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Functional pharmacology of human prostanoid EP_2 and EP_4 receptors

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Abstract

Prostanoid EP_2 and EP_4 receptor-mediated responses are difficult to distinguish pharmacologically because of the lack of potent, selective antagonists. We describe systematic agonist fingerprints for recombinant human prostanoid EP_2 and EP_4 receptors expressed in CHO and HEK293 cells, respectively. The rank orders of potency of endogenous prostaglandins were: prostanoid EP_2 receptors: prostaglandin $E_2 \gg$ prostaglandin D_2=prostaglandin $F_{2\alpha}$>prostaglandin I_2; prostanoid EP_4 receptors: prostaglandin $E_2 \gg$ prostaglandin I_2>prostaglandin D_2=prostaglandin $F_{2\alpha}$. Butaprost free acid (9-oxo-11α,16*R*-dihydroxy-17-cyclobutyl-prost-13*E*-en-1-oic acid) behaved as a highly selective partial agonist at prostanoid EP_2 receptors while butaprost methyl ester elicited small, low potency responses. The prostanoid EP_1 and EP_3 receptor agonists misoprostol (9-oxo-11α,16-dihydroxy-16-methyl-prost-13*E*-en-1-oic acid, methyl ester), sulprostone (*N*-(methylsulphonyl)-9-oxo-11α,15*R*-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-amide), and GR63799X ([1*R*-[1α(*Z*), 2β(*R**),3α]-(-)-4-benzoylamino)phenyl-7-[3-hydroxy-3-phenoxy-propoxy)-5-oxocyclopentyl]-4-heptenoate), and the prostanoid DP receptor agonist BW245C ((4*S*)-(3-[(3*R*,*S*)-3-cyclohexyl-3-hydropropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid), activated both prostanoid EP_2 and EP_4 receptors. Prostaglandin I_2, iloprost (6,9α-methylene-11α,15*S*-dihydroxy-16-methyl-prosta-5*E*,13*E*-dien-18-yn-1-oic acid, trometamol salt) and cicaprost (5-[(*E*)-(1*S*, 5*S*, 6*S*, 7*R*)-7-hydroxy-6-[(3*S*, 4*S*)-3-hydroxy-4-methylnona-1,6-diinyl]-bicyclo[3.3.0]octan-3-yliden]-3-oxapentanoic acid; ZK96480) were full agonists at prostanoid EP_4 receptors. Key differentiating agonists are: butaprost FA, 16,16-dimethyl-prostaglandin E_2, 19-(*R*)-hydroxy prostaglandin E_2, misoprostol, BW245C, prostaglandin $F_{2\alpha}$ and prostaglandin D_2.

Keywords: Prostaglandin; Receptor; Agonist; EP_2; EP_4

1. Introduction

Prostanoids are a group of lipid hormone mediators that are derived from C-20 fatty acids by the action of cyclo-oxygenases 1 and 2. They consist of the prostaglandins and the thromboxanes and they elicit a wide variety of biological responses through activation of G-protein-coupled receptors. Traditional classification schemes recognise eight prostanoid receptor proteins each being the product of an individual gene (Coleman et al., 1994b; Narumiya et al., 1999). These have been termed the prostanoid DP, EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors based on the natural prostanoid that displays the highest potency at each receptor. With the recent identification of prostaglandin D_2 as a potent agonist at the $CRTH_2$ (chemottractant receptor homologous molecule of TH2 cells) receptor (Hirai et al., 2001), the total number of prostanoid receptor subtypes is now nine.

Despite the existence of many agonists and antagonists with selectivity for one or more prostanoid receptors, the family is notorious for a paucity of high potency, truly selective ligands. This is particularly problematic in the study of prostanoid EP_2 and EP_4 receptor subtypes. These receptors are difficult to distinguish from each other since

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they are both coupled via $G\alpha_s$ to induce elevations in intracellular cAMP, are frequently co-located on the same cell or tissue types but differ in their sensitivity to inactivation mechanisms (Nishigaki et al., 1996). The most useful pharmacological tool for identifying which receptor is present is butaprost (9-oxo-11α,16*R*-dihydroxy-17-cyclobutyl-prost-13*E*-en-1-oic acid), a selective prostanoid EP_2 receptor agonist (Gardiner, 1986). However, care must be exercised when using butaprost since the commercially available methyl ester has been shown to be less selective than the free acid form of the molecule (Abramowitz et al., 2000). In addition, a compound established in the literature as a selective prostanoid EP_4 receptor antagonist, AH23848B (([1α(*Z*),2β5α]-(±)-7-[5[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-5-heptenoic acid); Brittain et al., 1985), has now been shown to possess little selectivity for this receptor (Abramowitz et al., 2000).

Pharmacological profiling of prostanoid receptors has been revolutionised with the cloning and expression of the receptor proteins in a variety of host cell backgrounds. To date, the most systematic prostanoid EP_2 and prostanoid EP_4 receptor profiles generated have been obtained from radioligand competition binding assays (Abramowitz et al., 2000; Kiriyama et al., 1997; Davis and Sharif, 2000) but little data exists describing the functional profiles of these prostanoid receptors. Therefore we have undertaken the systematic profiling of the human prostanoid EP_2 and EP_4 receptors using prostanoid receptor agonists by measuring cAMP accumulation in CHO and HEK293(T) cells stably expressing the human receptors. In this way, we have generated an agonist fingerprint for each receptor that will assist in receptor classification studies.

2. Materials and methods

2.1. Preparation of recombinant cell lines

2.1.1. CHO-EP_2

The entire coding region of the human prostanoid EP_2 receptor gene (nucleotides 1–1077 of GenBank accession number X83868) was cloned into pcDNA3 (Invitrogen, San Diego, CA, USA) at the *Bam*HI–*Not*I site. Following the incorporation of the vector using electroporation, cells were grown for 2 weeks under neomycin selection in Excel 301 medium containing 5% foetal bovine serum and 400 μg/ml neomycin (G418 or geneticin). Cells were separated using flow cytometry in order to isolate individual clones in the wells of 96-well plates. Each clone was expanded and pharmacologically characterised. A single clone displaying superior responses to prostaglandin E_2 was selected for further study. Subsequent culture was carried out in Dulbecco's modified Eagle's medium - Ham F12 mix (DMEM-F12), containing 5% heat-inactivated foetal bovine serum and 2 mM L-glutamine in 175-cm^2 flasks.

2.1.2. HEK-EP_4

HEK-293(T) cells expressing the recombinant human prostanoid EP_4 receptor were obtained from Receptor Biology (Beltsville, MD, USA). Cells were cultured in DMEM-F12, containing 10% heat-inactivated foetal bovine serum and 2 mM L-glutamine in 175-cm^2 flasks. Cells were either passaged into fresh medium or used in an assay once 90% confluency (determined visually) had been achieved.

2.2. CHO-hEP_2 and HEK-hEP_4 cAMP assay

Cells were harvested by treatment with Versene, resuspended in fresh culture medium and plated out to yield approximately 1×10^5 cells/well in a 96-well plate for overnight culture. For assay, the culture medium was replaced with assay medium (CHO-EP_2: DMEM-F12 containing 1 mM L-ascorbate and 300 μM isobutylmethylxanthine (IBMX); HEK-EP_4: DMEM-F12 containing 300 μM IBMX and 3 μM indomethacin) and incubated for 30 min. Following this, cells were incubated with agonists (CHO-EP_2: threefold dilution series; HEK-EP_4: fourfold) for 15 min. The reaction was stopped by the aspiration of the assay medium and the addition of ice-cold ethanol. All incubations were carried out at 37 °C in a 5% CO_2 atmosphere. Care was taken to ensure the constancy of IBMX, indomethacin and vehicle (dimethyl sulphoxide) concentrations throughout these experiments. The amount of cAMP in each well was then determined by [^{125}I]cAMP scintillation proximity assay using a proprietary kit (Amersham, Bucks., UK) according to the manufacturer's instructions.

2.3. Data analysis

Data from cAMP assays was the mean of two duplicate data points in each separate assay and was expressed as pmol cAMP per well. A four-parameter logistic equation of the form:

$$E = \frac{\alpha[\mathrm{A}]^{n_\mathrm{H}}}{\mathrm{EC}_{50}^{n_\mathrm{H}} + [\mathrm{A}]^{n_\mathrm{H}}}$$

was then fitted to E/[A] curve data in order to estimate maximum effect (α), curve midpoint (EC_{50}), and Hill slope parameter (n_H); other terms in the equation are effect (E) and concentration ([A]). Individual estimates of curve parameters were obtained from each curve and then averaged to provide mean data. Potency is expressed as the negative log of EC_{50} values (pEC_{50}). Quoted values are therefore the mean±standard deviation (S.D.) of n separate experiments, each derived from a separate cAMP assay. Because errors around slope estimates are log normally distributed, slope data is expressed as the geometric mean with 95% confidence intervals. Where curve parameters could not be estimated, the mean effect at

Table 1
Prostanoid agonist concentration–effect curve data determined in CHO-hEP$_2$ cells

Agonist	n	pEC$_{50}$	RP PGE$_2$=1.0	n_H	α (% PGE$_2$ max)	Max vs. PGE$_2$ P value
PGE$_1$	4	7.4±0.3	**1**	0.6 (0.5–0.8)	82±14	NS
PGE$_2$	7	7.5±0.3	**1**	0.8 (0.6–1.0)	100	
Butaprost FA	4	7.2±0.2	2	1.3 (1.1–1.4)	71±20	<0.001
16,16-Dimethyl-PGE$_2$	4	7.0±0.4	**3**	0.7 (0.4–1.4)	80±10	NS
19-(*R*)-Hydroxy-PGE$_2$	4	7.0±0.4	3	0.9 (0.4–1.8)	73±27	<0.05
11-Deoxy-PGE$_1$	4	6.6±0.3	8	1 (0.8–1.3)	63±9	<0.05
GR63799X	4	6.5 (n=1)	10 (n=1)	2.9 (n=1)	5±3	
Misoprostol	4	6.4±0.2	13	1.6 (0.9–2.9)	62±27	<0.05
BW245C	4	6.3±0.2	16	1.4 (1.0–2.0)	34±14	
PGF$_{2\alpha}$	4	6.2 (n=1)	20 (n=1)	2.6 (n=1)	14±3[a]	
PGD$_2$	4	6.1 (n=1)	25 (n=1)	2.6 (n=1)	6±1[a]	
Butaprost ME	4	<6.0	>30		29±3[b]	
17-Phenyl-ω-trinor PGE$_2$	4	5.7±0.5	63	1.6 (1.0–2.7)	40±20	
Iloprost	4	<5.5	>100		8±3a	
Sulprostone	4	<5.5	>100		−2±2a	
PGI$_2$	4	<5.0	**~320**		101±29[c]	
Cloprostenol	4	<5.0	**~320**		122±26c	
Cicaprost	3	<4.5	>1000		6±2c	
Fluprostenol	4	<4.5	>1000		9±4c	

Data are mean±S.D. or geometric mean (95% confidence intervals).
Full agonists are shown in bold type.
[a] Denotes max effect at 1 μM.
[b] Denotes max effect at 3 μM.
[c] Denotes max effect at 10 μM.

the maximum concentration tested is quoted. In Tables 1 and 2, the maximum effect of agonists is expressed as % of the prostaglandin E$_2$ E/[A] curve maximum generated on that plate. Data presented in Figs. 2 and 4 are mean pmol cAMP generated in different experiments. Hence, there are apparent differences between the maximum effect data quoted in the tables and shown in the figures. Statistical significance was determined with Student's t-test using P<0.05 to represent significance.

2.4. Drugs used

Indomethacin, prostaglandin E$_2$, prostaglandin I$_2$, prostaglandin D$_2$ and prostaglandin F$_{2\alpha}$ were purchased from

Table 2
Prostanoid agonist concentration–effect curve data determined in HEK 293(T)-hEP$_4$ cells

Agonist	n	pEC$_{50}$	RP PGE$_2$=1.0	n_H	α (% PGE$_2$ max)
PGE$_1$	4	10.1±0.1	**2**	2.1 (1.4–3.2)	102±24
PGE$_2$	9	10.3±0.1	**1**	2.1 (1.8–2.4)	100
11-Deoxy-PGE$_1$	3	9.9±0.1	**3**	2 (1.7–2.4)	147±58
16,16-Dimethyl-PGE$_2$	3	8.7±0.1	**40**	1.7 (1.0–2.9)	115±23
17-Phenyl-ω-trinor PGE$_2$	3	8.2±0.1	**120**	2.2 (0.9–5.3)	131±14
GR63799X	3	8.0±0.2	**200**	1.5 (0.6–4.1)	119±23
BW245C	3	8.0±0.2	**200**	1.5 (1.2–1.8)	138±45
19-(*R*)-Hydroxy-PGE$_2$	3	7.7±0.1	**400**	1.7 (1.0–3.0)	130±48
Cicaprost	4	7.4±0.1	**790**	1.1 (0.9–1.3)	108±21
PGI$_2$	3	6.9±0.7	**2500**	0.9 (0.3–2.7)	126±24
Misoprostol	3	6.8±0.2	**3200**	1.8 (1.3–2.7)	120±8
PGF$_{2\alpha}$	3	6.6±0.2	**5000**	1.6 (0.9–3.1)	121±16
PGD$_2$	3	6.5±0.4	**6300**	1.2 (0.2–5.8)	119±22
Iloprost	3	6.3±0.2	**10,000**	1.7 (1.3–2.2)	113±18
Sulprostone	3	5.8±0.1	**32,000**	1.1 (0.8–1.6)	135±22
Butaprost FA	4	<5.6	>50,000		15±7[a]
Fluprostenol	3	<5.5	**>63,000**		147±30[b]
Cloprostenol	3	<5.5	**>63,000**		195±37[b]
Butaprost ME	3	<4.0	>2,000,000		6±1[b]

Data are mean±S.D. or geometric mean (95% confidence intervals).
Full agonists are shown in bold type.
[a] Denotes max effect at 1 μM.
[b] Denotes max effect at 100 μM.

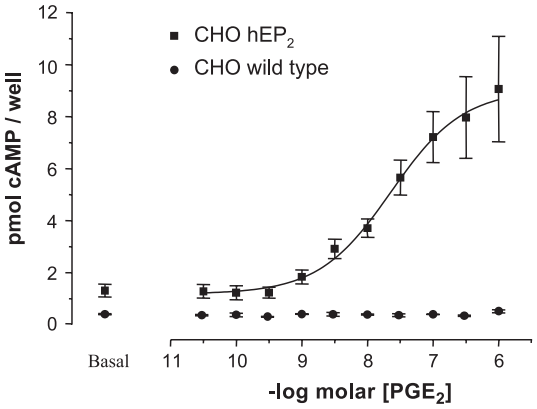

Fig. 1. Accumulation of cAMP in response to prostaglandin E_2 in CHO K1 cells stably expressing human prostanoid EP_2 receptors, and wild-type CHO K1 cells. Experiments were conducted as described in Materials and methods. Data are the mean (±standard deviation) of seven determinations, each performed in duplicate.

Sigma, Poole, Dorset, UK. Prostaglandin E_1, BW245C ((4*S*)-(3-[(3*R*,*S*)-3-cyclohexyl-3-hydropropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid), sulprostone (*N*-(methylsulphonyl)-9-oxo-11α,15*R*-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-amide), misoprostol (9-oxo-11α,16-dihydroxy-16-methyl-prost-13*E*-en-1-oic acid, methyl ester), butaprost (9-oxo-11α,16*R*-dihydroxy-17-cyclobutyl-prost-13*E*-en-1-oic acid, methyl ester), 17-phenyl trinor prostaglandin E_2, 11-deoxy prostaglandin E_1, 16,16-dimethyl prostaglandin E_2, 19-(*R*)-hydroxy prostaglandin E_2, fluprostenol ((±)-9α,11α,15*R*-trihydroxy-16-(3-(trifluoromethyl)phenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-oic acid), and cloprostenol (9α,11α,15*R*-trihydroxy-16-(3-(chlorophenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-oic acid, sodium salt) were purchased from Cayman Chemical, Ann Arbor, MI, USA. Iloprost (6,9α-methylene-11α,15*S*-dihydroxy-16-methyl-prosta-5*E*,13*E*-dien-18-yn-1-oic acid, trometamol salt) was purchased from Amersham. Cicaprost (5-[(*E*)-(1*S*, 5*S*, 6*S*, 7*R*)-7-hydroxy-6-[(3*S*, 4*S*)-3-hydroxy-4-methylnona-1,6-diinyl]-bicyclo[3.3.0]octan-3-yliden]-3-oxapentanoic acid; ZK96480) was the kind gift of Schering, Berlin, Germany. Butaprost free acid, GR32191B ([1*R*-[1α(*Z*),2β,3β,5α]]-(+)-7-[5-([1,1′-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptenoic acid), GR63799X ([1*R*-[1α(*Z*), 2β(*R**),3α]-(−)-4-benzoylamino)phenyl-7-[3-hydroxy-3-phenoxy-propoxy)-5-oxocyclopentyl]-4-heptenoate), and

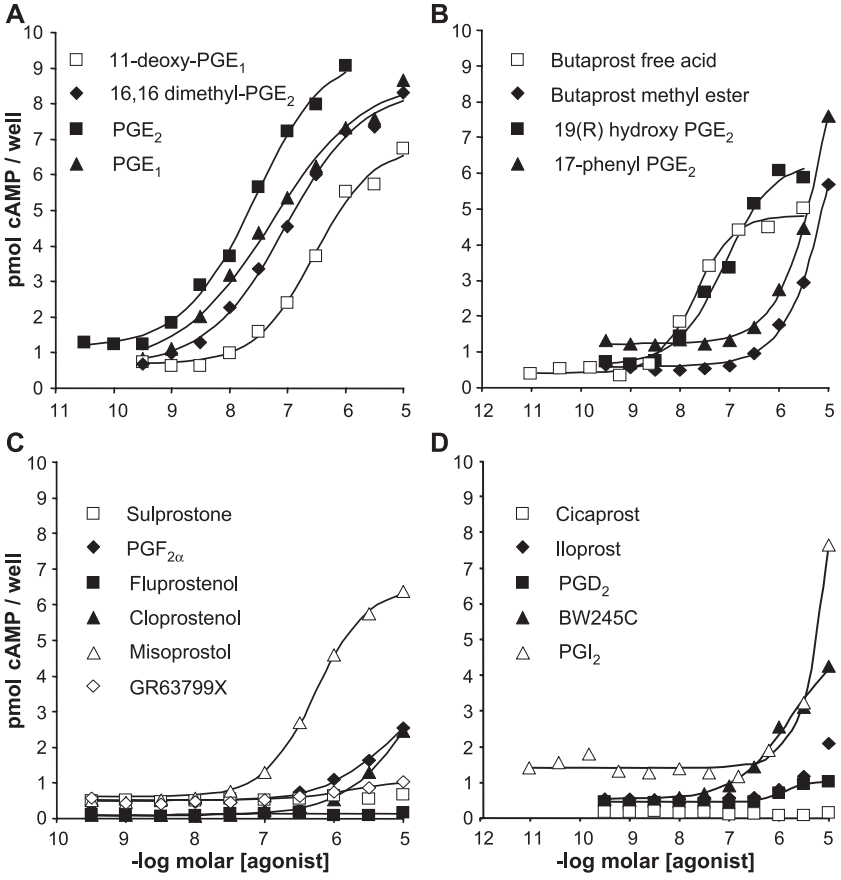

Fig. 2. Accumulation of cAMP in response to prostanoid agonists in CHO K1 cells stably expressing human prostanoid EP_2 receptors. Agonists are identified in the figure legends. Experiments were conducted as described in Materials and methods. Data are the mean (±standard deviation) of four determinations, each performed in duplicate. Error bars are omitted for clarity but at the highest concentrations tested standard deviations were in the range ±0.1–1.1 pmol cAMP except for prostaglandin E_2 (=2.0).

BWA868C (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) were prepared in the Department of Medicinal Chemistry, GlaxoWellcome Research and Development, Stevenage, UK.

Indomethacin and GR32191B were dissolved at 10 mM in dimethyl sulphoxide (DMSO). Iloprost was dissolved at 1 mM in Tris buffer pH 8.3. All other prostanoids were dissolved at 10 mM in 100% ethanol and stored at −20 °C. Compounds were freshly diluted into assay buffer as described above on each experimental occasion.

2.5. Other materials

Foetal bovine serum, heat-inactivated foetal bovine serum, neomycin and 200mM L-glutamine were purchased from Gibco-BRL, Paisley, UK. Excel 301 medium was obtained from JRH Biosciences, Lenexa, KS, USA. Dulbecco's modified Eagle's medium - Ham F12 mix (DMEM-F12) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma. IBMX was made up at 0.1 M in 0.1 M NaOH and added to DMEM-F12 for use.

