed under these conditions at the human $EP_{3\text{-I}}$ and $EP_{3\text{-II}}$ subtypes in the same study, and have been observed independently at the mouse $EP_{3\alpha}$ and $EP_{3\gamma}$ receptors (347, 531, 532). These

findings suggest that the high affinity conformation of the EP_3 receptor occurs in its uncoupled state, contrary to most GPCRs.

More recently, the affinities of various ligands for the eight recombinant mouse prostanoid receptors expressed in Chinese hamster ovary cells were reported (424). The rank order of affinity reported is PGD_2 > BW A868C = BW245C with K_i values of 21, 220, and 250 nM, respectively. These ligands have less affinity for the recombinant mDP compared to the recombinant hDP (0.6, 2.3, and 0.4 nM, respectively). Notably, BW245C exhibits a 500-fold difference in affinity between the 2 species homologues of DP, suggesting that this compound might be used as a chemical lead from which to make compounds for structure-activity relationship (SAR) assessments of the two receptors. Little is known about the SAR surrounding the DP receptor. A recent study of chimeric mDP/mIP receptors (533) suggests that the third transmembrane domain of mDP confers the selective binding of PGD_2 to this receptor. These results have yet to be extended to other DP agonists or other species homologues of DP.

Despite these differences, the inherent receptor cross-reactivity of the prostanoids is observed as an inter-species phenomenon. In the mouse system PGD_2 demonstrated only 2-fold higher selectivity for mDP relative to mFP, and in the human system the difference was 10-fold . The aforementioned observation that PGD_2-induced bronchoconstriction in the anesthetized dog may be mediated by the FP receptor (534) corroborates these findings. Together, these observations emphasize the necessity for the identification of selective compounds, such as L-644,698, to dissect the individual contributions of the prostanoid receptors in heterologous systems. The development of the system of recombinant human prostanoid receptors (423) was of paramount importance in the identification of L-644,698, and can be used as a screen to identify other novel, selective compounds.

However, the utility of such a panel of receptors is not limited to the prospective identification of novel compounds. The selectivity data that is consequentially generated for both selective and non-selective compounds can also be used to make retrospective inferences about data from prior studies. For instance, the original investigation into the DP-mediated activation of human platelet adenylate cyclase reported that PGD_2 and

BW245C were functioning in a biphasic manner, first through the hDP receptor and then through another receptor which was suggested to be the hIP receptor (535). This speculation is probably flawed, based on the data available from the panel of recombinant human prostanoid receptors, since neither PGD_2 and nor BW24C have affinity for the hIP receptor at the concentrations which were tested (up to 100 and 25 μM, respectively). Rather, it is likely that the second phase of activation is mediated through hEP receptors (probably hEP_4) since: i) both PGD_2 and BW245C have much higher affinities for hEP_4 than for hIP (PGD_2: 1500 and > 100 000 nM; BW245C: 132 and > 25 000 nM, respectively) and ii) the compounds used in the earlier study and deemed to be hIP selective actually demonstrate cross-reactivity with hEP receptors (i.e. carbacyclin is equipotent at hIP (290 nM) and hEP_4 (350 nM)). Moreover, the presence of EP_4 receptors on human platelets has been confirmed in a recent study (536).

The current work also addresses the activity of members of the PGJ series of compounds (PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2), which are metabolites of PGD_2 *in vivo*. These compounds, especially 15-deoxy-$\Delta^{12,14}$-PGJ_2 (hereafter 15d-PGJ_2), were recently identified as ligands for the γ isoform of the peroxisome proliferator-activated receptor (PPARγ) (537, 538). As part of the nuclear receptor family, the PPAR family of receptors (PPARα, PPARδ, PPARγ) function as ligand-activated transcription factors that regulate gene expression of enzymes involved in lipid homeostasis (539). Binding of 15d-PGJ_2 to PPARγ expressed in fibroblasts promotes their differentiation to adipocytes (537, 538). The concentration of 15d-PGJ_2 required to facilitate this process is 5-10 μM (EC_{50} = 7 μM) (538), which is ~ 20-fold higher than that shown here to stimulate cyclic AMP accumulation at hDP (300 nM). Although micromolar concentrations seem unreasonably high and physiological concentrations have not yet been determined for 15d-PGJ_2, PGJ series metabolites induce cell cycle arrest (540) and apoptosis (541) at similar concentrations. As well, the EC_{50} value for LTB_4 necessary for PPARα activation is in the same range, at 1-5 μM (539). When added exogenously, high ligand concentrations may be required to concentrate enough of the ligand intracellularly at the receptor site before an effect is observed. A specific plasma membrane transporter for

cyclopentenone PGs (PGA and PGJ series compounds) has been identified (542). Depending on concentrations of the PGJ series of compounds that exist extracellularly and/or the efficiency of the cyclopentenone transporter *in vivo*, the current data indicate that these compounds may also be physiologically active at the DP receptor. PGJ_2 and Δ^{12}-PGJ_2 might be particularly relevant, since they exhibited EC_{50} values of 1 and 90 nM in the stimulation of cyclic AMP accumulation, respectively. This is especially interesting in light of the recent evidence for the presence of DP receptors in the nuclear membrane fraction of porcine brain (543).

7.2. A novel biological role for prostaglandin D_2 is suggested by distribution studies of the rat DP prostanoid receptor

The DP receptor was the last known homologue of the prostanoid receptors to be cloned from the rat, making it a natural focus for investigators working in the prostanoid receptor field. Just prior to submission of the current work for publication, another group reported the cloning of rDP (544). However, this study did not demonstrate functional expression of the cloned cDNA, and instead the investigators turned their attention to an *in situ* hybridization analysis of rDP limited to the brain and the eye. As alluded to earlier, the reason for this may be that the investigators were unable to functionally express this cDNA. Comparison of the sequence identified in the current work with that of Gerashchenko et al. (1998) highlights two amino acid substitutions [D for H at position 71 and T for A at position 334 (position 338 when aligned to hDP)]. Based on homology to the cloned human DP receptor, for which hydropathy analysis has been performed (368), these residues lie in the second transmembrane domain and carboxyl tail of the receptor, respectively (see Figure 7.2.). The D residue is conserved amongst many GPCRs, as previously noted. The H for D substitution at this position is nonconservative, and has been shown to impair both G protein coupling and downstream signal transduction of the rat angiotension II type I receptor (545).

The importance of this conserved D residue is more apparent when a bioinformatics database is consulted, such as the TinyGRAP database of GPCR clinical and mutagenesis studies (http://www-grap.fagmed.uit.no/GRAP/homepage.html) (546). This database documents many studies of the D residue conserved at this position. The substitution of N for D (which is more conservative than H for D) has generally disruptive effects on different GPCRs. In fact, the N for D substitution impairs the G protein-coupling and signal transduction of a variety of receptors that couple to different G proteins and different effectors such as the rat ATII type I (545), human muscarinic M1 (547), rat neuropeptide Y type 1 (548), human PAF (549), human β_2-AR (550), and human serotonin 1A (551) receptors. In many cases this substitution effects the binding of agonists but not antagonists, implicating a general role for D in G protein coupling that may be independent of G protein subtype. This is highlighted by a study addressing the porcine α_2-AR, which normally couples to three different G proteins: $G\alpha_s$, $G\alpha_i$, and

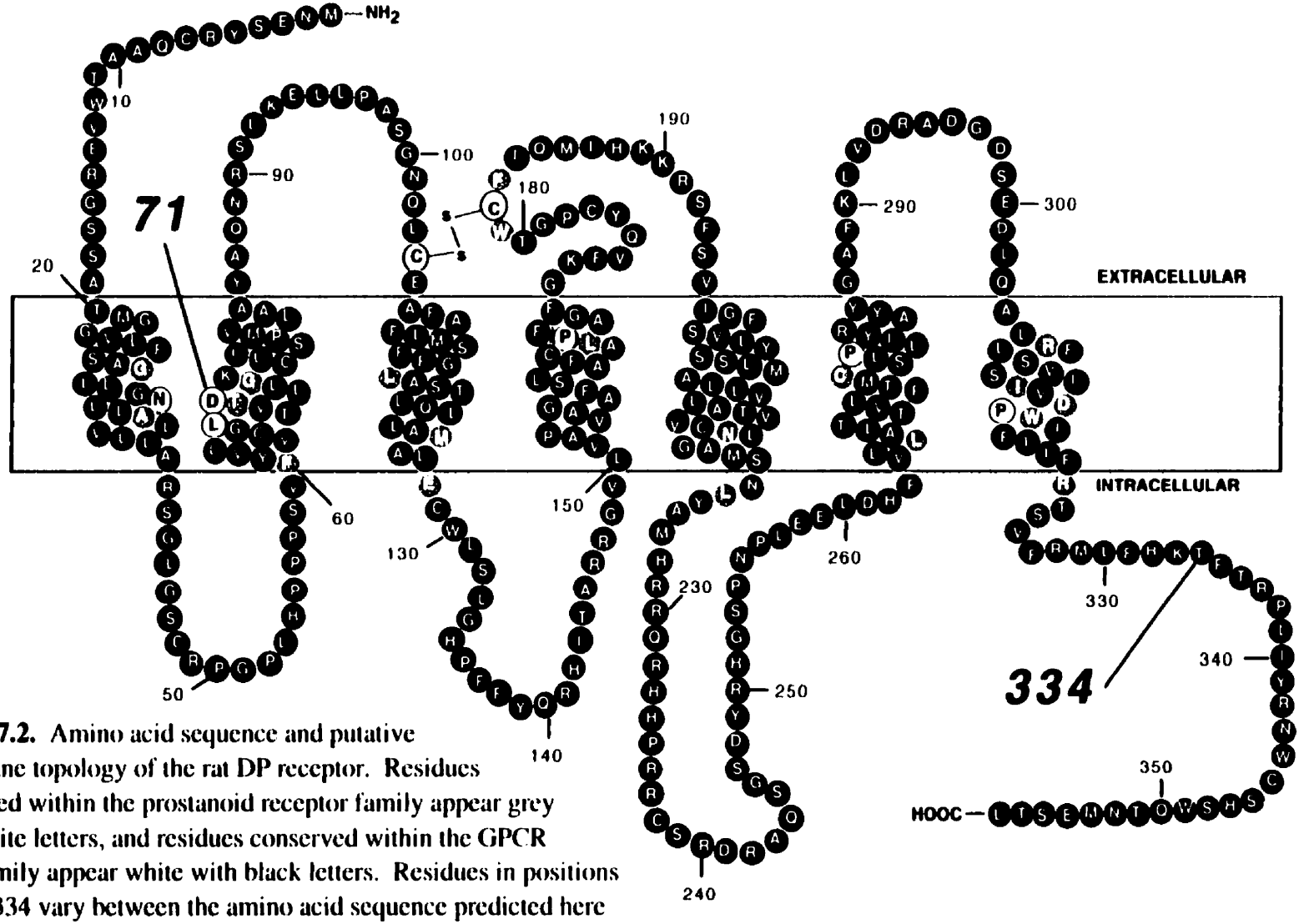

Figure 7.2. Amino acid sequence and putative membrane topology of the rat DP receptor. Residues conserved within the prostanoid receptor family appear grey with white letters, and residues conserved within the GPCR superfamily appear white with black letters. Residues in positions 71 and 334 vary between the amino acid sequence predicted here and a previously published sequence submitted to GenBank™ under accession number U92289 (see text for details).

$G\alpha_q$. The N for D substitution reduces the efficiency of coupling between α_2-AR and all three of these G proteins compared to the wildtype receptor (552). Even the conservative substitution of Q for D can be detrimental to the functional expression of some GPCRs, such as the rat neuropeptide Y type 1 (548) and the human chemokine CXCR2 (553) receptors. Thus, evidence presented here argues for a general role in GPCR structure and/or function for this conserved D in transmembrane domain II, which may extend to the rDP receptor. It is quite possible that the second substitution identified between the two rDP cDNAs (A for T) is also deleterious to the functional expression of the rDP receptor. However, this T residue is not conserved between GPCRs and has not been routinely studied.

Ligand binding and signal transduction (cyclic AMP accumulation) assays were performed as part of the characterization of the functional expression of rDP in this study, and hDP was used as a positive control in all experiments. Differences in the rank order of affinity were observed between the two receptors, specifically for the novel DP agonist L-644,698. For rDP and hDP, the rank orders of affinity were PGD_2 = BW245C > L-644,698 and L-644,698 > PGD_2 = BW245C, respectively. All the DP agonists demonstrated higher affinity for hDP than for rDP. PGD_2 and BW245C demonstrated a decreased affinity for rDP of 4 and 8-fold, respectively. However, L-644,698 demonstrated a decrease of ~180-fold in its affinity for rDP. A change in the rank order of affinity for species homologues of DP is also apparent for BW245C at mDP (424) when compared with hDP (423, 554), though the values are less disparate. BW245C has 2- to 4-fold more affinity than PGD_2 for hDP, but 12-fold less affinity than PGD_2 for mDP. In addition to rank order of potency, the degree of receptor selectivity exhibited by a ligand can also change between species homologues of receptors. BW245C is ~6-fold more selective for mDP over mFP. However, its selectivity changes to > 25 000-fold for hDP over hFP. It would be of obvious interest to determine the affinity between L-644,698 and the panel of recombinant mouse prostanoid receptors, to determine if its selectivity for DP is maintained in this system. Changes in ligand rank order of affinity have been observed for other receptors, both prostanoid and otherwise. For instance, the agonist M&B-28767 exhibits species-dependent differences in selectivity for EP

receptors between human and mouse (EP_3 >> EP_4 > EP_1 > EP_2 and EP_3 >>> EP_1 > EP_4 >> EP_2, respectively). The species selectivity of chemically distinct nonpeptide antagonists for the human neurokinin-1 receptor over the rat homologue (15-76-fold) has been shown to be dependent on two divergent residues. Interestingly, these residues do not effect the natural peptide ligand (555). Elsewhere, a single point mutation that increases the affinity of four human serotonin receptors (5-HT_{1D}, 5-HT_{1B}, 5-ht_{1e}, and 5-ht_{1f}) for β-AR antagonists (propanolol and pindolol) but leaves the affinity for serotonin unchanged from wildtype receptors (556) has been identified.

