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Characterization of [^{3}H]-prostaglandin E_2 binding to prostaglandin EP_4 receptors expressed with Semliki Forest virus

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1 The human prostaglandin EP_4 receptor has been expressed by use of the Semliki Forest virus system.

2 In cell membranes [^{3}H]-prostaglandin E_2 ([^{3}H]-PGE_2) bound to a high affinity site with a K_d of 1.12 ± 0.3 nM and a B_{max} of 3.1 ± 0.3 pmol mg^{-1} protein.

3 In competition studies the rank order of potency for prostaglandins was $PGE_2 = PGE_1 \gg PGF_{2\alpha} = PGI_2$.

4 The binding of [^{3}H]-PGE_2 to cell membranes was inhibited by approximately 60% by the addition of guanylnucleotides, suggesting that this proportion of the receptors was G-protein coupled.

5 [^{3}H]-PGE_2 binding was increased by greater than 200% by the addition of divalent cations, with little change in the IC_{50} of PGE_2.

6 In saturation studies removal of divalent cations and addition of GTPγS resulted in a 65% reduction in the B_{max} with no change in the K_d. These results are consistent with the ligand labelling two states of the receptor R*, a high affinity state and R*G, a high affinity G protein coupled state.

Keywords: Prostaglandin EP_4 receptor; G-protein; Semliki Forest virus

Introduction

Prostaglandin E_2 (PGE_2) produces a wide range of actions in many tissues as a result of binding to specific prostaglandin E receptors on the plasma membrane (Halushka *et al*., 1989; Negishi *et al*., 1995). PGE_2 is involved in the maintenance of local homeostasis acting to modify sodium and water reabsorption. In addition PGE_2 causes contraction or relaxation of vascular and nonvascular smooth muscle and modulates the release of neurotransmitters.

PGE_2 receptors are members of the G-protein coupled receptor superfamily with seven putative membrane spanning regions. Four receptor subtypes (EP_1, EP_2, EP_3 and EP_4) were originally described based on the pharmacology of a variety of agonist and antagonist ligands (Coleman *et al*., 1990). EP_1 receptors are blocked by the antagonists AH 6809 and SC-19220, EP_2 receptors activated by AY23626 but not by sulprostone, whilst both these compounds are agonists at EP_3 receptors. EP_1 receptors mediate their effects by producing a rapid increase in intracellular Ca^{2+} (Katoh *et al*., 1995). EP_2 and EP_3 receptors stimulate and inhibit adenylate cyclase activity, respectively. The EP_4 receptor was first identified on pig saphenous vein (Coleman *et al*., 1994), but is now known to be expressed in a variety of tissues including the lung, peripheral blood lymphocytes and the vasculature (An *et al*., 1993; Bastien *et al*., 1994). This receptor, like EP_2, also stimulates adenylate cyclase but differs in the pharmacology of a variety of ligands such as AH23848B which is a selective EP_4 antagonist.

All four receptors have now been cloned and expressed in cell lines. Some confusion arose with the cloning of the EP_4 receptor as this was initially classified as being the EP_2 receptor (Honda *et al*., 1993; An *et al*., 1993; Bastien *et al*., 1994). However, a subsequently cloned prostaglandin receptor was shown to have closer pharmacology to the classical EP_2 receptor and this led to the re-classification of the original EP_2 clones as EP_4 (Regan *et al*., 1994).

In this study we have used the Semliki Forest virus (SFV) system to obtain a high level of expression of EP_4 receptors which have allowed a detailed pharmacological characterization of the receptors by use of a radioligand binding assay. The pharmacology of a number of compounds has been compared to data previously obtained in the pig saphenous vein. In addition, we have examined the G protein coupling of the receptor, by investigating the effect of guanylnucleotides and divalent cations on the binding.

Human EP_4 receptor cDNA cloning

The EP_4 receptor was originally cloned in this laboratory as genomic DNA (Foord *et al*., 1996). The gene encoding the EP_4 receptor contains three exons. The first is non-coding with exon two representing the receptor from just 5′ of the initiation methionine to transmembrane 6 (289 amino acids) and exon three the remainder of the receptor (200 amino acids). Exon two was cloned as a HincII-BamHI fragment of genomic DNA. Exon three was cloned after amplification of cDNA (synthesized from peripheral blood lymphocyte mRNA) by the polymerase chain reaction (PCR). The primers chosen amplified the whole of exon three and extended over a unique SphI site present in exon two. The two halves of the cDNA sequence were sequenced and assembled by ligation at the common SphI site into pBluescript (Stratagene). The sequence was cloned into the Semliki Forest virus expression vector (pSFV1, Liljestrom & Garoff, 1991), as a BamHI fragment from pBluescript. This strategy leads to some non-coding genomic DNA being present at the 5′ end of the construct. The clone was shown to encode a functional EP_4 receptor by expression of transcribed cRNA in *Xenopus* oocytes with the cystic fibrosis transporter (CFTR) as a reporter of internal (adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels (essentially as described by Bastien *et al*., 1994). In subsequent experiments the genomic DNA sequences upstream of the EP_4 receptor initiation methionine were removed, but this made no significant difference to the number of EP_4 receptors expressed that could be detected by the specific binding of [^{3}H]-PGE_2.

Expression of the prostaglandin EP_4 receptor

In vitro RNA was transcribed with SP6 RNA polymerase from plasmids pSFV1-EP_4, pSFV3-LacZ and pSFV-Helper2 (Berglund *et al*., 1993) and electroporated into BHK cells as de-

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scribed previously (Liljestrom & Garoff, 1991). After 24 h the *in vivo* packaged virus particles were collected. The titre of the SFV-LacZ virus stock was determined as described previously (Lundstrom *et al*., 1994). Because the titres for the SFV-EP_4 virus stock could not be obtained directly, comparisons to the SFV-LacZ virus stocks were made. Since SFV-LacZ and SFV-EP_4 virus stocks were packaged in parallel, we assumed the titres to be of the same magnitude. This could also be confirmed from pulse-labelling experiments, where similar expression levels of the viral structural proteins were obtained.

In expression studies 70–80% confluent BHK cells were infected with the SFV-EP_4 virus stock at a multiplicity of infection of 10 and pulse-labelling carried out 16 h post-infection. Protein expression was verified by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), followed by autoradiography.

For preparation of the membrane fraction confluent Chinese hamster ovary (CHO) cells growing in T175 flasks were infected with SFV-EP_4 virus at a multiplicity of infection of 10 and cells harvested 16 h post-infection.

Membrane preparation

Cell pellets from SFV-EP_4 infected CHO cells were homogenized in assay buffer (10 mM MES, 10 mM $MnCl_2$, 1 mM EDTA, pH 6/NaOH) by an Ultra Turrax homogenizer and centrifuged at 48,000 *g* at 4°C for 30 min. The resulting supernatant was discarded and the pellet resuspended in assay buffer by homogenization. Membranes were stored in 0.5 ml aliquots (protein concentration = 876 μg ml^{-1}) at −70°C. Protein concentrations were determined by the method of Bradford (1976) with bovine serum album (BSA) as standard.

Radioligand binding

Binding assays were performed in a total volume of 0.25 ml assay buffer. Saturation studies were conducted with [^{3}H]-PGE_2 concentrations ranging from 0.2–2.5 nM and competition studies and kinetic experiments with a final concentration of 0.4 nM [^{3}H]-PGE_2. Assays were initiated by the addition of membrane (5 μg protein per tube). After 45 min incubation at room temperature, assays were terminated by rapid filtration of membranes through Whatman GF/B filters (presoaked in wash buffer: 10 mM MES plus 0.01% BSA, pH 6/NaOH), with a Brandel cell harvester. Filters were washed 4 times with 1 ml of ice-cold wash buffer and the residual [^{3}H]-PGE_2 bound to the filter was determined by liquid scintillation spectrometry. Non-specific binding was determined in the presence of 1 μM PGE_2.

Materials

[^{3}H]-PGE_2 (specific activity 183 $Cimmol^{-1}$) was obtained from Amersham International (Bucks., U.K.). PGE_2, PGE_1, $PGF_{2\alpha}$, PGI_2, guanosine 5′-triphosphate (GTP), guanosine 5′-*o*-(3-thiotriphosphate) (GTPγS) and 5′-guanylimidodiphosphate (GppNHp) were purchased from Sigma Chemical Co. (Dorset, U.K.). AH238348B ([1α(z), 2β5α]-(±)-7-[5[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-5-heptenoic acid) and AH 6809 (6-isopropoxy-9-oxoxanthense-2-carboxylic acid) were obtained from Glaxo Wellcome, U.K.

Data analysis

Experiments were performed, in duplicate or triplicate, a minimum of three times. Results are given as mean ± s.e.mean. [^{3}H]-PGE_2 saturation binding data were analysed by a nonlinear least-squares curve fitting procedure by use of the computer programme LIGAND (Munson & Rodbard, 1980) to obtain K_d and B_{max} values. Competition curves were analysed by use of the ALLFIT model (De Lean *et al*., 1977). IC_{50} values were derived from this analysis. IC_{50} values were converted to K_i values by use of the Cheng-Prusoff (1973) equation.

Results

Protein expression

The RNAs transcribed from the pSFV-Helper2, pSFV1-EP_4 and pSFV3-LacZ, respectively, were co-electroporated into BHK cells. Both the SFV recombinant and helper RNAs were expected to be replicated and translated into proteins. This was confirmed by the pulse-labellings where high levels of viral structural proteins, encoded by helper RNA, were detected by autoradiography five hours after electroporation (data not shown). At this early stage the prostaglandin EP_4 receptor was not clearly detected by pulse-labelling.

Although the SFV recombinant and helper RNAs were both replicated in the electroporated BHK cells only the SFV recombinant RNA should be packaged into virus particles. This is due to the presence of the packaging signal only in the SFV recombinant RNA but lacking from the helper RNA. Infections with the viral stocks are therefore expected to express only the genes carried by the SFV recombinant. This could be confirmed by pulse-labelling experiments. The generated SFV-LacZ virus stocks had titres of 10^9 to 10^{10} infective units ml^{-1} and the titres for the SFV-EP_4 virus stocks packaged in parallel were assumed to be of the same magnitude.

To provide material for binding studies confluent CHO cells in T175 flasks were infected at an estimated multiplicity of infection of 10 for 16 h. In parallel, pulse-labelling was carried out in 6 well plates to confirm the expression of the prostaglandin EP_4 receptor. A 100% infectivity of the CHO cell culture was achieved, which could also be detected by the change in the morphology and growth pattern of the cells.

Saturation binding

Saturation analysis of [^{3}H]-PGE_2 binding to EP_4 receptors showed that specific binding was saturable and of high affinity, $K_d = 1.12 \pm 0.3$ nM, slope = 1.0 ± 0.1 and $B_{max} = 3.1 \pm 0.3$ pmol mg^{-1} protein (Figure 1). Scatchard transformation of the isotherm data indicated that specific binding of [^{3}H]-PGE_2 was to a single site of high affinity (Figure 1). Specific [^{3}H]-PGE_2 binding was >80% of the total [^{3}H]-PGE_2 binding at the K_d value.

Kinetic analysis

The association time courses for the binding of [^{3}H]-PGE_2 to EP_4 receptors showed that specific binding was rapid ($t_{1/2} = 12.5 \times 1.29$ min), increased with time, reached equilibrium after 45 min at room temperature and remained stable for 90 min (Figure 2). As total binding did not exceed 10% of the amount of [^{3}H]-PGE_2 added, data were linearized according to a pseudo first order rate equation (Weiland & Molinoff, 1981). