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Pharmacological characterization of [^{3}H]-prostaglandin E_2 binding to the cloned human EP_4 prostanoid receptor

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1 Prostaglandin (PG) E_2 (PGE_2) is a potent prostanoid derived from arachidonic which can interact with EP_1, EP_2, EP_3 and EP_4 prostanoid receptor subtypes.

2 Recombinant human EP_4 receptors expressed in human embryonic kidney (HEK-293) cells were evaluated for their binding characteristics using [^{3}H]-PGE_2 and a broad panel of natural and synthetic prostanoids in order to define their pharmacological properties.

3 [^{3}H]-PGE_2 binding was optimal in 2-[N-Morpholino]ethanesulphonic acid (MES) buffer (pH 6.0) yielding $98 \pm 0.7\%$ specific binding. The receptor displayed high affinity ($K_d = 0.72 \pm 0.12$ nM; $n = 3$) for [^{3}H]-PGE_2 and interacted with a saturable number of binding sites ($B_{max} = 6.21 \pm 0.84$ pmol mg^{-1} protein).

4 In competition studies, PGE_2 ($K_i = 0.75 \pm 0.03$ nM; $n = 12$) and PGE_1 ($K_i = 1.45 \pm 0.24$ nM; $n = 3$) displayed high affinities, as did two derivatives of PGE_1, namely 11-deoxy-PGE_1 ($K_i = 1.36 \pm 0.34$ nM) and 13,14-dihydro-PGE_1 ($K_i = 3.07 \pm 0.29$ nM).

5 Interestingly, synthetic DP receptor-specific agonists such as BW245C ($K_i = 64.7 \pm 1.0$ nM; $n = 3$) and ZK118182 ($K_i = 425 \pm 42$ nM; $n = 4$), and the purported EP_3 receptor-specific ligand enprostil ($K_i = 43.1 \pm 4.4$ nM), also displayed high affinity for the EP_4 receptor.

6 Two known EP_4 receptor antagonists were weak inhibitors of [^{3}H]-PGE_2 binding akin to their known functional potencies, thus: AH23848 ($K_i = 2690 \pm 232$ nM); AH22921 ($K_i = 31{,}800 \pm 4090$ nM).

7 These studies have provided a detailed pharmacological characterization of the recombinant human EP_4 receptor expressed in HEK-293 cells.

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Keywords: EP_4 receptor; prostanoids; cloned human EP_4 receptor; ligand binding

Abbreviations: HEK-293, human embryonic kidney cells; IOP, intraocular pressure; PG, prostaglandin

Introduction

Prostanoids, including prostaglandins (PGs) and thromboxanes, exert diverse effects in biological systems, ranging from vasodilation and smooth muscle contraction or relaxation to platelet aggregation and immunoregulation (see Coleman *et al*., 1994b for review). This versatility is due, in part, to the number of prostanoid classes and their corresponding receptors (DP, EP, FP, IP, and TP) which are linked through G-proteins to different signalling pathways (cyclic AMP, phosphoinositide turnover, Ca^{2+} mobilization, etc.) (Coleman & Humphrey, 1993; Coleman *et al*., 1994b). Whilst prostanoids play important regulatory roles in foetal vascular development (Chemtob *et al*., 1996; Nguyen *et al*., 1997), maintenance of vascular tone (Schror, 1993), the immune response (Meja *et al*., 1997; Wise, 1998), and even neuroprotection (Akaike *et al*., 1994), they have also been implicated as possibly contributing to the pathogenesis of asthma (Wenzel, 1997), arthritis (Wittenburg *et al*., 1993), and immunosuppressive syndromes (Giacomini *et al*., 1998). Clinical interest in prostanoid agonists and antagonists has thus been stimulated due to their potential use as drugs to treat a variety of conditions. Recently, such compounds have also shown utility in reducing intraocular pressure (IOP), a risk factor associated with glaucoma (Bito *et al*., 1993). Studies on the distribution of prostanoid receptors in the eye have revealed receptors for EP, DP, and/or FP receptors in the ciliary muscle (Matsuo & Cynader, 1992; Sharif *et al*., 1999; Davis & Sharif, 1999) and trabecular meshwork (Anthony *et al*., 1998) structures which are key to the maintenance of IOP. As would be expected considering their repertoire of activities in other systems, different prostanoids serve to lower IOP by different mechanisms. Whereas $PGF_{2\alpha}$ appears to increase outflow *via* the uveoscleral route by promoting remodelling of the extracellular matrix in the ciliary muscle (Gabelt & Kaufman, 1989; Lindsey *et al*., 1996), PGE_1 may lower IOP by stimulating EP_4 and/or EP_2 receptors and promoting conventional outflow through the trabecular meshwork, though the exact mechanism is still unclear (Dijkstra *et al*., 1999). Even if limited to the E-series of prostanoids, a wide array of biological responses is still possible due to the presence of at least four subclasses of EP receptor (EP_1, EP_2, EP_3, and EP_4) (Coleman *et al*., 1994b) and splice variants within the EP_3 class (e.g. EP_{3a}, EP_{3b}, etc.; Ichikawa *et al*., 1996). These EP receptor subtypes have been cloned from mouse, rat, and human cDNA libraries, and the mechanisms of signal transduction for each subclass delineated (Coleman *et al*., 1994b).

The EP_4 subclass of EP receptor was first discovered in piglet saphenous vein (Coleman *et al*., 1994a,b) but has subsequently been detected in human leukocytes (Mori *et al*., 1996), human kidney (Morath *et al*., 1999), Chinese hamster ovary cells (Milne *et al*., 1994; Crider *et al*., 2000) and ocular tissues, namely the ciliary muscle and epithelium (Mukhopadhyay *et al*., 1997). Activation of the EP_4 receptor by PGE_2 increases intracellular cyclic AMP (Milne *et al*., 1994; Crider *et al*., 2000), and this leads to such responses as the relaxation of smooth muscle (Lydford *et al*., 1996). The initial characteriza-

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tion of the recombinant EP_4 receptors from the rat (Boie *et al*., 1997), mouse (Kiriyama *et al*., 1997) and human (Marshall *et al*., 1997) has been described using a limited number of prostanoids and related compounds. In the current studies, we aimed to employ a battery of 37 natural and synthetic prostanoids to pharmacologically characterize the receptor binding of [^{3}H]-PGE_2 binding to membranes prepared from human embryonic kidney (HEK-293) cells transfected with the recombinant human EP_4 receptor.

Methods

Recombinant human EP_4 receptor preparation

Cell membranes from HEK-293 cells expressing the recombinant human EP_4 receptor were obtained from Receptor Biology, Inc. (Beltsville, MD, U.S.A.). The membranes (Batch No. 1496, 3.8 pmole mg^{-1} protein) were stored in liquid nitrogen until use.

Radioligand

[^{3}H]-PGE_2 (NEN Life Science Products, Inc., Boston, MA, U.S.A.) was used in competitive binding studies. The radioligand (No. NET-428, lot 3281-134) had a specific activity of 200 Ci $mmol^{-1}$ and was supplied at 0.1 mCi ml^{-1}. This reagent was stored at $-40°C$ prior to use.

Chemicals and prostanoid compounds

Ethylenediaminetetraacetic acid (EDTA), 2-[N-morpholino]ethanesulphonic acid (MES), polyethylenimine (PEI), and manganese chloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Potassium hydroxide (KOH, 45%) was purchased from EM Sciences (Gibbstown, NJ, U.S.A.). The native prostanoids PGE_1, PGE_2, $PGF_{2\alpha}$, PGI_2, and PGD_2 as well as the following prostanoid compounds were obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.): latanoprost, cloprostenol, fluprostenol (racemic), iloprost, sulprostone, misoprostol, 17-phenyl-ω-trinor-PGE_2, 11-deoxy-16,16-dimethyl- PGE_2, 11-deoxy-PGE_1, 13,14-dihydro-PGE_1. An additional group of compounds was synthesized in-house at Alcon Research, Ltd. (Fort Worth, TX, U.S.A.): AL-5848 (+-isomer of fluprostenol), AL-6221 (isopropyl ester of AL-5848), PHXA85, enprostil, 16-R-Butaprost, BW245C, SQ27986, UFO-21, AL-6556 (13,14-dihydro-ZK118182) and AL-6598 (isopropyl ester of AL-6556) as well as the putative EP_4 receptor ligands (Burk, 1997) AL-24615 (15-methoxy-17-(2-furanyl)-18,19,20-trinor-$PGF_{2\alpha}$) and AL-24620 (17-(5-methyl-2-furanyl)-18,19,20-trinor-$PGF_{2\alpha}$) (refer to Figure 1 for structures of these compounds). The compounds AH6809 and SQ29548 were obtained from Tocris Cookson, Inc. (Ballwin, MO, U.S.A.), and Research Biochemicals Inc (Natick, MA, U.S.A.), respectively. ZK110841 and ZK118182 were generous gifts from Schering AG (Berlin, Germany). In addition, S-1033, SC19920, and RS93520 were kind generous gifts from Shionogi (Osaka, Japan), G.D. Searle (Skokie, IL, U.S.A.), and Hoffman-La Roche (Basel, Switzerland), respectively. The compounds AH22921X, AH23848B and BWA868C were generous gifts from Glaxo Wellcome, Inc. (Stevenage, U.K.). Chemical structures and the names of the most commonly used prostanoids can be found in the publications of Coleman & Humphrey (1993) and Coleman *et al*. (1994b).

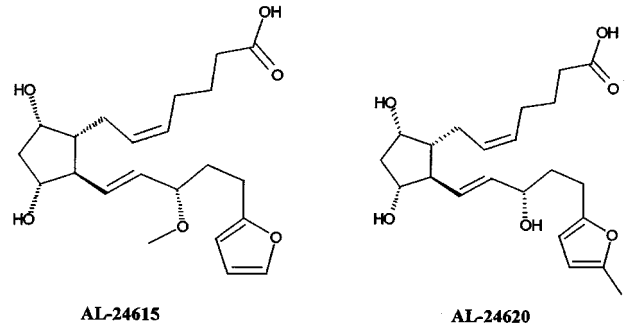

Figure 1 Chemical structures of two putative EP_4 receptor ligands evaluated in this study. Chemical names for each are given in the Methods section.

Competitive binding assays

Assays were conducted in 10 mM 2-[N-Morpholino]ethanesulphonic acid (MES) buffer containing 1 mM EDTA, 10 mM $MnCl_2$, adjusted to pH 6.0 with KOH. Initial tissue linearity studies were carried out using 0.5–160 μg of cell membranes containing the recombinant human EP_4 receptor per 0.5 ml total volume in 96-well deep well assay blocks (Matrix Technologies Corp., Hudson, NH, U.S.A.), using 300 pM [^{3}H]-PGE_2 (final concentration). Membranes were thawed quickly, diluted to the desired concentration in the binding buffer (see above), and mixed to a homogeneous suspension prior to dispensation. After addition of the radioligand, the assay mixtures were incubated at 23°C for 90 min on a rotary shaker (60 r.p.m.). The assay was terminated by rapid vacuum filtration on Whatman GF/B glass fibre filter mats (previously soaked in 0.5% polyetheleneimine) using cold 10 mM MES, 1 mM EDTA (pH 6). The filter mats were dried in a microwave oven for 3 min prior to being sealed in a plastic bag with 30 ml Wallac Betaplate Scint scintillation fluid (Wallac Oy, Turku, Finland). Bound radioligand was then quantitated by liquid scintillation spectrometry at 50% efficiency. For routine assays, the recombinant human EP_4 receptor preparation were used at 4 μg per 0.5 ml total volume. A series of competitive binding assays was carried out with unlabelled PGE_2 to determine the dissociation constant (K_d) and maximal ligand binding (B_{max}) values for the membrane preparation using the specific activity dilution methodology (McPherson, 1983). In these assays, unlabelled PGE_2 was diluted in 10 half log steps and 50 μl of each dilution was added to the assay block in duplicate using a Biomek® 2000 automated laboratory workstation (Beckman Instruments, Inc., Fullerton, CA, U.S.A.). This was followed by the addition of 400 μl of membranes and 50 μl of [^{3}H]-PGE_2 (final concentration 200 pM). Other assay parameters were as detailed above. For determination of inhibition constant (K_i) values, prostanoid compounds were diluted in five log steps and assayed in duplicate as described above. Nonspecific binding (NSB) in both assay formats was determined with 10 μM PGE_2 or 10 μM 11-deoxy-PGE_1, both yielding very similar results In some instances, NSB was defined by the highest concentration of the test compound. The amount of specific binding obtained from these types of studies were very similar and the data were pooled. Multiple experiments were performed with each compound.

Data analysis

Resulting disintegrations per min (d.p.m.) values of bound [^{3}H]-PGE_2 from individual assays were analysed with a non-

linear, iterative curve fitting computer program using logistic functions (Bowen & Jerman, 1995) to derive the inhibition constants (IC_{50}s) for the competing compounds. For derivation of compound inhibition constant (K_i) values, the method of Cheng & Prusoff (1973) was employed using the following equation: $K_i = IC_{50}/(1+[L]/K_d)$, where IC_{50} is the compound concentration causing 50% inhibition of the binding, L is the radioligand concentration used in the competition experiments, and K_d the dissociation constant of the radioligand. The K_d and B_{max} (apparent receptor density) values were computed from data obtained from additional 7-point competition curves of unlabelled PGE_2 competing for [^{3}H]-PGE_2 (using the specific activity dilution technique) using the 'KELL' (EBDA) software package (Biosoft, Cambridge, U.K.) as previously described (McPherson, 1983). All data were represented as the arithmetic mean ± s.e.mean.

Statistical analyses, when comparing the Hill coefficients of the different compounds competing for specific [^{3}H]-PGE_2 binding, involved a two tailed unpaired *t*-test with Welch correction with unequal variances. The data were assumed to follow Gaussian distribution. A *P* value of at least 0.05 was considered statistically significant.

Results

Tissue linearity

Binding of [^{3}H]-PGE_2 to HEK-293 cell membranes containing the recombinant human EP_4 receptor in the initial tissue linearity studies using the MES buffer is depicted in Figure 2. Specific binding was virtually indistinguishable from total binding with this membrane preparation, as the per cent specific binding values were 98.1 ± 0.7% ($n = 12$) across the entire titration range. In order to conserve membrane stocks and still preserve adequate signal-to-noise ratio, it was decided that 4 μg per 0.5 ml of the membrane preparation would be sufficient for subsequent assays. Krebs buffer was detrimental for specific [^{3}H]-PGE_2 binding (data not shown) and hence MES buffer was chosen for all subsequent studies.

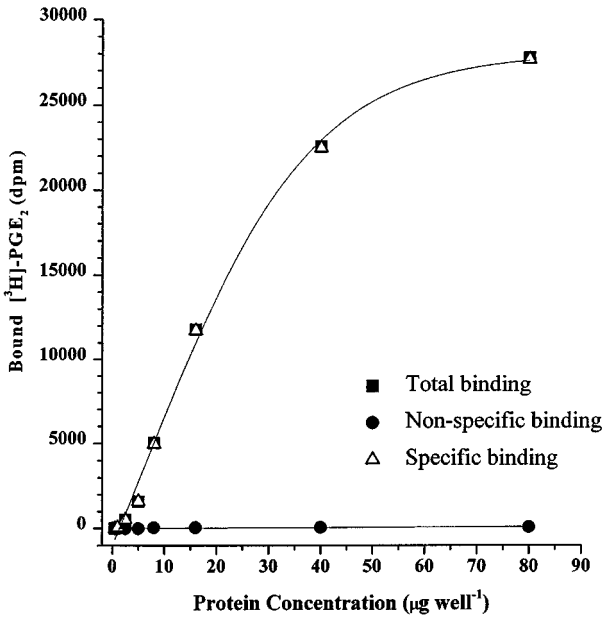

Figure 2 Tissue linearity of [^{3}H]-PGE_2 binding to HEK-293 cell membranes expressing the recombinant human EP_4 receptor. Membranes were added in varying amounts to 0.2 nM [^{3}H]-PGE_2 in a final volume of 500 μl and the binding assay performed as described in the Methods. Data from a representative experiment is shown. Note the very high level of specific binding found in this system.

Determination of K_d and B_{max} values

Unlabelled PGE_2 (seven concentrations) competed for [^{3}H]-PGE_2 binding in a concentration-dependent manner and analysis of these data produced a linear Scatchard plot (Figure 3). Computer analyses of these competition binding data by the specific activity dilution technique yielded affinity parameters such as the apparent dissociation constant (K_d) and the apparent receptor density (B_{max}) as follows:

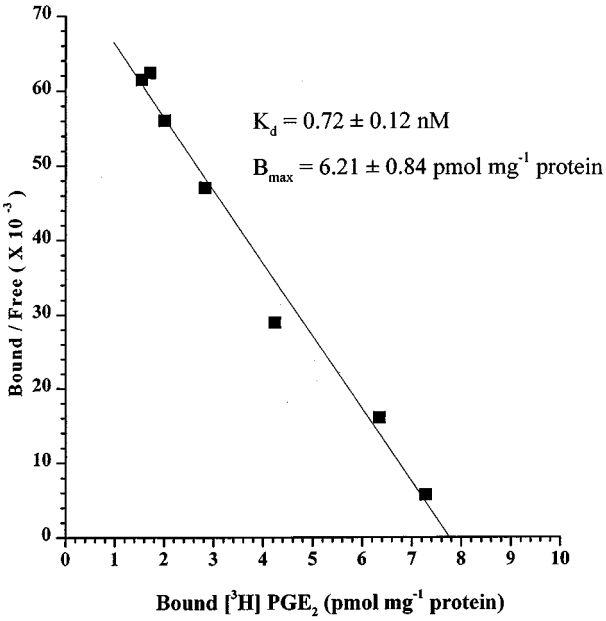

Figure 3 Scatchard analysis of [^{3}H]-PGE_2 binding to recombinant human EP_4 receptors. Data from a representative experiment of three is shown. The composite K_d and B_{max} values are shown.

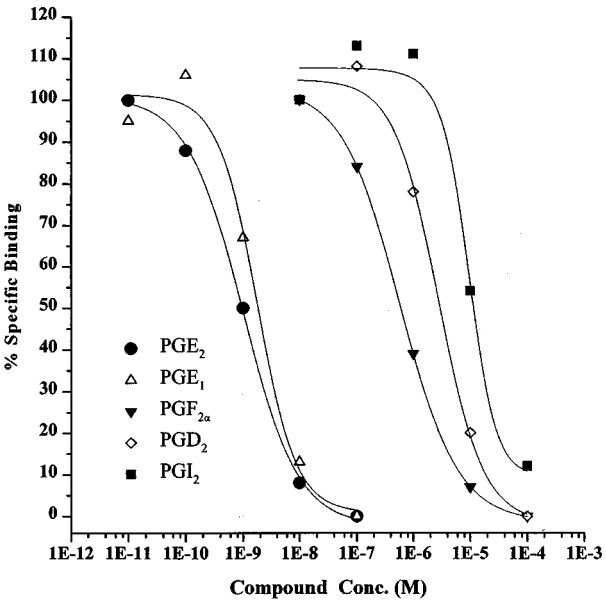

Figure 4 Competitive binding data for natural prostanoids at recombinant human EP_4 receptors. Data are means from 3–12 experiments. Error bars are omitted for clarity.

$K_d = 0.72 \pm 0.12$ nM and $B_{max} = 6.21 \pm 0.84$ pmol mg^{-1} protein ($n = 3$).

Determination of K_i *values for prostanoid compounds*

A broad range of prostanoid and related compounds with various prostanoid class specificities were allowed to compete against 0.2 nM [^{3}H]-PGE_2 to generate competition curves. Computer analyses of the binding data from these experiments produced inhibition constants (IC_{50}) values for each compound which were then converted to K_i values as described in the Methods section. The compounds could be divided into groups representing either their source (natural prostanoids as opposed to synthetic) or their relative specificity/affinity for specific prostanoid receptor classes as described in the literature (e.g. Coleman *et al.*, 1994b). Plots depicting such competition curves resulting from these experiments are presented in Figures 4 and 5, while summaries of the derived K_i data can be seen in Tables 1, 2, and 3.

A correlation of $-\log K_i$ (pK_i) values resulting from the current studies with those previously obtained by Boie *et al.* (1997) with recombinant rat EP_4 receptors expressed in HEK-293 cells yielded a linear regression fit with a correlation

Table 1 Competitive binding data for natural prostanoids competing for [^{3}H]-PGE_2 binding to cloned human EP_4 receptors

Prostanoid	K_i (nM)	*Hill coefficient* (n_H)	n
PGE_2	0.75 ± 0.03	1.03 ± 0.03	12
PGE_1	1.45 ± 0.24	1.26 ± 0.06*	3
$PGF_{2\alpha}$	433 ± 25	0.88 ± 0.05	3
PGD_2	2139 ± 180	1.21 ± 0.03**	3
PGI_2	8074 ± 254	1.25 ± 0.04**	3

Data are means $\pm$ s.e.mean from 3–12 experiments as shown, each performed in duplicate. Statistical significance: *$P < 0.03$; **$P < 0.01$ relative to PGE_2.

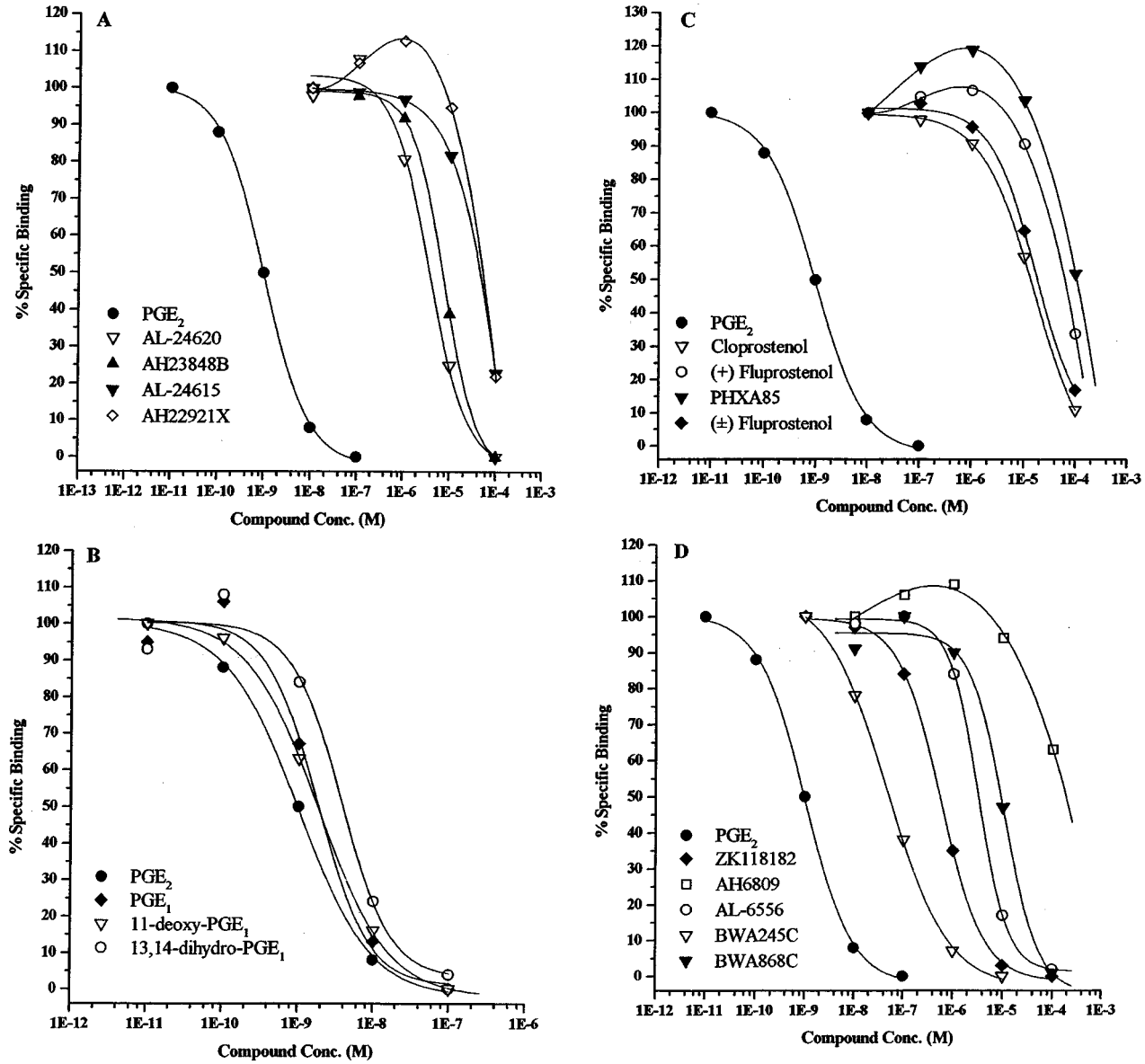

Figure 5 Competitive binding data for synthetic prostanoids, PGE_1, PGE_2 and putative EP_4 receptor agonists and antagonists. (A) shows data for PGE_2, two putative EP_4 receptor agonists (AL-24620 and AL-24615) and two known EP_4 receptor antagonists (AH23848B and AH22921X); (B) shows data for PGE_2, PGE_1 and its derivatives; (C) shows data for PGE_2 and FP receptor agonists; (D) shows data for PGE_2 and DP receptor ligands. Data are means from 3–12 experiments. Error bars are omitted for clarity.

Table 2 Ligand binding data for EP receptor-specific prostanoid compounds competing for [^{3}H]-PGE_2 binding to cloned human EP_4 receptors

Compound	*Reported Specificity*	K_i (nM)	*Hill coefficient* (n_H)	n
11-deoxy-PGE_1	$EP_2/EP_3/EP_4$	1.36±0.34	1.00±0.10	3
13, 14-dihydro-PGE_1	EP	3.07±0.29	1.29±0.13	3
17-phenyl-ω-trinor-PGE_2	EP_1/EP_3	34.5±14.1	1.17±0.33	3
11-deoxy-16, 16-dimethyl-PGE_2	EP	40.4±1.7	1.27±0.08*	5
Enprostil	EP_3	43.1±4.4	1.20±0.11	3
AH23848	EP_4	2690±232	1.23±0.06	3
AL-24620	EP_4	2720±425	1.15±0.06	3
Sulprostone	EP_1/EP_3	3310±126	0.96±0.03	3
Misoprostol	$EP_1/EP_3/EP_4$	4340±867	0.93±0.06	3
Butaprost	EP_2	6640±1140	1.46±0.19	3
AL-24615	EP_4	28,300±3200	1.20±0.06	3
AH22921	EP_4	31,800±4090	1.57±0.15	3
SC19220	EP_1	>100,000	ND	3

Data are means±s.e.mean from 3–5 experiments as shown, each performed in duplicate. Reported specifities have been taken from various publications (e.g. Coleman *et al*., 1994b). Statistical significance: $*P<0.03$ relative to PGE_2. ND=not determined.

Table 3 Ligand binding data for DP, FP, and IP receptor-specific compounds competing for [^{3}H]-PGE_2 binding to cloned human EP_4 receptors

Compound	*Reported specifity*	K_i (nM)	*Hill coefficient* (n_H)	n
BWA245C	DP	64.7±1.0	0.85±0.04*	3
ZK118182	DP	425±42	1.07±0.06	4
RS93520	DP	740±184	1.12±0.11	3
ZK110841	DP	862±45	1.85±0.47	4
AL-6556	DP	2870±409	1.23±0.08	4
SQ27986	DP	3050±98	1.34±0.40	3
S-1033	FP	6650±610	2.31±0.54	3
BWA868C	DP	8060±738	2.07±0.26*	4
Cloprostenol	FP	10,200±835	1.00±0.33	3
Fluprostenol	FP	14,400±1550	0.95±0.04	3
UFO-21	FP	15,200±3500	2.71±0.48	3
AL-6221	FP	18,700±6370	2.56±0.71	3
Ilprost	IP	22,800±1410	1.03±0.08	3
AL-6598	DP	28,900±11,50	0.95±0.26	3
AL-5848	FP	41,000±2590	1.20±0.05*	3
Latanoprost	FP	59,400±13,000	3.16±1.81	3
PHXA85	FP	75,100±2830	2.99±1.61	3
AH6809	EP/DP	119,000±10,800	0.91±0.21	3
SQ29548	TP	460,000±56,200	0.56±0.04***	3

Data are means±s.e.mean from 3–4 experients as shown, each performed in duplicate. Statistical significance: $*P<0.03$ and $***P<0.001$ relative to PGE_2.

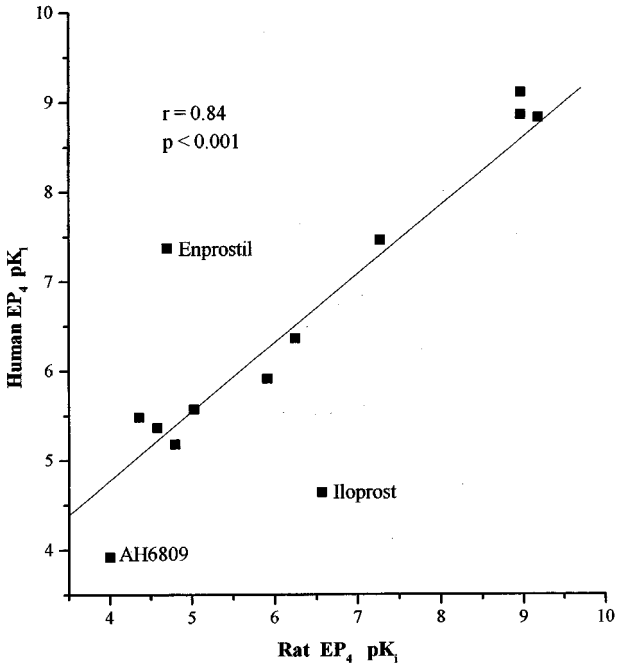

Figure 6 Correlation plot of recombinant human EP_4 receptor binding data for various prostanoids from this study and those reported by Boie *et al*. (1997) for the recombinant rat EP_4 receptor expressed in HEK-293 cells. Data points for compounds distinct from the regression line are individually labelled. Similar plots using data from mouse (Kiriyama *et al*., 1997) and human (Marshall *et al*., 1997) studies yielded correlation coefficients (r) of 0.76 and 0.98, respectively (data not shown). r=the correlation coefficient.

coefficient of 0.84 (Figure 6). Similar comparisons to previously published binding data for mouse (Kiriyama *et al*., 1997) and human EP_4 (Marshall *et al*., 1997) receptors and data from our current studies yielded correlation coefficients of 0.76 and 0.98, respectively (data not shown).

Discussion

The EP_4 receptor represents one of the four major EP prostanoid receptors with which the endogenous prostanoid PGE_2 can interact (Coleman *et al*., 1994a,b). Although EP_4 and EP_2 receptors are positively coupled to adenylyl cyclase and thus raise intracellular cyclic AMP levels (Ichikawa *et al*., 1996; Crider *et al*., 1998; 2000), there are distinct differences between the two subtypes. The EP_4 receptor responds more quickly to agonist-induced desensitization than the EP_2 receptor and the functional response to PGE_2 is more rapidly attenuated as the agonist is metabolized (Nishigaki *et al*., 1996). Functional responses elicited by the EP_4 receptor may be more finely regulated than those arising from EP_2 receptor stimulation (Nishigaki *et al*., 1996). Whereas the EP_2 receptor is apparently only incorporated into the cell surface membrane, the EP_4 receptor has been detected in the nuclear envelope of porcine brain and rat liver endothelial cells in addition to the cell surface membrane (Bhattacharya *et al*., 1999), suggesting a role in transcription. However, to the best of our knowledge, all the ligand binding studies reported to-date in the literature, and the current studies, employed cell membranes for [^{3}H]-PGE_2 binding.

Comparisons can be made with the K_d and B_{max} values generated in the present study to those generated in the three other studies utilizing recombinant human, mouse, or rat EP_4 receptors. Overall, the K_d values compared well: in the current study we obtained a K_d value of 0.72±0.12 nM compared to

1.12 ± 0.3 nM in the only other detailed study of the human EP_4 receptor to-date (Marshall *et al*., 1997). The B_{max} values in these two studies were also similar ($6.21 - 0.84$ pmol mg^{-1} protein here, versus 3.1 ± 0.3 pmol mg^{-1} in the study by Marshall *et al*., 1997). The historical K_d values for the rat and mouse recombinant EP_4 receptors are 1.1 ± 0.6 nM (Boie *et al*., 1997) and 2.5 ± 0.1 nM (Kiriyama *et al*., 1997), respectively. The B_{max} values for the two rodent preparations (0.9 ± 0.5 and 0.275 ± 0.01 pmol mg^{-1} protein) are much lower than those obtained with the recombinant human receptor preparations.

Initial characterization of the EP_4 receptor following its cloning from mouse (Kiriyama *et al*., 1997), rat (Boie *et al*., 1997), and human sources (Marshall *et al*., 1997) utilized only a small number of prostanoids and related compounds. To support future functional studies, we wished to define a prostanoid binding profile for the recombinant human EP_4 receptor with a wide range of compounds (37 in total) that differ in their currently defined prostanoid receptor specificity. The receptor binding parameters and compound affinity data we obtained for the natural prostanoids compared very well with those generated by other investigators for the mouse, rat, and human EP_4 receptors. Thus, we obtained a K_i value of 0.75 nM for PGE_2, versus values of 1.9 nM (Kiriyama *et al*., 1997), 1.1 nM (Boie *et al*., 1997), and 2.51 nM (Marshall *et al*., 1997), respectively. Similar points of comparison could be made for PGE_1 ($K_i = 1.45$ nM, versus 2.1, 0.66, and 3.16 nM), and the other natural prostanoids ($PGF_{2\alpha}$, PGD_2, and PGI_2) tested in this study. In the case of the synthetic prostanoids, results were mixed. For the EP receptor specific compounds, some differences seemed to be species-dependent. In these cases, our results compared well with those generated by Boie *et al*. (1997) with the rat EP_4 receptor (i.e. K_i for 11-deoxy-$PGE_1 = 1.45$ nM at recombinant human EP_4, versus 1.1 nM for the rat) but contrasted with those from the mouse EP_4 receptor studies ($K_i = 23$ nM) (Kiriyama *et al*., 1997). The same pattern is evident in the binding results for 17-phenyl-ω-trinor-PGE_2 ($K_i = 23$ nM at recombinant human EP_4 here, versus 54 nM for the rat, and 1000 nM for the mouse receptor) (Boie *et al*., 1997; Kiriyama *et al*., 1997). However, there were instances in which the human and rat EP_4 receptors yielded widely disparate results. This is most apparent in the data for sulprostone ($K_i = 3310$ nM here at recombinant human EP_4, versus 43,600 nM for the rat EP_4) and misoprostol ($K_i = 4340$ nM here at recombinant human EP_4, versus 26,300 nM for the rat EP_4). These comparisons can probably be ascribed to possible species differences for these particular compounds previously reported to have affinity for EP_3 and EP_1 receptors (Coleman *et al*., 1994b). However, it is worth noting that the EP_4 receptor-specific antagonist AH23848 yielded similar results to other studies using human EP_4 receptors ($K_i = 2690$ nM here, versus 3981 nM from Marshall *et al*. (1997)) as well as in the rat ($K_i = 9380$ nM; Boie *et al*., 1997). Furthermore, the recombinant human EP_4 receptor binding affinities of both the EP_4 receptor antagonists, AH22921 and AH23848, compared well with their functional antagonist potencies measured in the piglet saphenous vein ($K_b = 4 - 5$ μM; Coleman *et al*., 1994a,b) and in the cyclic AMP production assay in wild-type CHO cells ($K_b = 25.1$ μM and 5.01 μM, respectively; Crider *et al*., 2000). AL-24615 and AL-24620, which were reported to have IC_{25} values of 57 nM and 23 nM in the isolated rabbit jugular vein preparation (Burk, 1997), exhibited low micromolar affinities at the recombinant human EP_4 receptor in our studies (Table 2). Functional studies with the recombinant human EP_4 receptor with these compounds are necessary in order to determine whether these differences are species related.

Of the FP receptor-specific ligands tested in this study, only S-1033 yielded a K_i value less than 10 μM. This is somewhat surprising given the sub-micromolar affinity of $PGF_{2\alpha}$ in this system. However, $PGF_{2\alpha}$ is often regarded as a 'promiscuous' ligand, well known for binding to other prostanoid receptors (Coleman *et al*., 1994b). Thus, the K_i for $PGF_{2\alpha}$ at the recombinant human EP_4 receptor in our studies is 400 nM, comparable to the values obtained at the rat EP_4 receptor (570 nM; Boie *et al*., 1997) and the previous human EP_4 receptor study (794 nM; Marshall *et al*., 1997). This value is also comparable to that of S-1033, although the most specific FP receptor agonists to-date, cloprostenol and fluprostenol (Sharif *et al*., 1998; 1999), displayed low affinities for the recombinant human EP_4 receptor in our studies with K_i values greater than 10 μM.

Interestingly, some synthetic DP receptor-specific agonists showed a high affinity for the EP_4 receptor in this study. Several of these compounds displayed affinities in the nanomolar range, with BW245C ($K_i = 64.7$ nM) being the best example (Table 3). The affinity of PGD_2 itself in this system is only approximately 2 μM. These results are similar to those previously reported in another study of the human EP_4 receptor (Wright *et al*., 1998) with the exception that in our hands, ZK110841 yielded an approximate K_i of 845 nM, compared to the 41 nM value observed in their study. It is difficult to speculate on possible reasons for this difference, because Wright *et al*. (1998) provided virtually no details concerning their human EP_4 receptor preparation other than the fact that the receptor was expressed in HEK-293 cells. In this study, the K_i values noted for the synthetic DP agonists are somewhat surprising considering the low affinity of PGD_2 for recombinant human EP_4. Agonist data generated using the recombinant human DP receptor indicate that the K_i values for PGD_2, BW245C, and ZK110841 differ by only 2 fold or less (0.3 – 0.6 nM) (Wright *et al*., 1998). The DP antagonist BWA868C also demonstrated a high affinity in the above DP receptor preparation ($K_i = 2.3$ nM). However, the same cannot be said for BWA868C in regards to this present study with recombinant human EP_4 ($K_i = 8060$ nM), yet the synthetic DP agonists display K_i values in the nanomolar range. Both BW245C and BWA868C have been demonstrated to function as agonists and antagonists, respectively, at the EP_4 receptor in rabbit saphenous vein (Lydford *et al*., 1996). The ability of certain DP receptor-specific agonists to bind with relatively high affinity to the EP_4 receptor and to function in isolated tissue preparations poses some intriguing possibilities for drug development, as both receptors increase intracellular cyclic AMP levels. While an extensive functional study of the recombinant human EP_4 receptor remains to be done, it may be possible to develop bifunctional prostanoid compounds that elicit functional responses from both DP and EP_4 receptors.

Some of the differences observed between our compound affinity data for recombinant human EP_4 receptors expressed in HEK-293 cells and those from receptors expressed in CHO cells (Marshall *et al*., 1997; Kiriyama *et al*., 1997) may have resulted from the fact that since CHO cells express their own endogenous EP_4 receptors (Milne *et al*., 1994; Crider *et al*., 2000) the data obtained by Marshall *et al*. (1997) and Kiriyama *et al*. (1997) may reflect a composite of the endogenous CHO EP_4 receptors and the transfected human EP_4 receptors. These issues, together with the low expression levels cited by Kiriyama *et al*. (1997), may account for the low the Hill slopes (n_H) values reported in their study of the mouse EP_4 receptor (Kiriyama *et al*. 1997), and may further explain the discrepancies noted above between the human,

rat, and mouse binding data. When the n_H values of different studies are compared, only Marshall *et al.* (1997) and Kiriyama *et al.* (1997) cited n_H values for the limited number of compounds they evaluated on the cloned human and mouse EP_4 receptors, respectively. Unfortunately, n_H values for the rat EP_4 receptor binding study (Boie *et al.*, 1997) and another study employing human EP_4 receptors (Wright *et al.*, 1998) were not provided for comparison. Our results are generally in good agreement with those of Marshall *et al.* (1997) in that the majority of the few ligands they reported displayed n_H values $\geqslant 0.9$. In the only two cases where they report n_H values of 0.7, the K_i values are still in good agreement with those we obtained (for $PGF_{2\alpha}$, $K_i = 433$ nM in this study, versus 794 nM; for AH23848, $K_i = 2690$ nM here versus their K_i of 3981 nM).

In summary, a comprehensive evaluation of the binding characteristics of the recombinant human EP_4 receptor has been undertaken using a total of 37 different natural and synthetic prostanoids possessing different prostanoid receptor specificities. Our results are generally comparable to previous limited studies of the human, rat, and mouse EP_4 receptor, while providing additional data on a much greater number of compounds. Certain synthetic DP receptor agonists showed high affinity for the recombinant human EP_4 receptor, but the true significance of these findings awaits the results of future functional studies that are currently in progress.

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References

AKAIKE, A., KANEKO, S., TAMURA, Y., NAKATA, N., SHIOMI, H., USHIKUBI, F. & NARUMIYA, S. (1994). Prostaglandin E_2 protects cultured cortical neurons against N-methyl-D-aspartate receptor -mediated glutamate cytotoxicity. *Brain Res.,* **663,** 237 – 243.

ANTHONY, T.L., PIERCE, K.L., STAMER, W.D. & REGAN, J.W. (1998). Prostaglandin $F_{2\alpha}$ receptors in the human trabecular meshwork. *Invest. Ophthalmol. Vis. Sci.,* **39,** 315 – 321.

BHATTACHARYA, M., PERI, K., RIBEIRO-DA-SILVA, A., ALMAZAN, G., SHICHI, H., HOU, X., VARMA, D.R. & CHEMTOB, S. (1999). Localization of functional prostaglandin E_2 receptors EP_3 and EP_4 in the nuclear envelope. *J. Biol. Chem.,* **274,** 15719 – 15724.

BITO, L.Z., STJERNSCHANTZ, J., RESUL, B., MIRANDA, O.C. & BASU, S. (1993). The ocular effects of prostaglandin and the therapeutic potential of a new $PGF_{2\alpha}$ analog, PhXA41 (latanoprost) for glaucoma management. *J. Lipid Mediat.,* **6,** 535 – 543.

BOIE, Y., STOCCO, R., SAWYER, N., SLIPETZ, D.M., UNGRIN, M., NEUSCHAFER-RUBE, F., PUSCHEL, G., 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.

BOWEN, W.P. & JERMAN, J. (1995). Nonlinear regression using spreadsheets. *Trends. Pharmacol. Sci.,* **16,** 413 – 417.

BURK, R.M. (1997). Cyclopentane heptan(ene)oic acid, 2-heteroarylalkenyl derivatives as therapeutic agents. *International Patent Application,* WO 97/31895.

CHEMTOB, S., LI, D.Y., ABRAN, D., PERI, K.G. & VARMA, D.R. (1996). Regulation of cerebrovascular prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ receptors and their functions during development. *Semin. Perinatol.,* **20,** 164 – 172.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099 – 3108.

COLEMAN, R.A., GRIX, S.P., HEAD, S.A., LOUTTIT, J.B., MALLETT, A. & SHELDRICK, R.L. (1994a). A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostanoids,* **47,** 151 – 168.

COLEMAN, R.A. & HUMPHREY, P.P.A. (1993). *Prostanoid receptors: their function and classification.* London: Edward Arnold.

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

CRIDER, J.Y., GRIFFIN, B.W. & SHARIF, N.A. (2000). Endogenous EP_4 prostaglandin receptors coupled positively to adenylyl cyclase in Chinese hamster ovary cells: pharmacological characterization. *Prostagland, Leukotri. Essent. Fatty Acids,* **62,** 21 – 26.

CRIDER, J.Y., XU, S.X., GRIFFIN, B.W. & SHARIF, N.A. (1998). Use of a semi-automated, robotic radioimmunoassay to measure cAMP generated by activation of DP-, EP_2- and IP-prostaglandin receptors in human ocular and other cell-types. *Prostagland., Leukotri. Essent. Fatty Acids,* **59,** 77 – 82.

DAVIS, T.L. & SHARIF, N.A. (1999). Quantitative autoradiographic visualization and pharmacology of FP-prostaglandin receptors in human eyes using the novel phosphorimaging technology. *J. Ocular Pharmacol. Ther.,* **15,** 323 – 336.

DIJKSTRA, B.G., SCHEEMANN, A. & HOYNG, P.F. (1999). Flow after prostaglandin E_1 is mediated by receptor-coupled adenylyl cyclase in human anterior segments. *Invest. Ophthalmol. Vis. Sci.,* **40,** 2622 – 2626.

GABELT, B.T. & KAUFMAN, P.L. (1989). Prostaglandin $F_{2\alpha}$ increases uveoscleral outflow in the cynomolgus monkey. *Exp. Eye Res.,* **49,** 389 – 402.

GIACOMINI, E., GIORDANI, L., DI MODUGNO, F., CHERSI, A. & LUZZATI, A.L. (1998). Increased PGE_2 production meditates the in vitro inhibitory effect of human immunodeficiency virus P24 immunosuppressive heptapeptide Ch7. *Scand. J. Immunol.,* **48,** 248 – 253.

ICHIKAWA, A., SUGIMOTO, Y. & NEGISHI, M. (1996). Molecular aspects of the structures and functions of the prostanoid E receptors. *J. Lipid Mediat. Cell Signal,* **14,** 83 – 87.

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.

LINDSEY, J.D., KASHIWAGI, K., BOYLE, D., KASHIWAGI, F., FIRESTEIN, G.S. & WEINREB, R.N. (1996). Prostaglandin increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. *Curr. Eye Res.,* **15,** 869 – 875.

LYDFORD, S.J., MCKECHNIE, K.C. & DOUGALL, I.G. (1996). Pharmacological studies on prostanoid receptors in the rabbit isolated saphenous vein: a comparison with rabbit isolated ear artery. *Br. J. Pharmacol.,* **117,** 13 – 20.

MARSHALL, F.H., PATEL, K., LUNDSTROM, K., CAMACHO, J., FOORD, S.M. & LEE, M.G. (1997). Characterization of [^{3}H]-prostanoid E_2 binding to prostanoid EP_4 receptors expressed with Semliki Forest virus. *Br. J. Pharmacol.,* **121,** 1673 – 1678.

MATSUO, T. & CYNADER, M.S. (1992). Localization of prostaglandin $F_{2\alpha}$ and E_2 binding sites in the human eye. *Br. J. Opthalmol.,* **76,** 210 – 213.

MCPHERSON, G.A. (1983). A practical computer based approach to the analysis of radioligand binding experiments. *Computer Prog. Biomed.,* **17,** 107 – 114.

MEJA, K.K., BARNES, P.J. & GIEMBYCZ, M.A. (1997). Characterization of the prostanoid receptor(s) on human blood monocytes at which prostanoid E_2 inhibits lipopolysaccharide-induced tumour necrosis factor-alpha generation. *Br. J. Pharmacol.,* **122,** 149 – 157.

MILNE, S.A., LEE, J., ARMSTRONG, R.A. & WOODWARD, D.F. (1994). Human monocytes and cultured CHO cells both express EP_4 receptors positively coupled to adenylate cyclase. *Br. J. Pharmacol ,* **113** (suppl): 8P.

MORATH, R., KLEIN, T., SYBERTH, H.W. & NUSING, R.M. (1999). Immunolocalization of the four prostaglandin E_2 receptor proteins EP_1, EP_2, EP_3, and EP_4 in human kidney. *J. Am. Soc. Nephrol.,* **10,** 1851 – 1860.

MORI, K., TANAKA, I., KOTANI, M., MIYAOKA, F., SANDO, T., MURO, S., SASAKI, Y., NAKAGAWA, O., OGAWA, Y., USUI, T., OZAKI, S., ICHIKAWA, A., NAURMIYA, S. & NAKAO, K. (1996). Gene expression of the human prostaglandin E receptor EP_4 subtype: differential regulation in monocytoid and lymphoid lineage cells by phorbol ester. *J. Mol. Med.,* **74,** 333 – 336.

MUKHOPADHYAY, P., GEOGHEGAN, T.E., PATIL, R.V., BHATTACHERJEE, P. & PATERSON, C.A. (1997). Detection of EP_2, EP_4, and FP receptors in human ciliary epithelial and ciliary muscle cells. *Biochem. Pharmacol.,* **53,** 1249 – 1255.

NGUYEN, M., CAMENISCH, T., SNOUWAERT, J.N., HICKS, E., COFFMAN, T.M., ANDERSON, P.A.W., MALOUF, N.N. & KOLLER, B.H. (1997). The prostanoid receptor EP_4 triggers remodelling of the cardiovascular system at birth. *Nature,* **390,** 78 – 81.

NISHIGAKI, N., NEGISHI, M. & ICHIKAWA, A. (1996). Two G_2-coupled prostaglandin E receptor subtypes, EP_2 and EP_4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Mol. Pharmacol.,* **50,** 1031 – 1037.

SCHROR, K. (1993). The effect of prostaglandin and thromboxane A_2 on coronary vessel tone—mechanisms of action and therapeutic implications. *Eur. Heart J.,* **14** (Suppl I): 34 – 41.

SHARIF, N.A., DAVIS, T.L. & WILLIAMS, G.W. (1999). [^{3}H]AL-5848 ([^{3}H]9-β-[+]fluprostenol): carboxylic acid of travoprost (AL-6221), a novel FP prostaglandin to study the pharmacology and autoradiographic localization of the FP receptor. *J. Pharmac. Pharmacol.,* **51,** 685 – 694.

SHARIF, N.A, XU, S.X., WILLIAMS, G.W., GRIFFIN, B.W., CRIDER, J.Y. & DAVIS, T.L. (1998). Pharmacology of [^{3}H]Prostaglandin E_1/[^{3}H]Prostaglandin E_2 and [^{3}H]Prostaglandin $F_{2\alpha}$ binding to EP_3 and FP receptor binding sites in bovine corpus luteum: characterization and correlation with functional data. *J. Pharmacol. Expt. Ther.,* **286,** 1094 – 1102.

WENZEL, S.E. (1997). Arachidonic acid metabolites: mediators of inflammation in asthma. *Pharmacotherapy,* **17,** 3S – 12S.

WISE, H. (1998). Activation of the prostanoid EP_4-receptor subtype is highly coupled to inhibition of N-formyl-methionyl-leucyl-phenylalanine- stimulated rat neutrophil aggregation. *Prostanoid. Leukotri., Essent. Fatty Acids,* **58,** 77 – 84.

WITTENBURG, R.H., WILLBURGER, R.E., KLEEMEYER, K.S. & PESKAR, B.A. (1993). In vitro release of prostanoids and leukotrienes from synovial tissue, cartilage, and bone in degenerative joint diseases. *Arthritis Rheum.,* **36,** 1444 – 1450.

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.

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