The current work also addressed the cell-specific localization of DP mRNA in the CNS and GI tract of the rat. *In situ* hybridization identified positive signals in the brain, spinal cord, and various GI tract tissues. A rigorous discussion of the latter findings (those in the spinal cord and GI tract) is presented in the body of the current study. Therefore, the findings in the brain will be the focus of the present discussion. DP mRNA was localized in the rat brain to the leptomeninges and, for the first time, to the choroid plexus. These two structures are interconnected by foramina and are exposed to the CSF that is generated by the choroid plexus. Recently, the signal transduction of PGD_2 in promoting sleep has been studied in the rat. The immediate early gene Fos (considered a marker for distinguishing between wake- and sleep-active neurons (557)) was studied to neuroanatomically identify the neurons activated following PGD_2 infusion into the subarachinoid space of the rostral basal forebrain (558). Fos immunoreactivity was detected in the ventrolateral preoptic area (VLPO), which lies in the periphery of the brain next to the subarachinoid space, and was positively correlated with the preceding amount of sleep and negatively correlated with Fos expression in the tuberomammillary nucleus, which is the source of the histaminergic arousal system. The investigators surmised that PGD_2 induces sleep via peripheral leptomeningeal DP receptors with subsequent activation of the VLPO. However, this model (559) of PGD_2-regulated sleep is probably incomplete.

The choroid plexus is the major site of L-PGDS mRNA expression in the brain (560), though the L-PGDS protein is subsequently secreted into and circulates within the CSF (561). The presence of DP receptor mRNA in the choroid plexus observed here is

supported by earlier reports addressing the distribution of the DP receptor by ligand binding autoradiography (562, 563). The DP receptor of the choroid plexus is probably involved in sleep promotion since PGD_2 infusion into the subarachinoid space induces slow-wave sleep (SWS) without rapid eye movement (REM) sleep, while PGD_2 infusion into the brain ventricles that contain the choroid plexus induces both SWS and REM sleep (564, 565). Thus, depending on the site of PGD_2 administration there is a clear distinction in the type of sleep observed. A contribution by the choroid plexus toward sleep is further supported by studies demonstrating increased glucose utilization in this organ compared to the rest of the brain during sleep (566). The choroid plexus did not demonstrate any Fos immunoreactivity following PGD_2 administration into the subarachinoid space (558), suggesting perhaps a lack of cerebroventricular penetrance of the infused PGD_2 or that the choroid plexus uses another mechanism of action in the promotion of sleep.

7.3. The human prostanoid DP receptor stimulates mucin secretion in LS174T cells

Central to this body of work is the endogenous expression of the DP receptor in the human colonic adenocarcinoma cell line, LS174T. Pharmacological characterization of the endogenously expressed hDP receptor in LS174T cells involved ligand binding and signal transduction assays. The binding experiments clearly showed that hDP is endogenously expressed in these cells. However, in signal transduction assays all DP agonists exhibited EC_{50} values which were 100-fold less potent than those observed against the recombinant hDP receptor (554). Moreover, both L-644,698 and PGJ_2 performed as partial agonists. PGJ_2 functioned as a partial agonist in a previous study addressing activity against the DP receptor endogenously expressed in human non-pregnant myometrium (567). Low receptor number and poor receptor-G protein coupling in these cells could explain these observations. Indeed, L-644,698 and PGJ_2 may exhibit low intrinsic efficacy (568) and might be incapable of facilitating coupling as efficiently as PGD_2 or BW245C. Under this premise, L-644,698 and PGJ_2 would need to initiate more individual receptor-G protein interactions to function as full agonists. Although feasible in a high receptor expression system, such as that observed for recombinant hDP (554), this might be unachievable in a system of lower receptor expression due to an insufficient receptor reserve. This premise could be tested by artificially increasing the amount of receptor expressed in LS174T cells, and then reassessing the degree of efficacy exhibited by L-644,698 and PGJ_2 in the stimulation of cyclic AMP production. Alternatively, these compounds could be tested in a system where the recombinant hDP receptor is expressed at a variety of levels.

The ligand binding experiments clearly showed that hDP is endogenously expressed in LS174T cells. However, the value for L-644,698 in this regard was 10-fold lower than that demonstrated at the recombinant DP receptor in HEK 293(EBNA) cells. Differences in receptor number would not explain this observation, since high affinity binding as predicted by the ternary complex model for GPCRs (569) is independent of receptor number, instead being dependent on the ligand, receptor and G-protein under study. Based on this model, the disparity between the affinities observed in the two

systems could only have arisen from differences in either the receptor or G-protein, since the ligand remained unchanged. Differences at the level of the receptor might be explained by the existence of splice variants for hDP, for which the following evidence exists. In the human myometrium (567), the pK_B values for antagonism mediated by BW A868C were dependent on the agonist studied and its concentration. In the rabbit eye (570), BW A868C was observed to antagonize the increase in conjunctival microvascular permeability mediated by PGD_2 but not by BW245C. In the canine colon (571), BW245C did not mimic the inhibitory effects of PGD_2 on short-circuit current and was not significantly inhibited by BW A868C, but instead produced dose-dependent increases in short-circuit current. Interestingly, all these reports are based on observations made with synthetic ligands (usually BW245C), suggesting perhaps that the issue is the cross-reactivity of these ligands rather than a novel DP receptor subtype. After all, by definition this novel splice variant of DP would have to bind the endogenous ligand, PGD_2. Also, none of the receptors phylogenetically related to DP have been found to be subject to this form of receptor heterogeneity. However, particularly dissuasive in the argument for DP receptor splice variants is the fact that cyclic AMP production is observed to be the single, predominating signal transduction pathway reported for the DP receptor, both endogenous and recombinant alike. PGD_2 has been reported to increase intracellular Ca^{2+} concentrations, however, this was observed to occur through the FP receptor (572) or in a cyclic AMP-dependent, post receptor manner (573). Notably, none of the species homologues of recombinant DP have been reported to activate signal transduction mechanisms other than cyclic AMP production.

Thus, differences at the level of the G-protein most probably explain the dissimilarities observed in affinity and potency values between endogenous and recombinant DP. Specifically, this could be related to the cell-type specific spectrum of $G\beta$ and $G\gamma$ subunits with which the $G\alpha_s$ subunit can associate. If certain heterotrimeric G-protein complexes associate more effectively with the receptor, this could translate into a shift in affinity and potency values. Specific agonists (i.e. L-644,698) may be more or less sensitive to this, depending on their contact points on the receptor. The more general effects on the functional activity of the receptor, specifically the ~100-fold shift in the EC_{50} values observed for all the DP agonists studied, might also be explained at the level

of the G-protein. For instance, the expression of different isozymes of adenylate cyclase (for which at least 9 isoforms exist) or of different RGS proteins (regulators of G-protein signaling; responsible for GTPase activation) might explain the dissimilar potencies observed for all DP agonists between the two systems.

In the current work, BW A868C demonstrated insurmountable antagonism at the hDP receptor. This compound had previously been described as a competitive, surmountable antagonist of the DP receptor (535). As mentioned previously, this difference in antagonism may be a product of the kinetics of formation of the antagonist-receptor complex, which has been suggested to explain the antagonism by candesartan of the angiotensin II AT_1 receptor (574). Candesartan clearly exhibited insurmountable antagonism of the ATII-mediated accumulation of inositol phosphate. Kinetic experiments indicated that the antagonism mediated by candesartan increases with preincubation time and is optimal following prolonged incubation, while that mediated by the surmountable antagonist losartan is independent of preincubation time. Investigators demonstrated the attenuation of the effects of candesartan in the presence of losartan suggesting that they share the same binding site on the receptor, and therefore dismissed the possibility of candesartan inducing allosteric modulation of AT_1 receptors as its mechanism of action. Similar experiments would clarify the type of antagonism mediated by BW A868C. However, another well-characterized DP-specific antagonist has not yet become available.

Molecular biological and pharmacological confirmation of the endogenous expression of hDP validated the use of the LS174T cell line to study hDP in its ability to stimulate mucin secretion. An alternative to using a cultured cell line for this purpose is to use explanted tissue preparations from the organ(s) of interest. Both model systems have caveats associated with them (575). Explanted tissue preparations are inherently variable since they contain a mixture of cell types and they are derived from various animals. As well, the mechanical manipulation involved in the harvest of tissue explants can itself result in mucin secretion. In contrast, cultured cell lines are transformed and are perhaps not as representative of the relevant *in vivo* physiology. However, they

inherently require less manipulation prior to experimentation. As well, the number of stimuli to which most cultured cell lines respond and the response patterns observed suggest a high degree of similarity to the parent tissue (475). Human colonic cancer cell lines represent normal intestinal cells in many aspects such as cell polarity, restricted junctional permeability, enterocytic differentiation, as well as in water, ion and mucin secretion (576). Much of the progress in the field of mucin secretion has been made using cultured epithelial cells, such as LS174T, as *in vitro* model systems.

The measurement of the secreted mucin in the current work was facilitated by exposing cells to the radiolabelled mucin precursor [^{3}H]glucosamine for a period of 48 hours prior to experimentation. Immunological and microscopic techniques have also been used to assess mucin secretion, and each is better suited for certain endpoints (475). As mentioned previously, mucins from the same gene can be heterogeneous in nature due to variability in post-translational glycosylation. Immunological methods have been used to detect mucin secretion but are highly sensitive to this variability. They have found utility in the detection of certain mucin glycoforms as cancer markers. Classically, microscopic methods have been qualitative rather than quantitative, though computer-enhanced microscopy is now making quantitation a possibility. Microscopy has proven useful in assessing the aforementioned event of compound exocytosis. The current study involved the overall measurement of mucin secretion and the use of a general radiolabelled mucin precursor was judged to best facilitate this. Although glucosamine is a component of other glycoproteins such as proteoglycans, experiments performed here with and without gel filtration chromatography demonstrate that the vast majority of the radiolabelled protein collected in the media following mucin secretion is high molecular weight material, which corresponds to mucins. This has been previously documented (518) and has been corroborated by immunological methods (577).

Work in this laboratory provided the first evidence that the stimulation of mucin secretion might be mediated through the DP receptor (578). The current study confirmed this in an *in vitro* cell model. As noted in the body of the study, no prior results have demonstrated a link between gastrointestinal mucous secretion and either PGD_2 or the DP receptor. In fact, only two other studies suggest that PGD_2 may mediate mucin secretion.

In contrast to the current findings, both suggest the involvement of a receptor other than DP. In the rabbit conjunctiva, a decrease in the goblet cell population has been associated with PGD_2 and PGJ_2 administration (579). However, since administration of the DP-specific agonist BW245C did not stimulate goblet cell depletion, the investigators surmised that the mucin secretion induced by PGD_2 and PGJ_2 was not a DP receptor-mediated event. In another study, mucous secretion from human lung explants was not significantly enhanced by exogenous administration of PGD_2 except at a concentration of 100 μM, which the investigators themselves deemed to be a nonphysiological effect (580). It would be of interest to test L-644,698 in these preparations since PGD_2 has high affinity for the hFP receptor (423, 554) and $PGF_{2\alpha}$ is a potent mucin secretagogue (581), especially in the human lung (580). There is evidence for [^{3}H]PGD_2 binding to the serous cells of the bovine trachea (575), which contain antibacterial proteins (i.e. lysozyme). Perhaps the DP receptor has a more general role in exocytosis, which might include serous cell secretion.

This study does not provide a physiological context for DP-mediated mucin secretion, which would be the next logical extension of the current work. The DP receptor probably does not play a role in the mucin secretion of the normal resting colon, since PGI_2 is the major prostanoid formed under these conditions (582, 583). However, DP may function to heighten colonic mucin secretion as part of the host's response to gastrointestinal inflammation, such as during inflammatory bowel disease (IBD). This is based on the following analogy. A role for PGD_2 in both the immediate and late phases of allergic-type reactions (i.e. atopic rhinitis, bronchial asthma) is well documented in the literature (584). Central to its role in the allergic inflammation of the immediate phase reaction is the release of PGD_2 from mast cells, along with other potent preformed inflammatory mediators (i.e. histamine and LTs). A similar mechanism of action has been suggested to describe the role of eicosanoids in intestinal electrolyte transport [borrowed from Powell (1991) and modified by Eberhart & Dubois (1995)]. Here, primary stimuli (i.e. a microorganism) would stimulate the production of mediators of inflammation (i.e. PGs) from primary effector cells (i.e. mast cells). The inflammatory mediators formed would act to directly or indirectly influence ion flux (i.e. Cl^-). PGD_2-

mediated mucin secretion through the DP receptor might be explained through an extension of this model (see Figure 7.3.).

In support of this, the primary second messenger mediating Cl^- ion flux is cyclic AMP (585, 586), which is the signal transduction pathway activated by the DP receptor. Furthermore, cells may integrate Cl^- ion secretion and mucin granule exocytosis to maximize their secretory response (475). In parasite rejection models (i.e. *Nippostongylus brasiliensis*), a significant increase in the number of goblet cells and mast cells has been noted preceding expulsion of the parasite (587, 588). Mast cell degranulation has been noted at the point of expulsion in the parasite rejection model (588) and clinically, in the small intestine of Crohn's disease patients (589). The other preformed inflammatory mediators that are released following mast cell degranulation also provide corroborative evidence. Histamine is released in hypersensitivity reactions in the guinea pig intestinal mucosa, and enhances Cl^- ion secretion (590). Histamine also stimulates mucous secretion in the human colon (591). LTs are correlated with fluid hypersecretion in a number of species (592, 593), though they do not induce mucous secretion from rabbit intestinal goblet cells (594).

Many of the observations for the individual inflammatory mediators are undoubtedly species-specific. For instance, PGD_2 does not function as a mucin secretagogue in mouse mucosal explants (595). Additionally, the various contributions of genetic abnormalities and pathogenic antigens to IBD still need to be fully understood (596). In fact, it is not even clear whether mast cell-derived inflammatory mediators help or hinder the development of IBD. A recent report describes the suppression of the development of an experimental colitis model in genetically mast cell-deficient rats, suggesting that the mast cells themselves contribute to the development of the disease (597). As mentioned in the introduction, fluid and mucous hypersecretion may deplete the host's defense system, allowing the invading pathogen to colonize. In contrast, NSAIDs do not improve IBD but instead aggravate it (598, 599). Nonetheless, it is clear that eicosanoids can modulate the state of gastrointestinal inflammation (296). The current results warrant further investigation into a possible role *in vivo* for DP-mediated mucin secretion.

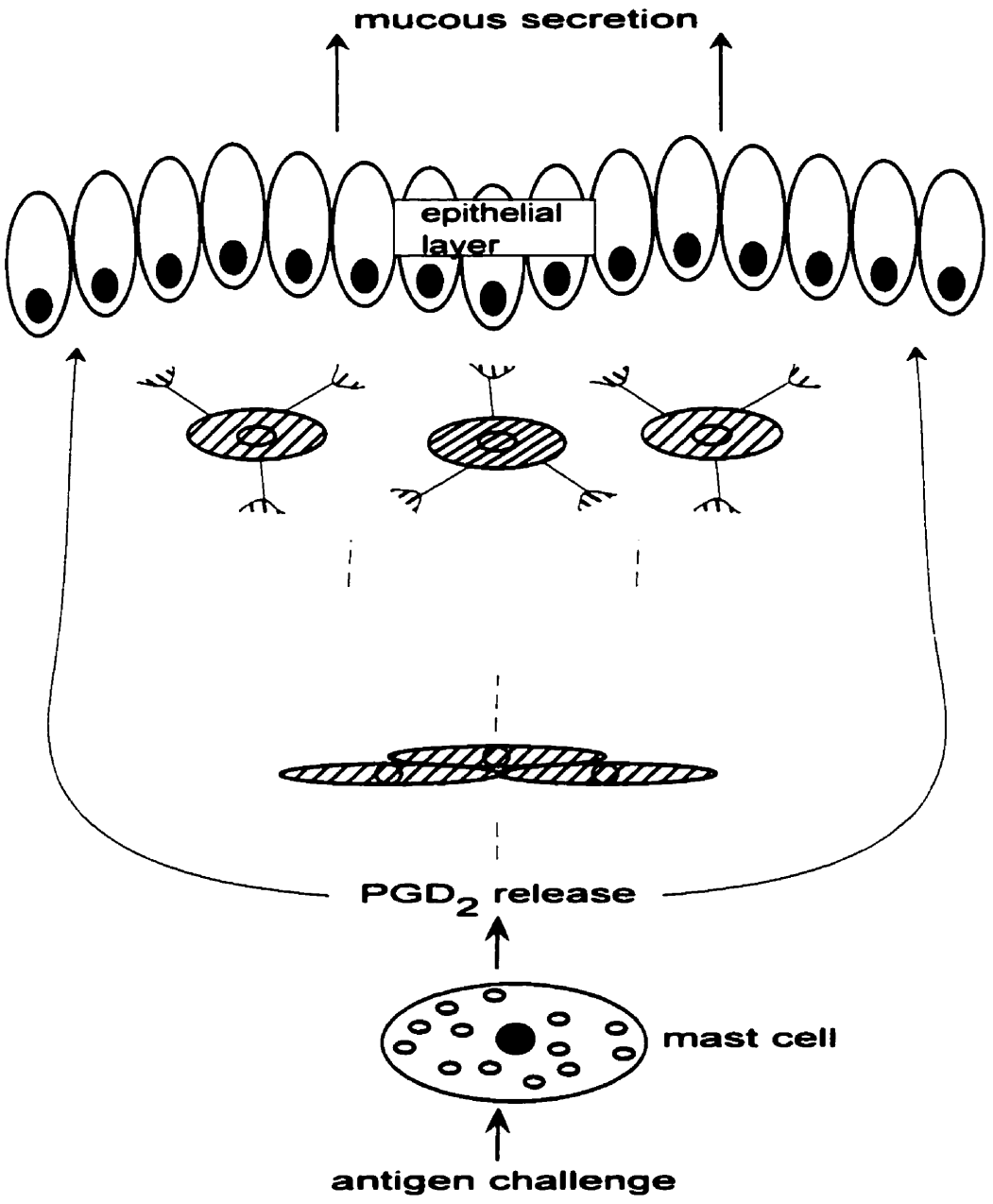

Figure 7.3.
A possible model for DP-mediated mucin secretion *in vivo*. The DP receptor may stimulate mucous secretion in a manner analogous to a model proposed by Powell (1991) for the role of eicosanoids in intestinal electrolyte transport. Here, mediators released by primary effector cells (i.e. mast cells) may directly stimulate epithelial cell secretions. An indirect pathway for intestinal electrolyte transport has also been demonstrated, whereby an elaboration of mediator release occurs due to stimulation of mesenchymal cells (i.e. fibroblasts). These mediators can act as neuromodulators of acetylcholine (Ach)-stimulated secretions. The *in vitro* observations made in the current research only lend support to the direct pathway of DP-mediated mucin secretion. Thus, the tentative applicability of the rest of the model to DP is symbolized by the sequence of hatched figures connected by dashes instead of arrows.

8. ORIGINAL CONTRIBUTIONS TO THE LITERATURE

Elements of the thesis that constitute original scholarship and an advancement of knowledge include the following:

1. Presented here is the first comprehensive comparison of the binding selectivity of a variety of the currently accepted DP-specific prostanoids and synthetic prostanoid analogues within a previously characterized system of recombinant human prostanoid receptors.

2. L-644,698 has been identified as a novel DP-specific agonist. Its pharmacological characterization within the aforementioned system of recombinant human prostanoid receptors has confirmed that it is one of the most highly selective DP-specific agonists identified to date, making it a valuable tool for discerning the contributions of the DP receptor in heterogeneous receptor systems.

3. The molecular cloning and functional expression of the rat DP receptor has been accomplished. Pharmacological characterization using prostanoids and synthetic prostanoid analogues, including L-644,698, has confirmed its identity as a species homologue of the DP receptor.

4. *In situ* hybridization using a cDNA representing the novel rat DP receptor has identified the cell-specific localization of mRNA corresponding to rat DP in the mucous-secreting goblet cells of various tissues of the gastrointestinal tract, including the stomach, small intestine, and colon. This led to the hypothesis of a novel biological activity for the DP receptor, namely the regulation of mucin secretion. Using these same *in situ* hybridization conditions in the central nervous system, rat DP-specific mRNA has been localized to the choroid plexus of the cerebroventricular system, as well as to the motor and sensory neurons of the lumbar spinal cord.

5. The endogenous functional expression of the human DP receptor has been demonstrated in LS174T cells, an established *in vitro* model for mucin secretion of colonic origin. The stimulation of mucin secretion has been confirmed to result from activation of the DP receptor. This has been demonstrated pharmacologically through the use of DP-specific agonists (including L-644,698) and a DP-specific antagonist.

9. REFERENCES & ENDNOTES

1. R. Kurzrok, C. C. Lieb, *Proc Soc Exp Biol Med* **28**, 268-72 (1930).

2. M. W. Goldblatt, *J Soc Chem Ind Lond* **52**, 1056-7 (1933).

3. U. S. v. Euler, *Arch Int Pharmacodyn Ther* **202 (suppl)**, 295-307 (1973).

4. J. Harkavy, *Arch Intern Med* **45**, 641-6 (1930).

5. S. Bergstrom, J. Sjovall, *Acta Chem Scand* **11**, 1086 (1957).

6. W. P. Schneider, J. E. Pike, F. P. Kupiecki, *Biochim Biophys Acta* **125**, 611-3 (1966).

7. E. W. Horton, in *Chemistry, biochemistry and pharmacological activity of prostanoids* S. M. Roberts, F. Scheinmann, Eds. (Pergamon Press, Oxford, U.K., 1979) pp. 1-16.

8. B. Samuelsson, *Fed Proc* **31**, 1442-50 (1972).

9. M. Hamberg, B. Samuelsson, *Proc Natl Acad Sci U S A* **70**, 899-903 (1973).

10. M. Hamberg, P. Hedqvist, K. Strandberg, J. Svensson, B. Samuelsson, *Life Sci* **16**, 451-62 (1975).

11. S. Moncada, R. Gryglewski, S. Bunting, J. R. Vane, *Nature* **263**, 663-5 (1976).

12. J. R. Vane, *Nat New Biol* **231**, 232-5 (1971).

13. F. A. Kuehl, Jr., J. L. Humes, *Proc Natl Acad Sci U S A* **69**, 480-4 (1972).

14. C. V. Rao, *Prostaglandins* **6**, 533-6 (1974).

15. W. S. Powell, S. Hammarstrom, B. Samuelsson, *Eur J Biochem* **41**, 103-7 (1974).

16. W. B. Campbell, in *Goodman and Gilman's the pharmacological basis of therapeutics* A. G. Gilman, T. W. Rall, A. S. Nies, P. Taylor, Eds. (Pergamon Press, New York, 1990) pp. 600-17.

17. A. L. Willis, *Nutr Rev* **39**, 289-301 (1981).

18. F. H. Chilton, A. N. Fonteh, M. E. Surette, M. Triggiani, J. D. Winkler, *Biochim Biophys Acta* **1299**, 1-15 (1996).

19. R. F. Irvine, *Biochem J* **204**, 3-16 (1982).

20. P. Needleman, J. Turk, B. A. Jakschik, A. R. Morrison, J. B. Lefkowith, *Annu Rev Biochem* **55**, 69-102 (1986).

21. W. L. Smith, *Biochem J* **259**, 315-24 (1989).

22. R. D. Burgoyne, A. Morgan, *Trends Biochem Sci* **15**, 365-6 (1990).

23. G. Cirino, *Biochem Pharmacol* **55**, 105-11 (1998).

24. J. Balsinde, M. A. Balboa, P. A. Insel, E. A. Dennis, *Annu Rev Pharmacol Toxicol* **39**, 175-89 (1999).

25. W. L. Smith, *Am J Physiol* **263**, F181-91 (1992).

26. A. J. Habenicht, et al., *Nature* **345**, 634-6 (1990).

27. J. Axelrod, R. M. Burch, C. L. Jelsema, *Trends Neurosci* **11**, 117-23 (1988).

28. C. C. Leslie, *J Biol Chem* **272**, 16709-12 (1997).

29. E. A. Dennis, *Trends Biochem Sci* **22**, 1-2 (1997).

30. T. S. Kim, et al., *J Biol Chem* **272**, 2542-50 (1997).

31. A. Gassama-Diagne, J. Fauvel, H. Chap, *J Biol Chem* **264**, 9470-5 (1989).

32. M. Hattori, H. Arai, K. Inoue, *J Biol Chem* **268**, 18748-53 (1993).

33. L. W. Tjoelker, et al., *Nature* **374**, 549-53 (1995).

34. J. A. Tischfield, *J Biol Chem* **272**, 17247-50 (1997).

35. M. Murakami, et al., *J Biol Chem* **273**, 14411-23 (1998).

36. J. Ishizaki, et al., *J Biol Chem* **274**, 24973-9 (1999).

37. P. Vadas, J. Browning, J. Edelson, W. Pruzanski, *J Lipid Mediat* **8**, 1-30 (1993).

38. L. Arbibe, et al., *J Clin Invest* **102**, 1152-60 (1998).

39. J. Takasaki, et al., *FEBS Lett* **440**, 377-81 (1998).

40. J. Pfeilschifter, C. Schalkwijk, V. A. Briner, H. van den Bosch, *J Clin Invest* **92**, 2516-23 (1993).

41. M. Murakami, T. Kambe, S. Shimbara, I. Kudo, *J Biol Chem* **274**, 3103-15 (1999).

42. G. Atsumi, et al., *Biochim Biophys Acta* **1349**, 43-54 (1997).

43. R. S. Koduri, et al., *J Biol Chem* **273**, 32142-53 (1998).

44. J. D. Sharp, D. L. White, *J Lipid Mediat* **8**, 183-9 (1993).

45. K. Takayama, et al., *FEBS Lett* **282**, 326-30 (1991).

46. C. S. Ramesha, D. L. Ives, *Biochim Biophys Acta* **1168**, 37-44 (1993).

47. W. G. Hoeck, C. S. Ramesha, D. J. Chang, N. Fan, R. A. Heller, *Proc Natl Acad Sci U S A* **90**, 4475-9 (1993).

48. J. D. Clark, N. Milona, J. L. Knopf, *Proc Natl Acad Sci U S A* **87**, 7708-12 (1990).

49. C. C. Leslie, D. R. Voelker, J. Y. Channon, M. M. Wall, P. T. Zelarney, *Biochim Biophys Acta* **963**, 476-92 (1988).