3. Results

3.1. CHO-hEP$_2$

Prostaglandin E_2 produced a concentration-related increase in cAMP accumulation (pEC_{50}=7.5±0.3; n=7; Fig. 1). Wild-type CHO cells were devoid of any response to prostaglandin E_2. Eighteen other prostanoid receptor agonists also elicited elevations in cAMP (Fig. 2; Tables 1 and 3). The rank order of agonist potency was (full agonists shown in bold type, partial agonists in italics, agonists that failed to reach a maximum response in ordinary type): **prostaglandin E_2=prostaglandin E_1**>*butaprost free acid (9-oxo-11α,16R-dihydroxy-17-cyclobutyl-prost-13E-en-1-oic acid)*≥**16,16-dimethyl prostaglandin E_2**=*19(R)-hydroxy-prostaglandin E_2*>*11-deoxy-prostaglandin E_1*>*misoprostol (9-oxo-11α,16-dihydroxy-16-methyl-prost-13E-en-1-oic acid, methyl ester)*=*BW245C((4S)-(3-[(3R,S)-3-cyclohexyl-3-hydropropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid)*>*17-phenyl-ω-trinor prostaglandin E_2*>prostaglandin D_2=prostaglandin $F_{2\alpha}$=GR63799X ([1*R*-[1α(*Z*),2β(*R**),3α]-(−)-4-benzoylamino)-phenyl-7-[3-hydroxy-3-phenoxy-propoxy)-5-oxocyclopentyl]-4-heptenoate)>iloprost (6,9α-methylene-11α,15*S*-dihydroxy-16-methyl-prosta-5*E*,13*E*-dien-18-yn-1-oic acid, trometamol salt)=cicaprost (5-[(*E*)-(1*S*, 5*S*, 6*S*, 7*R*)-7-hydroxy-6-[(3*S*, 4*S*)-3-hydroxy-4-methylnona-1,6-diinyl]-bicyclo[3.3.0]octan-3-yliden]-3-oxapentanoic acid, ZK96480)=**prostaglandin I_2=cloprostenol (9α,11α,15*R*-trihydroxy-16-(3-(chlorophenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-oic acid, sodium salt)**=fluprostenol ((±)-9α,11α,15*R*-trihydroxy-16-(3-(trifluoromethyl)phenoxy)-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-oic acid)=sulprostone (*N*-(methylsulphonyl)-9-oxo-11α,15*R*-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5*Z*,13*E*-dien-1-amide)=butaprost methyl ester (9-oxo-11α,16*R*-dihydroxy-17-cyclobutyl-prost-13*E*-en-1-oic acid, methyl ester). Where complete concentration–effect (*E*/[A]) curves were generated, relative activities compared to PGE_2 were between 0.34±0.14 (BW245C) and 0.82±0.14 (prostaglandin E_1). For butaprost free acid, the maximum stimulation of cAMP production was significantly less than that of prostaglandin E_2 (4.9±0.3 pmol/well and 7.3±0.3 pmol/well respectively; P<0.001). Hill slopes were in the range 0.7 (0.4–1.4; 16,16-dimethyl prostaglandin E_2) to 1.6 (1.0–2.7; 17-phenyl-ω-trinor prostaglandin E_2).

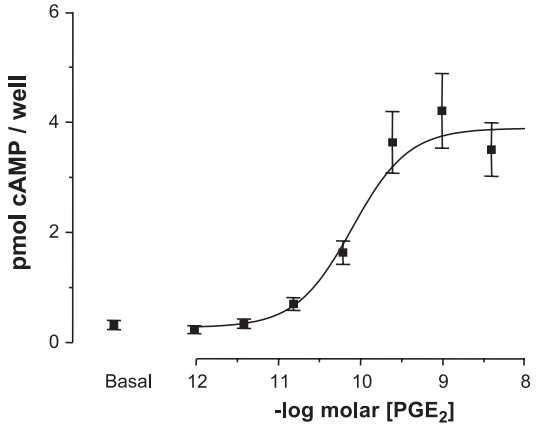

Fig. 3. Accumulation of cAMP in HEK 293(T) cells stably expressing human prostanoid EP_4 receptors in response to prostaglandin E_2. Experiments were conducted as described in Materials and methods. Data are the mean (±standard deviation) of nine determinations, each performed as a single replicate.

Table 3
Prostanoid agonist relative potency data determined in CHO-hEP$_2$ and HEK 293(T)-hEP$_4$ cells

Agonist	hEP$_2$ RP PGE_2=1.0	hEP$_4$ RP PGE_2=1.0
PGE_1	**1**	**2**
PGE_2	**1**	**1**
11-Deoxy-PGE_1	8	**3**
16,16-Dimethyl-PGE_2	**3**	**40**
17-Phenyl-ω-trinor PGE_2	63	**120**
GR63799X	10 (n=1)	**200**
BW245C	16	**200**
19-(*R*)-Hydroxy-PGE_2	3	**400**
Cicaprost	>1000	**790**
PGI_2	**~320**	**2500**
Misoprostol	13	**3200**
$PGF_{2\alpha}$	20 (n=1)	**5000**
PGD_2	25 (n=1)	**6300**
Iloprost	>100	**10,000**
Sulprostone	>100	**32,000**
Butaprost FA	2	>50,000
Fluprostenol	>1000	**>63,000**
Cloprostenol	**~320**	**>63,000**
Butaprost ME	>30	>2,000,000

Data shown are mean values. Full agonists are shown in bold type.

3.2. HEK-hEP$_4$

Prostaglandin E_2 produced a concentration-related increase in cAMP accumulation (pEC_{50}=10.3±0.1; n=9; Fig. 3). The host cell background into which the receptor had been transfected could not be obtained. The same panel of 18 prostanoid receptor agonists elicited elevations in cAMP (Fig. 4; Tables 2 and 3) but with a profile distinct to that obtained at prostanoid EP_2 receptors. The rank order of agonist potency was: **prostaglandin E_2=prostaglandin E_1>11-deoxy-prostaglandin E_1>16,16-dimethyl prostaglandin E_2>17-phenyl-ω-trinor prostaglandin E_2=BW245C=GR63799>misoprostol>cicaprost>prostaglan-prostaglandin I_2=19(*R*)-hydroxy prostaglandin E_2>prostaglandin D_2=prostaglandin $F_{2\alpha}$>iloprost>sulprostone>cloprostenol=fluprostenol**=butaprost methyl ester=butaprost free acid. Where complete concentration–effect (*E*/[A]) curves were generated, relative activities compared to prostaglandin E_2 were between 1.02±0.24 (prostaglandin E_1) and 1.47±0.58 (11-deoxy prostaglandin E_1). Hill slopes were in the range 0.9 (0.3–2.7; prostaglandin I_2) to 2.2 (0.9–5.3; 17-phenyl-ω-trinor prostaglandin E_2).

4. Discussion

The ability of a functional assay system to detect agonism in a compound is a function of the intrinsic efficacy of the molecule, the level of receptor expression in the test system ([R_o]), and the stimulus–response coupling in that system (Kenakin, 1993). Where the same agonist is used in several assay systems, the potency that it displays will be determined by receptor concentration and stimulus–response coupling in these systems. Here, prostaglandin E_2 displayed extremely high potency at prostanoid EP_4 receptors expressed in human embryonic kidney (HEK) 293(T) cells. In saturation binding studies at this receptor in commercially obtained membranes derived from this same cell line, Davis and Sharif have reported a K_d estimate of 0.72±0.12 nM. These data, coupled with the very high

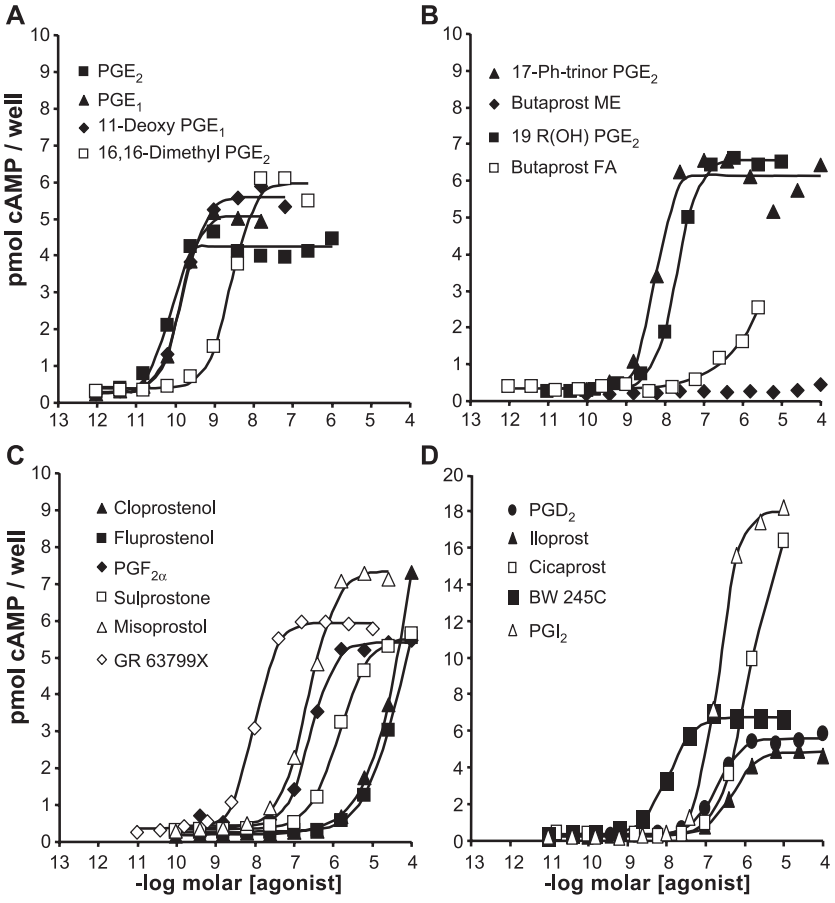

Fig. 4. Accumulation of cAMP in response to prostanoid agonists in HEK 293 (T) cells stably expressing human prostanoid EP_4 receptors. Agonists are identified in the figure legends. Experiments were conducted as described in Materials and methods. Data are the mean of three determinations, each performed in duplicate. Error bars are omitted for clarity but at the highest concentrations tested standard deviations were in the range ±0.1–1.2 pmol cAMP except for misoprostol (=3.3). NB: Data for prostaglandin I_2 and cicaprost were taken from a separate experiment where max prostaglandin E_2 response=22 pmol cAMP/well.

potency observed here for prostaglandin E_2, suggest that this cell line possesses a large receptor reserve resulting in the production of clearly measurable agonist concentration–effect curves, even for agonists with very low intrinsic efficacy. Most compounds behaved as full agonists including sulprostone which had a potency 32,000-fold lower than that of prostaglandin E_2. In contrast, at prostanoid EP_2 receptors expressed in Chinese hamster ovary (CHO) cells, prostaglandin E_2 elicited responses with moderate potency. The relatively low potency of prostaglandin E_2 could be indicative of low receptor expression, but may also be related to poor receptor–effector coupling in these cells. While adequate for the purposes of this investigation, we expect our ability to detect agonism at hEP_2 in molecules with either low intrinsic efficacy or with higher efficacy but low affinity at hEP_2 to have been severely limited.

Prostaglandin E_2 and prostaglandin E_1 curve slopes in CHO-hEP_2 cells were flat (0.8 and 0.6, respectively). In contrast to published findings (Crider et al., 2000), we found our wild-type CHO cells to be devoid of responses to prostaglandin E_2. Therefore, it seems unlikely that activation of a second receptor type is responsible. Indeed, synthetic prostanoid agonists generally produced curve slopes nearer to 1.0 with the notable exception of 16,16-dimethyl prostaglandin E_2 (n_H=0.7). Curve slopes in HEK hEP_4 cells (0.9–2.2) were steeper and more consistent with activation of a single receptor type. We were unable to obtain the same host background used to express the prostanoid EP_4 receptor and could not rule out the presence of additional prostanoid receptors. However, if an endogenous prostanoid EP_4 receptor was present, it would be human in origin and would therefore not invalidate these results. Furthermore, we have demonstrated that a selective competitive prostanoid EP_4 receptor antagonist, GW627368X, displaces prostaglandin E_2 *E*/[A] curves in a manner consistent with interaction at a single receptor population (Giblin et al., 2002a,b; Wilson et al., 2003) so it seems reasonable to assume that a single population of prostanoid EP_4 receptors exists in these cells.

Before considering the agonist fingerprints that will discriminate between human prostanoid EP_2 and EP_4 receptors, we wish to review some of the individual agonist responses. In the discussion that follows, we have used relative potency estimates to make comparisons in the selectivity of agonist molecules between prostanoid EP_2 and EP_4 receptors. Robust relative potency (RP) values should only be generated for full agonists compared with a full reference agonist. In CHO-EP_2 cells, some comparisons have been made between a partial test agonist and a full reference agonist and should be treated with appropriate caution.

In accord with the binding data recently published by Abramowitz et al., butaprost FA was highly selective for human prostanoid EP_2 receptors (hEP_2 RP=2; hEP_4 RP>200,000). In accord with the Abramowitz observations of affinity, we have found that the potency of butaprost depends upon the precise form of the molecule used. Butaprost free acid was only twofold less potent than prostaglandin E_2 but the methyl ester was about 100 times less potent. Examination of agonist relative potencies suggests that butaprost methyl ester is also selective for prostanoid EP_2 receptors over prostanoid EP_4 receptors (hEP_2 RP>30; hEP_4 RP>200,000). The free acid form is not commercially available so care must be exercised in interpreting results obtained using butaprost and in functional studies it is vital that relative potencies are used. In addition, our data also shows for the first time that butaprost free acid has lower intrinsic efficacy than prostaglandin E_2 (maximum effect=62±27% of prostaglandin E_2 response) and may therefore behave as a partial agonist in some systems.

The hydantoin, BW245C was originally identified as a selective prostanoid DP receptor agonist (Whittle et al., 1983). Isolated tissue studies later found additional activities in this molecule which were postulated to be prostanoid EP_2 agonism (Giles et al., 1989). Our data clearly demonstrates that BW245C is an agonist at both human prostanoid EP_4 (RP=200) and EP_2 receptors (RP=16; α=34%). In keeping with these data Abramowitz et al. (2000) and Davis and Sharif (2000), have demonstrated high affinity BW245C binding to the human prostanoid EP_4 receptors in agonist radioligand binding experiments (pK_i=6.9 and 7.2, respectively) and to human prostanoid EP_2 receptors (pK_i=6.7). Consequently, in tissues or cells containing mixed receptor populations, an agonist response generated by BW245C cannot be assumed to have arisen by interaction with prostanoid DP receptors.

Three agonists claimed to have selectivity for prostanoid EP_1/EP_3 receptors, sulprostone, misoprostol and GR63799X, were agonists at human prostanoid EP_4 receptors. Only misoprostol produced significant agonism at prostanoid EP_2 receptors, a feature of its pharmacology that has long been recognised (see Coleman et al., 1994b, for review). The ability of sulprostone to activate porcine prostanoid EP_4 receptors has been previously observed (Coleman et al., 1994a) but it is not a widely recognised property of this molecule. The current data show that sulprostone has low potency at prostanoid EP_4 receptors (pEC_{50}=5.8; RP=32,000), which is in agreement with quoted pK_i values of 5.5 and 5.1 (Davis and Sharif, 2000; Abramowitz et al., 2000, respectively). Therefore, in systems containing mixed receptor populations, it is possible that sulprostone could exert opposing effects, possibly resulting in bell-shaped *E*/[A] curves. The finding of moderately potent agonism by GR63799X at human prostanoid EP_4 receptors (RP=200) has not previously been observed although it has been shown to have low affinity for murine prostanoid EP_4 receptors (pK_i=6.3; Kiriyama et al., 1997). This molecule was originally identified as a selective prostanoid EP_3 receptor agonist (Bunce et al., 1991) but the moderate potency at prostanoid EP_4 receptors shown by this compound may explain the biphasic *E*/[A] curves seen in rabbit ductus arteriosus (Smith and McGrath, 1995) and the

reduced uterine-stimulation potential of this compound in vivo.

It has been suspected that selective prostanoid IP receptor agonists have some activity at porcine prostanoid EP_4 receptors (Jones et al., 2000) and we have confirmed this. Indeed, in our experiments, iloprost and cicaprost were full agonists at human prostanoid EP_4 receptors with maximal effects not significantly different to those of prostaglandin E_2, albeit with much lower potency than the latter. This extends the binding data from Abramowitz et al. (2000) who noted binding of iloprost (pK_i=6.6) and cicaprost (pK_i=7.4) at prostanoid EP_4 receptors and lower affinity binding at prostanoid EP_2 receptors (pK_i=5.7 and <5.9, respectively). Prostaglandin I_2 was also an agonist at human prostanoid EP_4 receptors so it would appear that there is a common structural motif in prostanoid IP receptor agonists leading to prostanoid EP_4 receptor agonism. Clearly, these findings are of relevance to the use of prostanoid IP receptor agonists to discriminate between prostanoid IP and EP Gs-coupled receptors where mixed receptor populations exist.

In addition to butaprost, selectivity for prostanoid EP_2 receptors has been claimed for a number of other prostanoid agonists including 19-(*R*)-hydroxy prostaglandin E_2 (Woodward et al., 1993) and 11-deoxy prostaglandin E_1 (Chen and Woodward, 1992). We examined these agonists along with a nonselective agonist, 16,16-dimethyl-prostaglandin E_2 (Coleman et al., 1994b, for review), and an agonist reported to be selective for prostanoid EP_1 and EP_3 receptors, 17-phenyl-ω-trinor-prostaglandin E_2 (Miller et al., 1975). The relative potencies of 19-(*R*)-hydroxy prostaglandin E_2 for human prostanoid EP_2 (RP=3) and EP_4 (RP=400) receptors confirm that it is indeed a prostanoid EP_2 receptor selective agonist. However, our data suggest that 11-deoxy prostaglandin E_1 has no selectivity for prostanoid EP_2 receptors (hEP_2 RP=8; hEP_4 RP=3) confirming observations by De Vries et al. (1995). Indeed, it probably has a higher relative potency for prostanoid EP_4 receptors since it behaved as a partial agonist at human prostanoid EP_2 receptors (α=63%). which means that our RP value at prostanoid EP_2 receptors is probably an underestimate where it acts as a full agonist. In contrast with published functional data, our data suggest that 16,16-dimethyl-prostaglandin E_2 possesses modest selectivity for human prostanoid EP_2 receptors (hEP_2 RP=3; hEP_4 RP=40) and also that 17-phenyl-ω-trinor-prostaglandin E_2 is not selective for prostanoid EP_1 and EP_3 receptors but can also activate both prostanoid EP_2 and EP_4 receptors (hEP_2 RP=63; hEP_4 RP=120).

Our data confirms and extends many previous observations that most prostanoid agonists do not have absolute selectivity and such claims should be viewed with extreme caution. However, it is possible to discriminate between human prostanoid EP_2 and EP_4 receptors by careful study of relative agonist (and antagonist) potencies. Endogenous prostaglandins activated human prostanoid EP_2 receptors with a rank order of potency: prostaglandin E_2≫prostaglandin D_2=prostaglandin $F_{2\alpha}$>prostaglandin I_2. At human prostanoid EP_4 receptors, the rank order of agonist potency was: prostaglandin E_2≫prostaglandin I_2>prostaglandin $F_{2\alpha}$=prostaglandin D_2. We suggest that the following agonists are the most important for studies seeking to discriminate between human prostanoid EP_2 and EP_4 receptors (EP_2 RP/EP_4 RP): butaprost FA (2/>50,000), 16,16-dimethyl-prostaglandin E_2 (3/40), 19(*R*)-hydroxy-prostaglandin E_2 (3/400), misoprostol (13/3,200), BW245C (16/200), prostaglandin $F_{2\alpha}$ (20/5000), prostaglandin D_2 (25/6300). The key discriminator is butaprost FA but because butaprost is a partial agonist it may appear inactive in some systems. Interestingly, all these key compounds are relatively more potent at prostanoid EP_2 receptors than at prostanoid EP_4 receptors.

The original classification of prostanoid receptors (Kennedy et al., 1982) was based on the rank order of potency of the primary naturally occurring prostaglandins and thromboxanes obtained using a range of whole tissue bioassay techniques. However, once the human receptors were cloned and expressed using recombinant expression systems (An et al., 1993; Bastien et al., 1994; Regan et al., 1994; Boie et al., 1997; Marshall et al., 1997; Davis and Sharif, 2000), radioligand competition became the method of choice by which relative prostaglandin binding affinities were established. In the field of receptor research, many authors have sought to correlate binding potency (IC_{50}) or affinity (pK_i) with functional pEC_{50} values. There is no a priori reason to expect functional potency to correlate with binding affinity because the relationship between these parameters is efficacy dependent (Kenakin, 2000). Therefore, the functional agonist fingerprints established here will be invaluable in receptor classification studies.

Difficulties can still arise where functional data is used for classification purposes without reference to systematically obtained agonist fingerprints. For example, the original attribution of functional responses in human nonpigmented ciliary epithelial cells to activation of prostanoid EP_2 receptors (Crider et al., 1998a,b) may not have been made if the data presented here were available. Indeed, the authors later redefined these responses as being due to activation of a mixed prostanoid EP_2/EP_4 receptor population (Crider and Sharif, 2001) based on evidence obtained with the prostanoid EP_4 receptor antagonist AH23848B. Careful application of the agonist fingerprints we have generated would have led to the same conclusion by comparison with the earlier agonist data. Interestingly, Crider et al. found a high potency response in human nonpigmented ciliary epithelial cells to butaprost methyl ester. This raises the possibility that these cells possess significant esterase activity that could convert the ester to the more potent free acid, and highlights a further complicating factor that may arise with the use of certain prostanoid agonists.

These results demonstrate the care that must be exercised when attempting to classify prostanoid receptors that are pharmacologically similar such as prostanoid EP_2 and EP_4 receptors. We have shown that a number of ligands regarded

as selective for particular types of prostanoid receptor have additional activities at human prostanoid EP_2 and EP_4 receptors that can complicate their use and prevent reliable conclusions being drawn. In the absence of truly selective and potent antagonists, discrimination between these receptors can only be achieved through careful agonist fingerprinting and comparison with data generated systematically at recombinant receptors, with the caveat that nonhuman receptor pharmacology may differ.

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