50. J. D. Clark, A. R. Schievella, E. A. Nalefski, L. L. Lin, *J Lipid Mediat Cell Signal* **12**, 83-117 (1995).

51. L. L. Lin, A. Y. Lin, D. L. DeWitt, *J Biol Chem* **267**, 23451-4 (1992).

52. T. Nakamura, L. L. Lin, S. Kharbanda, J. Knopf, D. Kufe, *Embo J* **11**, 4917-22 (1992).

53. L. L. Lin, A. Y. Lin, J. L. Knopf, *Proc Natl Acad Sci U S A* **89**, 6147-51 (1992).

54. E. Rodewald, U. Tibes, G. Maass, W. Scheuer, *Eur J Biochem* **223**, 743-9 (1994).

55. M. Gijon, Z. Oiu, J. Kyriakis, C. Leslie, *Faseb J* **10**, A978 (1996).

56. M. Mosior, D. A. Six, E. A. Dennis, *J Biol Chem* **273**, 2184-91 (1998).

57. M. A. Lemmon, K. M. Ferguson, J. Schlessinger, *Cell* **85**, 621-4 (1996).

58. J. Y. Channon, C. C. Leslie, *J Biol Chem* **265**, 5409-13 (1990).

59. E. A. Nalefski, et al., *J Biol Chem* **269**, 18239-49 (1994).

60. A. R. Schievella, M. K. Regier, W. L. Smith, L. L. Lin, *J Biol Chem* **270**, 30749-54 (1995).

61. C. N. Serhan, J. Z. Haeggstrom, C. C. Leslie, *Faseb J* **10**, 1147-58 (1996).

62. M. R. Sierra-Honigmann, J. R. Bradley, J. S. Pober, *Lab Invest* **74**, 684-95 (1996).

63. L. L. Lin, et al., *Cell* **72**, 269-78 (1993).

64. X. X. Xu, C. O. Rock, Z. H. Qiu, C. C. Leslie, S. Jackowski, *J Biol Chem* **269**, 31693-700 (1994).

65. M. E. Doerfler, J. Weiss, J. D. Clark, P. Elsbach, *J Clin Invest* **93**, 1583-91 (1994).

66. M. Murakami, et al., *J Biol Chem* **270**, 22653-6 (1995).

67. N. Uozumi, et al., *Nature* **390**, 618-22 (1997).

68. J. V. Bonventre, et al., *Nature* **390**, 622-5 (1997).

69. S. L. Hazen, R. W. Gross, *J Biol Chem* **268**, 9892-900 (1993).

70. J. Tang, et al., *J Biol Chem* **272**, 8567-75 (1997).

71. E. J. Ackermann, E. S. Kempner, E. A. Dennis, *J Biol Chem* **269**, 9227-33 (1994).

72. J. Balsinde, I. D. Bianco, E. J. Ackermann, K. Conde-Frieboes, E. A. Dennis, *Proc Natl Acad Sci U S A* **92**, 8527-31 (1995).

73. J. Balsinde, E. A. Dennis, *J Biol Chem* **272**, 16069-72 (1997).

74. K. Karimi, M. R. Lennartz, *J Immunol* **155**, 5786-94 (1995).

75. M. J. Wolf, R. W. Gross, *J Biol Chem* **271**, 20989-92 (1996).

76. I. Fuse, T. Iwanaga, H. H. Tai, *J Biol Chem* **264**, 3890-5 (1989).

77. L. C. McPhail, C. C. Clayton, R. Snyderman, *Science* **224**, 622-5 (1984).

78. J. D. Morrow, et al., *Proc Natl Acad Sci U S A* **87**, 9383-7 (1990).

79. J. D. Morrow, T. M. Harris, L. J. D. Roberts, *Anal Biochem* **184**, 1-10 (1990).

80. L. J. Roberts, 2nd, R. G. Salomon, J. D. Morrow, C. J. Brame, *Faseb J* **13**, 1157-68 (1999).

81. D. R. Harder, A. R. Lange, D. Gebremedhin, E. K. Birks, R. J. Roman, *J Vasc Res* **34**, 237-43 (1997).

82. M. Rahman, J. T. Wright, Jr., J. G. Douglas, *Am J Hypertens* **10**, 356-65 (1997).

83. S. Yamamoto, *Biochim Biophys Acta* **1128**, 117-31 (1992).

84. C. D. Funk, *Biochim Biophys Acta* **1304**, 65-84 (1996).

85. H. Kuhn, B. J. Thiele, *FEBS Lett* **449**, 7-11 (1999).

86. S. Narumiya, J. A. Salmon, F. H. Cottee, B. C. Weatherley, R. J. Flower, *J Biol Chem* **256**, 9583-92 (1981).

87. J. Y. Vanderhoek, in *Lipoxygenases and their products* S. T. Crooke, A. Wong, Eds. (Academic Press, Inc., San Diego, CA, 1991) pp. 16175.

88. E. Sigal, et al., *Biochem Biophys Res Commun* **157**, 457-64 (1988).

89. E. Sigal, S. Dicharry, E. Highland, W. E. Finkbeiner, *Am J Physiol* **262**, L392-8 (1992).

90. K. van Leyen, R. M. Duvoisin, H. Engelhardt, M. Wiedmann, *Nature* **395**, 392-5 (1998).

91. M. I. Siegel, R. T. McConnell, N. A. Porter, P. Cuatrecasas, *Proc Natl Acad Sci U S A* **77**, 308-12 (1980).

92. Y. Takahashi, N. Ueda, S. Yamamoto, *Arch Biochem Biophys* **266**, 613-21 (1988).

93. P. Walstra, J. Verhagen, M. A. Vermeer, G. A. Veldink, J. F. Vliegenthart, *Biochim Biophys Acta* **921**, 312-9 (1987).

94. H. Kuhn, L. Chan, *Curr Opin Lipidol* **8**, 111-7 (1997).

95. S. J. Feinmark, J. A. Cornicelli, *Biochem Pharmacol* **54**, 953-9 (1997).

96. B. M. Vonakis, J. Y. Vanderhoek, *J Biol Chem* **267**, 23625-31 (1992).

97. H. Herbertsson, S. Hammarstrom, *Biochim Biophys Acta* **1244**, 191-7 (1995).

98. P. Borgeat, B. Samuelsson, *J Biol Chem* **254**, 7865-9 (1979).

99. C. H. Kellaway, E. R. Trethewie, *J Exp Physiol* **30**, 121-45 (1940).

100. C. A. Rouzer, S. Kargman, *J Biol Chem* **263**, 10980-8 (1988).

101. M. Abramovitz, et al., *Eur J Biochem* **215**, 105-11 (1993).

102. J. A. Mancini, et al., *FEBS Lett* **318**, 277-81 (1993).

103. T. Matsumoto, et al., *Proc Natl Acad Sci U S A* **85**, 26-30 (1988).

104. R. A. Dixon, et al., *Proc Natl Acad Sci U S A* **85**, 416-20 (1988).

105. K. M. Metters, *J Lipid Mediat Cell Signal* **12**, 413-27 (1995).

106. T. Yokomizo, T. Izumi, K. Chang, Y. Takuwa, T. Shimizu, *Nature* **387**, 620-4 (1997).

107. K. R. Lynch, et al., *Nature* **399**, 789-93 (1999).

108. C. N. Serhan, M. Hamberg, B. Samuelsson, *Proc Natl Acad Sci U S A* **81**, 5335-9 (1984).

109. C. N. Serhan, M. Hamberg, B. Samuelsson, J. Morris, D. G. Wishka, *Proc Natl Acad Sci U S A* **83**, 1983-7 (1986).

110. C. N. Serhan, et al., *J Biol Chem* **261**, 16340-5 (1986).

111. M. A. Wasserman, E. F. Smith III, D. C. Underwood, M. A. Barnette, in *Lipoxygenases and their products* S. T. Crooke, A. Wong, Eds. (Academic Press, Inc., San Diego, CA, 1991) pp. 1-36.

112. A. R. Brash, W. E. Boeglin, M. S. Chang, *Proc Natl Acad Sci U S A* **94**, 6148-52 (1997).

113. W. E. Boeglin, R. B. Kim, A. R. Brash, *Proc Natl Acad Sci U S A* **95**, 6744-9 (1998).

114. D. L. DeWitt, W. L. Smith, *Proc Natl Acad Sci U S A* **85**, 1412-6 (1988).

115. J. P. Merlie, D. Fagan, J. Mudd, P. Needleman, *J Biol Chem* **263**, 3550-3 (1988).

116. C. Yokoyama, T. Takai, T. Tanabe, *FEBS Lett* **231**, 347-51 (1988).

117. A. Raz, A. Wyche, N. Siegel, P. Needleman, *J Biol Chem* **263**, 3022-8 (1988).

118. A. Raz, A. Wyche, P. Needleman, *Proc Natl Acad Sci U S A* **86**, 1657-61 (1989).

119. J. Y. Fu, J. L. Masferrer, K. Seibert, A. Raz, P. Needleman, *J Biol Chem* **265**, 16737-40 (1990).

120. J. L. Masferrer, B. S. Zweifel, K. Seibert, P. Needleman, *J Clin Invest* **86**, 1375-9 (1990).

121. D. A. Kujubu, B. S. Fletcher, B. C. Varnum, R. W. Lim, H. R. Herschman, *J Biol Chem* **266**, 12866-72 (1991).

122. M. K. O'Banion, H. B. Sadowski, V. Winn, D. A. Young, *J Biol Chem* **266**, 23261-7 (1991).

123. T. Hla, K. Neilson, *Proc Natl Acad Sci U S A* **89**, 7384-8 (1992).

124. D. A. Jones, D. P. Carlton, T. M. McIntyre, G. A. Zimmerman, S. M. Prescott, *J Biol Chem* **268**, 9049-54 (1993).

125. R. N. Dubois, et al., *Faseb J* **12**, 1063-73 (1998).

126. W. L. Smith, R. M. Garavito, D. L. DeWitt, *J Biol Chem* **271**, 33157-60 (1996).

127. J. R. Vane, Y. S. Bakhle, R. M. Botting, *Annu Rev Pharmacol Toxicol* **38**, 97-120 (1998).

128. W. Xie, H. R. Herschman, *J Biol Chem* **270**, 27622-8 (1995).

129. J. Barnett, et al., *Biochim Biophys Acta* **1209**, 130-9 (1994).

130. W. E. Lands, J. Sauter, G. W. Stone, *Prostaglandins Med* **1**, 117-20 (1978).

131. D. Picot, P. J. Loll, R. M. Garavito, *Nature* **367**, 243-9 (1994).

132. W. L. Smith, D. L. DeWitt, in *Advances in Immunology* F. J. Dixon, Ed. (Academic Press, Orlando, FL, 1996), vol. 62, pp. 167-215.

133. C. C. Chan, et al., *J Pharmacol Exp Ther* **274**, 1531-7 (1995).

134. R. A. Copeland, et al., *Proc Natl Acad Sci U S A* **91**, 11202-6 (1994).

135. N. Futaki, et al., *Prostaglandins* **47**, 55-9 (1994).

136. J. K. Gierse, et al., *J Biol Chem* **271**, 15810-4 (1996).

137. E. Wong, C. Bayly, H. L. Waterman, D. Riendeau, J. A. Mancini, *J Biol Chem* **272**, 9280-6 (1997).

138. G. P. O'Neill, et al., *Mol Pharmacol* **45**, 245-54 (1994).

139. J. A. Mancini, D. Riendeau, J. P. Falgueyret, P. J. Vickers, G. P. O'Neill, *J Biol Chem* **270**, 29372-7 (1995).

140. M. E. Hemler, W. E. Lands, *J Biol Chem* **255**, 6253-61 (1980).

141. Y. Ren, D. S. Loose-Mitchell, R. J. Kulmacz, *Arch Biochem Biophys* **316**, 751-7 (1995).

142. Y. Ren, et al., *Arch Biochem Biophys* **323**, 205-14 (1995).

143. I. Song, W. L. Smith, *Arch Biochem Biophys* **334**, 67-72 (1996).

144. J. C. Otto, W. L. Smith, *J Biol Chem* **269**, 19868-75 (1994).

145. S. T. Reddy, H. R. Herschman, *J Biol Chem* **271**, 186-91 (1996).

146. M. Murakami, R. Matsumoto, Y. Urade, K. F. Austen, J. P. Arm, *J Biol Chem* **270**, 3239-46 (1995).

147. J. W. Han, H. Sadowski, D. A. Young, I. G. Macara, *Proc Natl Acad Sci U S A* **87**, 3373-7 (1990).

148. M. G. O'Sullivan, F. H. Chilton, E. M. Huggins, Jr., C. E. McCall, *J Biol Chem* **267**, 14547-50 (1992).

149. J. Sirois, D. L. Simmons, J. S. Richards, *J Biol Chem* **267**, 11586-92 (1992).

150. D. L. DeWitt, E. A. Meade, *Arch Biochem Biophys* **306**, 94-102 (1993).

151. A. Ristimaki, K. Narko, T. Hla, *Biochem J* **318**, 325-31 (1996).

152. T. S. Brannon, A. J. North, L. B. Wells, P. W. Shaul, *J Clin Invest* **93**, 2230-5 (1994).

153. K. Yamagata, K. I. Andreasson, W. E. Kaufmann, C. A. Barnes, P. F. Worley, *Neuron* **11**, 371-86 (1993).

154. A. I. Schafer, *J Clin Pharmacol* **35**, 209-19 (1995).

155. E. J. Zambraski, *Semin Nephrol* **15**, 205-13 (1995).

156. M. A. Trevethick, I. Oakley, N. M. Clayton, P. Strong, *Gen Pharmacol* **26**, 1455-9 (1995).

157. C. D. Breder, D. Dewitt, R. P. Kraig, *J Comp Neurol* **355**, 296-315 (1995).

158. I. Chakraborty, S. K. Das, J. Wang, S. K. Dey, *J Mol Endocrinol* **16**, 107-22 (1996).

159. L. Koehler, R. Hass, D. L. DeWitt, K. Resch, M. Goppelt-Struebe, *Biochem Pharmacol* **40**, 1307-16 (1990).

160. C. J. Smith, J. D. Morrow, L. J. D. Roberts, L. J. Marnett, *Biochem Biophys Res Commun* **192**, 787-93 (1993).

161. R. C. Harris, et al., *J Clin Invest* **94**, 2504-10 (1994).

162. R. M. Nusing, T. Klein, J. Pfeilschifter, V. Ullrich, *Biochem J* **313**, 617-23 (1996).

163. C. Cao, K. Matsumura, K. Yamagata, Y. Watanabe, *Brain Res* **733**, 263-72 (1996).

164. N. J. Gardiner, S. Giblett, B. D. Grubb, *Br J Pharmacol* **120**, 71P (1997).

165. M. Tsujii, R. N. DuBois, *Cell* **83**, 493-501 (1995).

166. M. K. Bauer, et al., *Eur J Biochem* **243**, 726-31 (1997).

167. C. E. Eberhart, et al., *Gastroenterology* **107**, 1183-8 (1994).

168. A. Ristimaki, N. Honkanen, H. Jankala, P. Sipponen, M. Harkonen, *Cancer Res* **57**, 1276-80 (1997).

169. M. L. Parrett, et al., *Int J Oncol* **10**, 503-7 (1997).

170. M. Oshima, et al., *Cell* **87**, 803-9 (1996).

171. S. M. Prescott, R. L. White, *Cell* **87**, 783-6 (1996).

172. R. Langenbach, et al., *Cell* **83**, 483-92 (1995).

173. S. G. Morham, et al., *Cell* **83**, 473-82 (1995).

174. J. E. Dinchuk, et al., *Nature* **378**, 406-9 (1995).

175. T. Shimizu, S. Yamamoto, O. Hayaishi, *J Biol Chem* **254**, 5222-8 (1979).

176. E. Christ-Hazelhof, D. H. Nugteren, *Biochim Biophys Acta* **572**, 43-51 (1979).

177. M. Ujihara, S. Tsuchida, K. Satoh, K. Sato, Y. Urade, *Arch Biochem Biophys* **264**, 428-37 (1988).

178. E. Christ-Hazelhof, D. H. Nugteren, D. A. Van Dorp, *Biochim Biophys Acta* **450**, 450-61 (1976).

179. Y. Urade, K. Watanabe, O. Hayaishi, *J Lipid Mediat Cell Signal* **12**, 257-73 (1995).

180. A. Nagata, et al., *Proc Natl Acad Sci U S A* **88**, 4020-4 (1991).

181. Y. Urade, et al., *J Biol Chem* **262**, 15132-6 (1987).

182. Y. Urade, et al., *Proc Natl Acad Sci U S A* **90**, 9070-4 (1993).

183. K. Watanabe, Y. Urade, M. Mader, C. Murphy, O. Hayaishi, *Biochem Biophys Res Commun* **203**, 1110-6 (1994).

184. T. Tanaka, et al., *J Biol Chem* **272**, 15789-95 (1997).

185. Y. Eguchi, et al., *Proc Natl Acad Sci U S A* **94**, 14689-94 (1997).

186. N. Eguchi, et al., *Proc Natl Acad Sci U S A* **96**, 726-30 (1999).

187. M. Ujihara, et al., *Arch Biochem Biophys* **260**, 521-31 (1988).

188. Y. Urade, et al., *J Biol Chem* **265**, 371-5 (1990).

189. Y. Urade, N. Fujimoto, M. Ujihara, O. Hayaishi, *J Biol Chem* **262**, 3820-5 (1987).

190. Y. Kanaoka, et al., *Cell* **90**, 1085-95 (1997).

191. T. Watanabe, S. Narumiya, T. Shimizu, O. Hayaishi, *J Biol Chem* **257**, 14847-53 (1982).

192. T. Ogorochi, M. Ujihara, S. Narumiya, *J Neurochem* **48**, 900-9 (1987).

193. K. Watanabe, K. Kurihara, Y. Tokunaga, O. Hayaishi, *Biochem Biophys Res Commun* **235**, 148-52 (1997).

194. P. J. Jakobsson, S. Thoren, R. Morgenstern, B. Samuelsson, *Proc Natl Acad Sci U S A* **96**, 7220-5 (1999).

195. K. Watanabe, et al., *Proc Natl Acad Sci U S A* **83**, 1583-7 (1986).

196. O. A. Barski, K. Watanabe, *FEBS Lett* **320**, 107-10 (1993).

197. K. Watanabe, et al., *Proc Natl Acad Sci U S A* **85**, 11-5 (1988).

198. W. Kuchinke, O. Barski, K. Watanabe, O. Hayaishi, *Biochem Biophys Res Commun* **183**, 1238-46 (1992).

199. Y. Urade, K. Watanabe, N. Eguchi, Y. Fujii, O. Hayaishi, *J Biol Chem* **265**, 12029-35 (1990).

200. H. Hayashi, Y. Fujii, K. Watanabe, Y. Urade, O. Hayaishi, *J Biol Chem* **264**, 1036-40 (1989).

201. T. Suzuki, et al., *J Biol Chem* **274**, 241-8 (1999).

202. N. Whittaker, et al., *Prostaglandins* **12**, 915-28 (1976).

203. M. Hamberg, J. Svensson, B. Samuelsson, *Proc Natl Acad Sci U S A* **72**, 2994-8 (1975).

204. D. L. DeWitt, W. L. Smith, *J Biol Chem* **258**, 3285-93 (1983).

205. S. Hara, et al., *J Biol Chem* **269**, 19897-903 (1994).

206. R. F. Shen, H. H. Tai, *J Biol Chem* **261**, 11592-9 (1986).

207. K. Ohashi, K. H. Ruan, R. J. Kulmacz, K. K. Wu, L. H. Wang, *J Biol Chem* **267**, 789-93 (1992).

208. I. Siegle, et al., *FEBS Lett* **347**, 221-5 (1994).

209. R. M. Tuder, et al., *Am J Respir Crit Care Med* **159**, 1925-32 (1999).

210. M. W. Geraci, et al., *J Clin Invest* **103**, 1509-15 (1999).

211. T. Tanabe, V. Ullrich, *J Lipid Mediat Cell Signal* **12**, 243-55 (1995).

212. R. Nusing, V. Ullrich, *Eicosanoids* **3**, 175-80 (1990).

213. S. H. Ferreira, J. R. Vane, *Nature* **216**, 868-73 (1967).

214. U. Diczfalusy, *Prog Lipid Res* **33**, 403-28 (1994).

215. S. E. Barrow, et al., *Prostaglandins* **28**, 743-54 (1984).

216. T. Sago, R. Nakayama, T. Okumura, K. Saito, *Biochim Biophys Acta* **879**, 330-8 (1986).

217. Y. Kikawa, S. Narumiya, M. Fukushima, H. Wakatsuka, O. Hayaishi, *Proc Natl Acad Sci U S A* **81**, 1317-21 (1984).

218. F. A. Fitzpatrick, M. A. Wynalda, *J Biol Chem* **258**, 11713-8 (1983).

219. E. Granstrom, M. Kumlin, in *Prostaglandins and related substances: a practical approach* C. Benedetto, R. G. McDonald-Gibson, S. Nigam, T. F. Slater, Eds. (IRL Press Ltd., Oxford, U.K., 1987) pp. 5-27.

220. B. Samuelsson, E. Granstrom, K. Green, M. Hamberg, S. Hammarstrom, *Annu Rev Biochem* **44**, 669-95 (1975).

221. E. Granstrom, M. Hamberg, G. Hansson, H. Kindahl, *Prostaglandins* **19**, 933-57 (1980).

222. E. Granstrom, B. Samuelsson, *J Am Chem Soc* **94**, 4380-1 (1972).

223. L. J. D. Roberts, A. R. Brash, J. A. Oates, *Adv Prostaglandin Thromboxane Leukot Res* **10**, 211-25 (1982).

224. L. J. D. Roberts, B. J. Sweetman, J. A. Oates, *J Biol Chem* **256**, 8384-93 (1981).

225. P. Westlund, E. Granstrom, M. Kumlin, A. Nordenstrom, *Prostaglandins* **31**, 929-60 (1986).

226. P. Piper, J. Vane, *Ann N Y Acad Sci* **180**, 363-85 (1971).

227. A. Hassid, *Am J Physiol* **243**, C205-11 (1982).

228. S. Hyslop, G. De Nucci, *Prostaglandins Leukot Essent Fatty Acids* **49**, 723-60 (1993).

229. R. Bicknell, B. L. Vallee, *Proc Natl Acad Sci U S A* **85**, 5961-5 (1988).

230. S. Xue, D. E. Brockman, D. M. Slater, L. Myatt, *Prostaglandins* **49**, 351-69 (1995).

231. A. Tay, P. Maxwell, Z. G. Li, H. Goldberg, K. Skorecki, *Biochem J* **304**, 417-22 (1994).

232. W. L. Smith, D. L. DeWitt, *Semin Nephrol* **15**, 179-94 (1995).

233. H. R. Herschman, *Biochim Biophys Acta* **1299**, 125-40 (1996).

234. M. Murakami, R. Matsumoto, K. F. Austen, J. P. Arm, *J Biol Chem* **269**, 22269-75 (1994).

235. W. R. Hansen, J. A. Keelan, S. J. Skinner, M. D. Mitchell, *Prostaglandins Other Lipid Mediat* **57**, 243-57 (1999).

236. F. Berthiaume, J. A. Frangos, *FEBS Lett* **308**, 277-9 (1992).

237. J. Kalman, et al., *Neurochem Res* **17**, 167-72 (1992).

238. H. Kawaguchi, et al., *Endocrinology* **135**, 1157-64 (1994).

239. N. L. Brown, S. A. Alvi, M. G. Elder, P. R. Bennett, M. H. Sullivan, *Placenta* **19**, 625-30 (1998).

240. L. Z. Bito, R. A. Baroody, *Am J Physiol* **229**, 1580-4 (1975).

241. V. L. Schuster, *Annu Rev Physiol* **60**, 221-42 (1998).

242. J. C. McGiff, et al., *Nature* **223**, 742-5 (1969).

243. P. J. Piper, J. R. Vane, J. H. Wyllie, *Nature* **225**, 600-4 (1970).

244. S. Moncada, R. Korbut, S. Bunting, J. R. Vane, *Nature* **273**, 767-8 (1978).

245. C. Robinson, J. R. Hoult, *Biochem Pharmacol* **31**, 633-8 (1982).

246. T. E. Eling, H. J. Hawkins, M. W. Anderson, *Prostaglandins* **14**, 51-60 (1977).

247. L. Z. Bito, R. A. Baroody, M. E. Reitz, *Am J Physiol* **232**, E383-7 (1977).

248. B. R. Pitt, J. R. Forder, C. N. Gillis, *J Pharmacol Exp Ther* **227**, 531-7 (1983).

249. M. W. Anderson, T. E. Eling, *Prostaglandins* **11**, 645-77 (1976).

250. W. Dawson, S. J. Jessup, W. McDonald-Gibson, P. W. Ramwell, J. E. Shaw, *Br J Pharmacol* **39**, 585-98 (1970).

251. L. Z. Bito, *Prostaglandins* **12**, 639-46 (1976).

252. U. Diczfalusy, S. E. Alexson, *J Lipid Res* **31**, 307-14 (1990).

253. J. M. D. Irish, *Am J Physiol* **237**, F268-73 (1979).

254. L. Z. Bito, *J Physiol (Lond)* **221**, 371-87 (1972).

255. J. F. Glatz, M. M. Vork, D. P. Cistola, G. J. van der Vusse, *Prostaglandins Leukot Essent Fatty Acids* **48**, 33-41 (1993).

256. A. K. Dutta-Roy, N. Gopalswamy, D. V. Trulzsch, *Eur J Biochem* **162**, 615-9 (1987).

257. H. Raza, J. R. Pongubala, S. Sorof, *Biochem Biophys Res Commun* **161**, 448-55 (1989).

258. N. Kanai, et al., *Science* **268**, 866-9 (1995).

259. U. S. von Euler, R. Eliasson, in *Prostaglandins* G. DeStevens, Ed. (Academic Press, New York, 1967), vol. 8, pp. 167.

260. S. Itoh, et al., *Mol Pharmacol* **50**, 738-42 (1996).

261. R. Lu, N. Kanai, Y. Bao, V. L. Schuster, *J Clin Invest* **98**, 1142-9 (1996).

262. M. Peters-Golden, A. Feyssa, *Am J Physiol* **264**, L438-47 (1993).

263. A. J. Marcus, B. B. Weksler, E. A. Jaffe, M. J. Broekman, *J Clin Invest* **66**, 979-86 (1980).

264. S. Karim, A. Habib, S. Levy-Toledano, J. Maclouf, *J Biol Chem* **271**, 12042-8 (1996).

265. S. L. Pfister, D. D. Deinhart, W. B. Campbell, *Hypertension* **31**, 206-12 (1998).

266. O. P. Barry, D. Pratico, J. A. Lawson, G. A. FitzGerald, *J Clin Invest* **99**, 2118-27 (1997).

267. A. J. Marcus, *Prog Hemost Thromb* **8**, 127-42 (1986).

268. J. Maclouf, et al., *Haemostasis* **26 Suppl 4**, 28-36 (1996).

269. R. A. Coleman, I. Kennedy, P. P. A. Humphrey, K. Bunce, P. Lumley, in *Membranes and receptors* P. G. Sammes, J. B. Taylor, Eds. (Pergamon Press, Oxford, U.K., 1990), vol. 3, pp. 644-714.

270. R. A. Coleman, in *Prostaglandins adn related substances: a practical approach* C. Benedetto, R. G. McDonald-Gibson, S. Nigam, T. F. Slater, Eds. (IRL Press Ltd., Oxford, U.K., 1987) pp. 267-78.

271. R. A. Lewis, et al., *J Immunol* **129**, 1627-31 (1982).

272. S. Ito, S. Narumiya, O. Hayaishi, *Prostaglandins Leukot Essent Fatty Acids* **37**, 219-34 (1989).

273. T. Ogorochi, et al., *J Neurochem* **43**, 71-82 (1984).

274. H. Giles, P. Leff, *Prostaglandins* **35**, 277-300 (1988).

275. M. Negishi, Y. Sugimoto, A. Ichikawa, *J Lipid Mediat Cell Signal* **12**, 379-91 (1995).

276. B. B. Fredholm, P. Hedqvist, *Br J Pharmacol* **47**, 711-8 (1973).

277. D. E. MacIntyre, J. L. Gordon, *Nature* **258**, 337-9 (1975).

278. M. Greaves, K. J. Ibbotson, D. Atkins, T. J. Martin, *Clin Sci* **58**, 201-10 (1980).

279. J. J. Reeves, R. Stables, *Br J Pharmacol* **86**, 677-84 (1985).

280. M. W. Musch, M. Field, R. J. Miller, J. S. Stoff, *Am J Physiol* **252**, G120-7 (1987).

281. R. A. Coleman, I. Kennedy, *Br J Pharmacol* **68**, 533-9 (1980).

282. M. Yamamoto, J. Hashimoto, H. Takaba, K. Miyake, *J Urol* **137**, 345-8 (1987).

283. G. L. Steele, P. C. Leung, *J Lipid Mediat* **6**, 509-13 (1993).

284. M. Hirata, F. Ushikubi, S. Narumiya, *J Lipid Mediat Cell Signal* **12**, 393-404 (1995).

285. H. Wise, R. L. Jones, *Trends Pharmacol Sci* **17**, 17-21 (1996).

286. P. V. Halushka, C. J. Allan, K. L. Davis-Bruno, *J Lipid Mediat Cell Signal* **12**, 361-78 (1995).

287. K. F. Chung, T. W. Evans, P. D. Graf, J. A. Nadel, *Eur J Pharmacol* **117**, 373-5 (1985).

288. K. T. Bunce, N. M. Clayton, *Br J Pharmacol* **91**, 23-9 (1987).

289. A. Bennett, *Nature* **231**, 536 (1971).

290. A. Robert, J. E. Nezamis, J. P. Phillips, *Am J Dig Dis* **12**, 1073-6 (1967).

291. A. Robert, J. E. Nezamis, J. P. Phillips, *Gastroenterology* **55**, 481-7 (1968).

292. A. Bennett, I. F. Stamford, H. L. Stockley, *Br J Pharmacol* **61**, 579-86 (1977).

293. A. Aly, C. Johansson, P. Slezak, K. Green, *Biochem Med* **31**, 319-31 (1984).

294. C. J. Hawkey, D. S. Rampton, *Gastroenterology* **89**, 1162-88 (1985).

295. G. S. Smith, G. Warhurst, L. A. Turnberg, *Biochim Biophys Acta* **713**, 684-7 (1982).

296. C. E. Eberhart, R. N. Dubois, *Gastroenterology* **109**, 285-301 (1995).

297. K. Seibert, et al., *Proc Natl Acad Sci U S A* **91**, 12013-7 (1994).

298. S. Kargman, et al., *Gastroenterology* **111**, 445-54 (1996).

299. Y. Kishimoto, et al., *Life Sci* **60**, L127-33 (1997).

300. B. K. Reuter, S. Asfaha, A. Buret, K. A. Sharkey, J. L. Wallace, *J Clin Invest* **98**, 2076-85 (1996).

301. A. Robert, *Adv Prostaglandin Thromboxane Res* **2**, 507-20 (1976).

302. A. Robert, *Gastroenterology* **77**, 761-7 (1979).

303. J. S. Redfern, E. Lee, M. Feldman, *Gastroenterology* **92**, 969-77 (1987).

304. M. Feldman, *Am J Med Sci* **300**, 116-32 (1990).

305. A. K. Banerjee, J. Phillips, W. M. Winning, *Nat New Biol* **238**, 177-9 (1972).

306. G. L. Kauffman, Jr., B. J. Whittle, D. Aures, J. R. Vane, M. I. Grossman, *Gastroenterology* **77**, 1301-6 (1979).

307. M. Feldman, T. J. Colturi, *Gastroenterology* **87**, 1339-43 (1984).

308. P. W. Collins, *J Med Chem* **29**, 437-43 (1986).

309. M. Feldman, *J Clin Invest* **72**, 295-303 (1983).

310. U. Seidler, K. Knafla, R. Kownatzki, K. F. Sewing, *Gastroenterology* **95**, 945-51 (1988).

311. T. H. Lanthorn, R. G. Bianchi, W. E. Perkins, *Drug Dev Res* **34**, 35 (1995).

312. B. J. Whittle, *Br J Pharmacol* **60**, 455-60 (1977).

313. E. B. Chang, M. W. Musch, L. Mayer, *Gastroenterology* **98**, 1518-24 (1990).

314. E. Beubler, K. Bukhave, J. Rask-Madsen, *Gastroenterology* **90**, 1972-7 (1986).

315. K. McGowan, G. Piver, J. S. Stoff, M. Donowitz, *Gastroenterology* **98**, 873-80 (1990).

316. T. Frieling, C. Rupprecht, A. B. Kroese, M. Schemann, *Am J Physiol* **266**, G132-9 (1994).

317. K. J. Goerg, C. Diener, M. Diener, W. Rummel, *Am J Physiol* **260**, G904-10 (1991).

318. D. W. Powell, in *Handbook of physiology* S. G. Schultz, M. Field, R. A. Frizzell, B. B. Rauner, Eds. (American Physiological Society, Bethesda, MD, 1991), vol. 4.

319. A. T. Blikslager, M. C. Roberts, J. M. Rhoads, R. A. Argenzio, *J Clin Invest* **100**, 1928-33 (1997).

320. D. J. McCool, M. A. Marcon, J. F. Forstner, G. G. Forstner, *Biochem J* **267**, 491-500 (1990).

321. T. E. Phillips, C. M. Stanley, J. Wilson, *Prostaglandins Leukot Essent Fatty Acids* **48**, 423-8 (1993).

322. G. J. Sanger, A. Jackson, A. Bennett, *Eur J Pharmacol* **81**, 141-3 (1982).

323. H. Mizuno, et al., *Gastroenterology* **112**, 387-97 (1997).

324. M. Takahashi, et al., *J Clin Invest* **98**, 2604-11 (1996).

325. M. J. Thun, M. M. Namboodiri, C. W. Heath, Jr., *N Engl J Med* **325**, 1593-6 (1991).

326. L. Rosenberg, et al., *J Natl Cancer Inst* **83**, 355-8 (1991).

327. H. Sheng, et al., *J Clin Invest* **99**, 2254-9 (1997).

328. K. Watanabe, et al., *Cancer Res* **59**, 5093-6 (1999).

329. W. D. M. Payton, in *Drug receptors and their function* R. Porter, M. O'Connor, Eds. (Churchill, New York, 1970) pp. 3.

330. V. R. Pickles, in *Prostaglandins* S. Bergstrom, B. Samuelsson, Eds. (Almquist & Wicksell, Stockholm, 1967), vol. 2, pp. 79-83.

331. P. J. Gardiner, H. O. Collier, *Prostaglandins* **19**, 819-41 (1980).

332. N. H. Andersen, P. W. Ramwell, *Arch Intern Med* **133**, 30-50 (1974).

333. I. Kennedy, R. A. Coleman, P. P. Humphrey, G. P. Levy, P. Lumley, *Prostaglandins* **24**, 667-89 (1982).

334. R. F. Furchgott, in *Handbook of experimental pharmacology, catecholamines* H. Blaschko, E. Muscholl, Eds. (Springer Verlag, New York, 1972), vol. 33, pp. 283-335.

335. T. P. Kenakin, *Pharmacol Rev* **36**, 165-222 (1984).

336. O. V. Miller, R. R. Gorman, *J Pharmacol Exp Ther* **210**, 134-40 (1979).

337. B. J. Whittle, S. Moncada, J. R. Vane, *Prostaglandins* **16**, 373-88 (1978).

338. N. H. Andersen, et al., *Prostaglandins* **22**, 841-56 (1981).

339. R. A. Coleman, I. Kennedy, R. L. Sheldrick, *Adv Prostaglandin Thromboxane Leukot Res* **17A**, 467-70 (1987).

340. R. A. Coleman, I. Kennedy, R. L. G. Sheldrick, *Br J Pharmacol* **91**, 323P (1987).

341. R. A. Coleman, et al., *Prostaglandins* **47**, 151-68 (1994).

342. M. Adam, et al., *FEBS Lett* **338**, 170-4 (1994).

343. M. Kotani, et al., *Genomics* **40**, 425-34 (1997).

344. J. W. Regan, et al., *Br J Pharmacol* **112**, 377-85 (1994).

345. A. Irie, et al., *Eur J Biochem* **217**, 313-8 (1993).

346. M. Negishi, Y. Sugimoto, A. Irie, S. Narumiya, A. Ichikawa, *J Biol Chem* **268**, 9517-21 (1993).

347. Y. Sugimoto, et al., *J Biol Chem* **268**, 2712-8 (1993).

348. K. Takeuchi, T. Abe, N. Takahashi, K. Abe, *Biochem Biophys Res Commun* **194**, 885-91 (1993).

349. T. Namba, et al., *Nature* **365**, 166-70 (1993).

350. R. M. Breyer, et al., *J Biol Chem* **269**, 6163-9 (1994).

351. M. K. Raychowdhury, et al., *J Biol Chem* **269**, 19256-61 (1994).

352. E. Okuda-Ashitaka, et al., *J Biol Chem* **271**, 31255-61 (1996).

353. K. L. Pierce, et al., *J Biol Chem* **272**, 883-7 (1997).

354. J. B. Findlay, D. J. Pappin, *Biochem J* **238**, 625-42 (1986).

355. R. A. Dixon, et al., *Nature* **321**, 75-9 (1986).

356. J. Nathans, D. S. Hogness, *Cell* **34**, 807-14 (1983).

357. R. Henderson, et al., *J Mol Biol* **213**, 899-929 (1990).

358. D. Donnelly, M. S. Johnson, T. L. Blundell, J. Saunders, *FEBS Lett* **251**, 109-16 (1989).

359. C. D. Strader, T. M. Fong, M. R. Tota, D. Underwood, R. A. Dixon, *Annu Rev Biochem* **63**, 101-32 (1994).

360. T. Kenakin, *Pharmacol Rev* **48**, 413-63 (1996).

361. J. Wess, *Faseb J* **11**, 346-54 (1997).

362. T. Schoenberg, G. Schultz, T. Gudermann, *Mol Cell Endocrinol* **151**, 181-93 (1999).

363. P. V. Avdonin, I. V. Svitina-Ulitina, V. L. Leytin, V. A. Tkachuk, *Thromb Res* **40**, 101-12 (1985).

364. R. Murray, G. A. FitzGerald, *Proc Natl Acad Sci U S A* **86**, 124-8 (1989).

365. F. Ushikubi, et al., *J Biol Chem* **264**, 16496-501 (1989).

366. M. Hirata, et al., *Nature* **349**, 617-20 (1991).

367. M. Hirata, A. Kakizuka, M. Aizawa, F. Ushikubi, S. Narumiya, *Proc Natl Acad Sci U S A* **91**, 11192-6 (1994).

368. Y. Boie, N. Sawyer, D. M. Slipetz, K. M. Metters, M. Abramovitz, *J Biol Chem* **270**, 18910-6 (1995).

369. A. Watabe, et al., *J Biol Chem* **268**, 20175-8 (1993).

370. Y. Boie, et al., *Eur J Pharmacol* **340**, 227-41 (1997).

371. C. D. Funk, et al., *J Biol Chem* **268**, 26767-72 (1993).

372. M. Katsuyama, et al., *FEBS Lett* **372**, 151-6 (1995).

373. J. W. Regan, et al., *Mol Pharmacol* **46**, 213-20 (1994).

374. S. An, J. Yang, M. Xia, E. J. Goetzl, *Biochem Biophys Res Commun* **197**, 263-70 (1993).

375. L. Bastien, N. Sawyer, R. Grygorczyk, K. M. Metters, M. Adam, *J Biol Chem* **269**, 11873-7 (1994).

376. A. Honda, et al., *J Biol Chem* **268**, 7759-62 (1993).

377. T. Sando, et al., *Biochem Biophys Res Commun* **200**, 1329-33 (1994).

378. M. Abramovitz, et al., *J Biol Chem* **269**, 2632-6 (1994).

379. P. E. Graves, et al., *Endocrinology* **136**, 3430-6 (1995).

380. J. Kitanaka, et al., *Prostaglandins* **48**, 31-41 (1994).

381. K. Sakamoto, et al., *J Biol Chem* **269**, 3881-6 (1994).

382. Y. Sugimoto, et al., *J Biol Chem* **269**, 1356-60 (1994).

383. Y. Boie, et al., *J Biol Chem* **269**, 12173-8 (1994).

384. M. Katsuyama, et al., *FEBS Lett* **344**, 74-8 (1994).

385. O. Nakagawa, et al., *Circulation* **90**, 1643-7 (1994).

386. T. Namba, et al., *J Biol Chem* **269**, 9986-92 (1994).

387. Y. Sasaki, et al., *Biochim Biophys Acta* **1224**, 601-5 (1994).

388. T. Abe, et al., *J Clin Invest* **96**, 657-64 (1995).

389. S. Muck, A. A. Weber, J. Meyer-Kirchrath, K. Schror, *Naunyn Schmiedebergs Arch Pharmacol* **357**, 10-6 (1998).

390. T. Namba, et al., *Biochem Biophys Res Commun* **184**, 1197-203 (1992).

391. T. M. Savarese, C. M. Fraser, *Biochem J* **283**, 1-19 (1992).

392. H. G. Dohlman, J. Thorner, M. G. Caron, R. J. Lefkowitz, *Annu Rev Biochem* **60**, 653-88 (1991).

393. L. Audoly, R. M. Breyer, *J Biol Chem* **272**, 13475-8 (1997).

394. N. Chiang, W. M. Kan, H. H. Tai. *Arch Biochem Biophys* **334**, 9-17 (1996).

395. D. D. D'Angelo, J. J. Eubank, M. G. Davis, G. W. Dorn, 2nd. *J Biol Chem* **271**, 6233-40 (1996).

396. G. W. Dorn, 2nd. *J Biol Chem* **265**, 4240-6 (1990).

397. N. Chiang, H. H. Tai. *Arch Biochem Biophys* **352**, 207-13 (1998).

398. D. E. Mais, T. A. True, M. J. Martinelli. *Eur J Pharmacol* **227**, 267-74 (1992).

399. W. P. Hausdorff, M. G. Caron, R. J. Lefkowitz. *Faseb J* **4**, 2881-9 (1990).

400. S. S. Ferguson, L. S. Barak, J. Zhang, M. G. Caron. *Can J Physiol Pharmacol* **74**, 1095-110 (1996).

401. R. J. Lefkowitz. *J Biol Chem* **273**, 18677-80 (1998).

402. S. Narumiya, Y. Sugimoto, F. Ushikubi. *Physiol Rev* **79**, 1193-226 (1999).

403. F. Ushikubi, M. Hirata, S. Narumiya. *J Lipid Mediat Cell Signal* **12**, 343-59 (1995).

404. L. Audoly, R. M. Breyer. *Mol Pharmacol* **51**, 61-8 (1997).

405. C. Huang, H. H. Tai. *Biochem J* **307**, 493-8 (1995).

406. T. Hirata, et al.. *J Clin Invest* **94**, 1662-7 (1994).

407. H. Toh, A. Ichikawa, S. Narumiya. *FEBS Lett* **361**, 17-21 (1995).

408. T. O. Ishikawa, et al.. *Genomics* **32**, 285-8 (1996).

409. M. Taketo, et al.. *Genomics* **19**, 585-8 (1994).

410. A. M. Duncan, L. L. Anderson, C. D. Funk, M. Abramovitz, M. Adam. *Genomics* **25**, 740-2 (1995).

411. R. M. Nusing, et al.. *J Biol Chem* **268**, 25253-9 (1993).

412. B. Batshake, C. Nilsson, J. Sundelin. *Eur J Biochem* **231**, 809-14 (1995).

413. S. M. Foord, et al.. *Genomics* **35**, 182-8 (1996).

414. K. Hasumoto, et al.. *Genes Cells* **2**, 571-80 (1997).

415. Y. Ogawa, et al., *Genomics* **27**, 142-8 (1995).

416. T. Arakawa, et al., *J Biol Chem* **271**, 29569-75 (1996).

417. M. Katsuyama, et al., *Biochem J* **330**, 1115-21 (1998).

418. S. L. Smock, et al., *Gene* **237**, 393-402 (1999).

419. K. L. Pierce, J. W. Regan, *Life Sci* **62**, 1479-83 (1998).

420. M. Nakajima, et al., *Biochem Biophys Res Commun* **158**, 958-65 (1989).

421. B. T. Kinsella, D. J. O'Mahony, G. A. FitzGerald, *J Biol Chem* **269**, 29914-9 (1994).

422. N. Takahashi, et al., *Biochem Biophys Res Commun* **244**, 489-93 (1998).

423. M. Abramovitz, et al., *Biochim Biophys Acta* **1483**, 285-293 (2000).

424. M. Kiriyama, et al., *Br J Pharmacol* **122**, 217-24 (1997).

425. R. A. Coleman, W. L. Smith, S. Narumiya, *Pharmacol Rev* **46**, 205-29 (1994).

426. R. W. Butcher, E. W. Sutherland, *Ann N Y Acad Sci* **139**, 849-59 (1967).

427. E. W. Dunham, M. K. Haddox, N. D. Goldberg, *Proc Natl Acad Sci U S A* **71**, 815-9 (1974).

428. H. E. Hamm, *J Biol Chem* **273**, 669-72 (1998).

429. S. Ito, E. Okuda, K. Sugama, M. Negishi, O. Hayaishi, *Br J Pharmacol* **99**, 13-4 (1990).

430. W. K. Sonnenburg, J. H. Zhu, W. L. Smith, *J Biol Chem* **265**, 8479-83 (1990).

431. H. Hashimoto, M. Negishi, A. Ichikawa, *Prostaglandins* **40**, 491-505 (1990).

432. B. Simon, H. Kather, B. Kommerell, *Adv Prostaglandin Thromboxane Res* **8**, 1617-20 (1980).

433. S. Ito, et al., *Biochem Biophys Res Commun* **200**, 756-62 (1994).

434. V. Raymond, P. C. Leung, F. Labrie, *Biochem Biophys Res Commun* **116**, 39-46 (1983).

435. A. Shenker, P. Goldsmith, C. G. Unson, A. M. Spiegel, *J Biol Chem* **266**, 9309-13 (1991).

436. H. Arita, T. Nakano, K. Hanasaki, *Prog Lipid Res* **28**, 273-301 (1989).

437. T. Hirata, F. Ushikubi, A. Kakizuka, M. Okuma, S. Narumiya, *J Clin Invest* **97**, 949-56 (1996).

438. B. R. Creese, M. A. Denborough, *Clin Exp Pharmacol Physiol* **9**, 145-55 (1982).

439. M. Negishi, et al., *J Biol Chem* **263**, 6893-900 (1988).

440. M. Negishi, et al., *J Biol Chem* **268**, 26067-70 (1993).

441. M. Negishi, H. Hasegawa, A. Ichikawa, *FEBS Lett* **386**, 165-8 (1996).

442. H. Katoh, A. Watabe, Y. Sugimoto, A. Ichikawa, M. Negishi, *Biochim Biophys Acta* **1244**, 41-8 (1995).

443. N. Nishigaki, M. Negishi, A. Ichikawa, *Mol Pharmacol* **50**, 1031-7 (1996).

444. L. Zeng, S. An, E. J. Goetzl, *Immunology* **86**, 620-8 (1995).

445. F. Neuschafer-Rube, M. Oppermann, U. Moller, U. Boer, G. P. Puschel, *Mol Pharmacol* **56**, 419-28 (1999).

446. S. An, J. Yang, S. W. So, L. Zeng, E. J. Goetzl, *Biochemistry* **33**, 14496-502 (1994).

447. M. Bastepe, B. Ashby, *Br J Pharmacol* **126**, 365-71 (1999).

448. A. Krane, J. MacDermot, M. Keen, *Biochem Pharmacol* **47**, 953-9 (1994).

449. A. Krane, J. Malkhandi, L. Mercy, M. Keen, *Biochim Biophys Acta* **1206**, 203-7 (1994).

450. E. J. Adie, I. Mullaney, F. R. McKenzie, G. Milligan, *Biochem J* **285**, 529-36 (1992).

451. S. D. Tachado, Y. Zhang, A. A. Abdel-Latif, *Invest Ophthalmol Vis Sci* **34**, 2023-32 (1993).

452. J. C. Lamsa, R. A. Cushman, M. G. Nay, J. A. McCracken, *Prostaglandins* **43**, 165-79 (1992).

453. A. K. Okwu, D. E. Mais, P. V. Halushka, *Biochim Biophys Acta* **1221**, 83-8 (1994).

454. G. Losonczy, et al., *Am J Physiol* **272**, R1734-9 (1997).

455. I. Elalamy, S. Emadi, B. B. Vargaftig, M. Hatmi, *Br J Haematol* **99**, 190-6 (1997).

456. J. L. Parent, P. Labrecque, M. J. Orsini, J. L. Benovic, *J Biol Chem* **274**, 8941-8 (1999).

457. T. Doetschman, et al., *Nature* **330**, 576-8 (1987).

458. M. R. Capecchi, *Science* **244**, 1288-92 (1989).

459. F. Ushikubi, et al., *Nature* **395**, 281-4 (1998).

460. L. P. Audoly, M. Nguyen, B. H. Koller, T. M. Coffman, *Faseb J* **13**, A1549 (1999).

461. C. R. Kennedy, et al., *Nat Med* **5**, 217-20 (1999).

462. S. L. Tilley, et al., *J Clin Invest* **103**, 1539-45 (1999).

463. L. P. Audoly, et al., *Am J Physiol* **277**, H924-30 (1999).

464. H. Hizaki, et al., *Proc Natl Acad Sci U S A* **96**, 10501-6 (1999).

465. E. F. Fleming, et al., *Am J Physiol* **275**, F955-61 (1998).

466. K. Takeuchi, et al., *Gastroenterology* **117**, 1128-35 (1999).

467. M. Nguyen, et al., *Nature* **390**, 78-81 (1997).

468. E. Segi, et al., *Biochem Biophys Res Commun* **246**, 7-12 (1998).

469. Y. Sugimoto, et al., *Science* **277**, 681-3 (1997).

470. G. A. Gross, et al., *Proc Natl Acad Sci U S A* **95**, 11875-9 (1998).

471. T. Murata, et al., *Nature* **388**, 678-82 (1997).

472. D. W. Thomas, et al., *J Clin Invest* **102**, 1994-2001 (1998).

473. G. Hecht, *Am J Physiol* **277**, C351-8 (1999).

474. S. J. Gendler, A. P. Spicer, *Annu Rev Physiol* **57**, 607-34 (1995).

475. J. F. Forstner, G. G. Forstner, in *Physiology of the gastrointestinal tract* L. R. Johnson, Ed. (Raven Press, New York, 1994) pp. 1255-1283.

476. J. R. Gum, Jr., *Biochem Soc Trans* **23**, 795-9 (1995).

477. S. J. Gendler, et al., *J Biol Chem* **265**, 15286-93 (1990).

478. J. R. Gum, Jr., J. W. Hicks, N. W. Toribara, B. Siddiki, Y. S. Kim, *J Biol Chem* **269**, 2440-6 (1994).

479. V. Guyonnet Duperat, et al., *Biochem J* **305**, 211-9 (1995).

480. L. W. Klomp, L. Van Rens, G. J. Strous, *Biochem J* **308**, 831-8 (1995).

481. D. Meezaman, et al., *J Biol Chem* **269**, 12932-9 (1994).

482. J. H. van de Bovenkamp, et al., *Biochem Biophys Res Commun* **245**, 853-9 (1998).

483. L. A. Bobek, H. Tsai, A. R. Biesbrock, M. J. Levine, *J Biol Chem* **268**, 20563-9 (1993).

484. A. Velcich, L. Palumbo, L. Selleri, G. Evans, L. Augenlicht, *J Biol Chem* **272**, 7968-76 (1997).

485. J. R. Gum, Jr., *Am J Respir Cell Mol Biol* **7**, 557-64 (1992).

486. G. J. Strous, J. Dekker, *Crit Rev Biochem Mol Biol* **27**, 57-92 (1992).

487. N. W. Toribara, et al., *J Biol Chem* **268**, 5879-85 (1993).

488. J. A. Hanover, W. J. Lennarz, J. D. Young, *J Biol Chem* **255**, 6713-6 (1980).

489. J. P. Briand, S. P. Andrews, Jr., E. Cahill, N. A. Conway, J. D. Young, *J Biol Chem* **256**, 12205-7 (1981).

490. D. J. Thornton, et al., *Biochem J* **265**, 179-86 (1990).

491. N. Jentoft, *Trends Biochem Sci* **15**, 291-4 (1990).

492. K. M. Tytgat, et al., *Gastroenterology* **107**, 1352-63 (1994).

493. S. K. Chang, et al., *Gastroenterology* **107**, 28-36 (1994).

494. N. W. Toribara, et al., *J Clin Invest* **88**, 1005-13 (1991).

495. J. R. Gum, et al., *Biochem Biophys Res Commun* **171**, 407-15 (1990).

496. J. R. Gum, Jr., et al., *J Biol Chem* **272**, 26678-86 (1997).

497. N. Porchet, et al., *Biochem Soc Trans* **23**, 800-5 (1995).

498. V. C. Nguyen, et al., *Hum Genet* **86**, 167-72 (1990).

499. C. De Bolos, M. Garrido, F. X. Real, *Gastroenterology* **109**, 723-34 (1995).

500. N. W. Toribara, et al., *J Biol Chem* **272**, 16398-403 (1997).

501. D. J. McCool, J. F. Forstner, G. G. Forstner, *Biochem J* **302**, 111-8 (1994).

502. J. K. Sheehan, et al., *Biochem J* **315**, 1055-60 (1996).

503. M. A. McGuckin, P. L. Devine, B. G. Ward, *Biochem Cell Biol* **74**, 87-93 (1996).

504. S. Munro, H. R. Pelham, *Cell* **48**, 899-907 (1987).

505. G. Forstner, *Annu Rev Physiol* **57**, 585-605 (1995).

506. P. G. Wheater, *Functional histology: a text and colour atlas*, P. G. Wheater, H. G. Burkitt, V. G. Daniels, Eds. (Churchill Livingstone, New York, ed. 2nd, 1990).

507. A. Belley, K. Keller, M. Gottke, K. Chadee, M. Goettke, *Am J Trop Med Hyg* **60**, 10-5 (1999).

508. R. D. Specian, M. R. Neutra, *Gastroenterology* **87**, 1313-25 (1984).

509. R. D. Specian, M. G. Oliver, *Am J Physiol* **260**, C183-93 (1991).

510. M. G. Oliver, R. D. Specian, *Am J Physiol* **259**, G991-7 (1990).

511. R. D. Specian, M. R. Neutra, *Am J Physiol* **242**, G370-9 (1982).

512. C. R. Boland, G. D. Deshmukh, *Gastroenterology* **98**, 1170-7 (1990).

513. P. X. Xing, J. Prenzoska, G. T. Layton, P. L. Devine, I. F. McKenzie, *J Natl Cancer Inst* **84**, 699-703 (1992).

514. S. B. Ho, et al., *Cancer Res* **53**, 641-51 (1993).

515. K. M. Tytgat, F. J. Opdam, A. W. Einerhand, H. A. Buller, J. Dekker, *Gut* **38**, 554-63 (1996).

516. C. Hanski, et al., *J Pathol* **188**, 304-311 (1999).

517. K. Kyo, et al., *Hum Mol Genet* **8**, 307-11 (1999).

518. K. Chadee, W. A. Petri, Jr., D. J. Innes, J. I. Ravdin, *J Clin Invest* **80**, 1245-54 (1987).

519. K. Chadee, M. L. Johnson, E. Orozco, W. A. Petri, Jr., J. I. Ravdin, *J Infect Dis* **158**, 398-406 (1988).

520. K. Chadee, E. Meerovitch, *Am J Trop Med Hyg* **34**, 283-91 (1985).

521. G. Lundblad, *Comp Biochem Physiol* **68 B**, 71 (1981).

522. J. P. Noel, H. E. Hamm, P. B. Sigler, *Nature* **366**, 654-63 (1993).

523. S. R. Sprang, *Annu Rev Biochem* **66**, 639-78 (1997).

524. D. G. Lambright, J. P. Noel, H. E. Hamm, P. B. Sigler, *Nature* **369**, 621-8 (1994).

525. E. Lee, R. Taussig, A. G. Gilman, *J Biol Chem* **267**, 1212-8 (1992).

526. E. Hanski, P. C. Sternweis, J. K. Northup, A. W. Dromerick, A. G. Gilman, *J Biol Chem* **256**, 12911-9 (1981).

527. P. C. Sternweis, J. K. Northup, M. D. Smigel, A. G. Gilman, *J Biol Chem* **256**, 11517-26 (1981).

528. F. J. Rojas, L. Birnbaumer, *J Biol Chem* **260**, 7829-35 (1985).

529. A. G. Gilman, *Annu Rev Biochem* **56**, 615-49 (1987).

530. E. J. Neer, *Cell* **80**, 249-57 (1995).

531. M. Negishi, et al., *Biochim Biophys Acta* **1175**, 343-50 (1993).

532. A. Irie, et al., *Eur J Biochem* **224**, 161-6 (1994).

533. T. Kobayashi, et al., *J Biol Chem* **272**, 15154-60 (1997).

534. R. A. Coleman, L. Feniuk, I. Kennedy, *Br J Pharmacol* **74**, 913P (1981).

535. H. Giles, P. Leff, M. L. Bolofo, M. G. Kelly, A. D. Robertson, *Br J Pharmacol* **96**, 291-300 (1989).

536. B. Z. Paul, B. Ashby, S. B. Sheth, *Br J Haematol* **102**, 1204-11 (1998).

537. B. M. Forman, et al., *Cell* **83**, 803-12 (1995).

538. S. A. Kliewer, et al., *Cell* **83**, 813-9 (1995).

539. P. R. Devchand, et al., *Nature* **384**, 39-43 (1996).

540. M. Fukushima, *Eicosanoids* **3**, 189-99 (1990).

541. I. K. Kim, J. H. Lee, H. W. Sohn, H. S. Kim, S. H. Kim, *FEBS Lett* **321**, 209-14 (1993).

542. S. Narumiya, M. Fukushima, *J Pharmacol Exp Ther* **239**, 500-5 (1986).

543. M. Bhattacharya, et al., *Proc Natl Acad Sci U S A* **95**, 15792-7 (1998).

544. D. Gerashchenko, et al., *J Neurochem* **71**, 937-45 (1998).

545. L. Hunyady, A. J. Baukal, T. Balla, K. J. Catt, *J Biol Chem* **269**, 24798-804 (1994).

546. M. W. Beukers, I. Kristiansen, I. J. AP, I. Edvardsen, *Trends Pharmacol Sci* **20**, 475-7 (1999).

547. J. Lameh, et al., *J Biol Chem* **267**, 13406-12 (1992).

548. C. Robin-Jagerschmidt, et al., *Mol Cell Endocrinol* **139**, 187-98 (1998).

549. J. L. Parent, C. Le Gouill, M. Rola-Pleszczynski, J. Stankova, *Biochem Biophys Res Commun* **219**, 968-75 (1996).

550. F. Z. Chung, C. D. Wang, P. C. Potter, J. C. Venter, C. M. Fraser, *J Biol Chem* **263**, 4052-5 (1988).

551. B. Y. Ho, A. Karschin, T. Branchek, N. Davidson, H. A. Lester, *FEBS Lett* **312**, 259-62 (1992).

552. O. Chabre, B. R. Conklin, S. Brandon, H. R. Bourne, L. E. Limbird, *J Biol Chem* **269**, 5730-4 (1994).

553. W. Yang, W. P. Schraw, S. G. Mueller, A. Richmond, *Biochemistry* **36**, 15193-200 (1997).

554. D. H. Wright, K. M. Metters, M. Abramovitz, A. W. Ford-Hutchinson, *Br J Pharmacol* **123**, 1317-24 (1998).

555. C. J. Jensen, N. P. Gerard, T. W. Schwartz, U. Gether, *Mol Pharmacol* **45**, 294-9 (1994).

556. N. Adham, et al., *Neuropharmacology* **33**, 387-91 (1994).

557. S. M. Sagar, F. R. Sharp, T. Curran, *Science* **240**, 1328-31 (1988).

558. T. Scammell, et al., *Proc Natl Acad Sci U S A* **95**, 7754-9 (1998).

559. Y. Urade, O. Hayaishi, *Biochim Biophys Acta* **1436**, 606-15 (1999).

560. B. Blodorn, et al., *Neurosci Lett* **209**, 117-20 (1996).

561. A. Hoffmann, et al., *J Neurochem* **61**, 451-6 (1993).

562. A. Yamashita, Y. Watanabe, O. Hayaishi, *Proc Natl Acad Sci U S A* **80**, 6114-8 (1983).

563. Y. U. Watanabe, Y. Watanabe, O. Hayaishi, in *Biomedical imaging* O. Hayaishi, K. Torizuka, Eds. (Academic Press, Tokyo, 1986) pp. 227-38.

564. R. Ueno, K. Honda, S. Inoue, O. Hayaishi, *Proc Natl Acad Sci U S A* **80**, 1735-7 (1983).

565. H. Onoe, et al., *Proc Natl Acad Sci U S A* **85**, 4082-6 (1988).

566. P. Bobillier, et al., *Brain Res* **240**, 359-63 (1982).

567. B. Fernandes, D. Crankshaw, *Eur J Pharmacol* **283**, 73-81 (1995).

568. T. Kenakin, in *Pharmacologic analysis of drug-receptor interaction* M. A. McLaughlin, Ed. (Lippincott-Raven, Philadelphia, 1997) pp. 291.

569. D. Colquhoun, *Trends Pharmacol Sci* **6**, 197 (1985).

570. D. F. Woodward, et al., *Eur J Pharmacol* **230**, 327-33 (1993).

571. P. K. Rangachari, P. A. Betti, E. T. Prior, L. J. Roberts, 2nd, *J Pharmacol Exp Ther* **275**, 611-7 (1995).

572. N. Miwa, H. Sugino, R. Ueno, O. Hayaishi, *J Neurochem* **50**, 1418-24 (1988).

573. E. Okuda-Ashitaka, K. Sakamoto, H. Giles, S. Ito, O. Hayaishi, *Biochim Biophys Acta* **1176**, 148-54 (1993).

574. P. M. Vanderheyden, F. L. Fierens, J. P. De Backer, N. Fraeyman, G. Vauquelin, *Br J Pharmacol* **126**, 1057-65 (1999).

575. C. B. Basbaum, J. M. Madison, C. P. Sommerhoff, J. K. Brown, W. E. Finkbeiner, *Am Rev Respir Dis* **141**, S141 (1990).

576. C. Laboisse, et al., *Biochem Soc Trans* **23**, 810-3 (1995).

577. A. Belley, K. Keller, J. Grove, K. Chadee, *Gastroenterology* **111**, 1484-92 (1996).

578. D. H. Wright, F. Nantel, K. M. Metters, A. W. Ford-Hutchinson, *Eur J Pharmacol* **377**, 101-15 (1999).

579. D. F. Woodward, et al., *Invest Ophthalmol Vis Sci* **31**, 138-46 (1990).

580. Z. Marom, J. H. Shelhamer, M. Kaliner, *J Clin Invest* **67**, 1695-702 (1981).

581. M. T. Lopez-Vidriero, I. Das, A. P. Smith, R. Picot, L. Reid, *Thorax* **32**, 734-9 (1977).

582. L. E. LeDuc, P. Needleman, *Adv Prostaglandin Thromboxane Res* **8**, 1515-7 (1980).

583. D. Y. Lee, J. R. Lupton, R. S. Chapkin, *Prostaglandins* **43**, 143-64 (1992).

584. R. Pawankar, *Allergol Int* **48**, 171 (1999).

585. D. R. Halm, G. R. Rechkemmer, R. A. Schoumacher, R. A. Frizzell, *Am J Physiol* **254**, C505-11 (1988).

586. A. Weymer, P. Huott, W. Liu, J. A. McRoberts, K. Dharmsathaphorn, *J Clin Invest* **76**, 1828-36 (1985).

587. H. R. Miller, Y. Nawa, C. R. Parish, *Int Arch Allergy Appl Immunol* **59**, 281-5 (1979).

588. H. R. Miller, R. G. Woodbury, J. F. Huntley, G. Newlands, *Immunology* **49**, 471-9 (1983).

589. A. M. Dvorak, R. A. Monahan, J. E. Osage, G. R. Dickersin, *Lancet* **1**, 498 (1978).

590. S. Poonyachoti, D. R. Brown, *Eur J Pharmacol* **379**, 81-5 (1999).

591. D. R. Halm, S. T. Halm, *Am J Physiol* **277**, C501-22 (1999).

592. M. W. Musch, R. J. Miller, M. Field, M. I. Siegel, *Science* **217**, 1255-6 (1982).

593. P. L. Smith, D. P. Montzka, G. P. McCafferty, M. A. Wasserman, J. D. Fondacaro, *Am J Physiol* **255**, G175-83 (1988).

594. T. E. Phillips, W. F. Stenson, M. R. Neutra, *Prostaglandins Leukot Essent Fatty Acids* **37**, 51-5 (1989).

595. V. L. Cohan, A. L. Scott, C. A. Dinarello, R. A. Prendergast, *Cell Immunol* **136**, 425-34 (1991).

596. R. S. Blumberg, L. J. Saubermann, W. Strober, *Curr Opin Immunol* **11**, 648-56 (1999).

597. Y. Araki, A. Andoh, Y. Fujiyama, T. Bamba, *Clin Exp Immunol* **119**, 264-9 (2000).

598. H. J. Kaufmann, H. L. Taubin, *Ann Intern Med* **107**, 513-6 (1987).

599. I. Bjarnason, et al., *Gastroenterology* **93**, 480-9 (1987).

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10. APPENDICES

Appendix A: Future directions for this research

Below are listed some of the possible future directions that stem from the research for this thesis. This list is not in order of importance and is not meant to be exhaustive.

1. The most obvious question to be answered is whether the DP receptor plays a role in the regulation of gastrointestinal mucin secretion *in vivo*. Animal models for the *in vivo* study of mucin secretion are established, however, the protocols are complicated and the results are difficult to assess. One method that could be used is the colonic loop method. Here, segments of colon are surgically isolated *in situ*, in the anesthetized animal. Compounds of interest are applied to the preparation and the mucins secreted are collected for analysis. This technique has been used successfully for a number of mucin secretagogues, including PGE_2.

2. As alluded to earlier, the DP receptor has been shown to modulate chloride ion secretion in the T84 colonic cell line. As well, it has been proposed that cells may integrate electrolyte secretion and mucin exocytosis to maximize their secretory response. This has been shown for a number of mucin secretagogues, including PGE_1. The regulation of choride transport by the DP receptor and its effect on DP-mediated mucin secretion could be investigated in the LS174T cell line by first measuring electrolyte transport in response to DP-specific agonists and then blocking chloride secretion. Previous studies have demonstrated that chloride secretion can be repressed by Ca^{2+} channel inhibitors (i.e. Ba^{2+}). The interdependence between DP-mediated chloride transport and mucin secretion could also be assessed using these inhibitors.

3. Outside of the gastrointestinal tract, the next most obvious question is whether the DP receptor plays a role in the regulation of mucin secretion in other mucosecretory tracts (tracheobronchial, reproductive). As discussed previously, there is some evidence for a role for PGD_2-mediated mucin secretion in the eye and the tracheobronchial tract. However, the results from these studies suggest that a receptor other than DP is responsible for mediating the mucin response in these tissues. *In situ* hybridization studies, similar to those performed here, and/or immunocytochemical experiments would help clarify whether the DP receptor is present on goblet cells in these tissues. Classically, tissue explants have been used to study mucin secretion from these tissues *in vitro*, and could be applied here.

4. Although it does not stem from this research, an interesting observation is that the L-PGDS is a retinoid transporter and the PGJ series of compounds require receptor heterodimerization between PPARγ and a retinoid receptor (RXR) to mediate biological activity. Effectively, the L-PGDS isoform might be able to modulate the signal transduction of the PGJ series of compounds by regulating receptor recruitment, in addition to being involved in their synthesis. Thus, an interesting question is whether these processes are linked. In this regard, it might be useful to see if L-PGDS and PPARγ co-localize. Furthermore, it would be interesting to see how a change in L-PGDS synthetic activity (PGD_2 synthesis) effects retinoid transport, retinoid receptor activity, and ultimately PGJ-mediated activity.

Appendix B: Copyright waiver forms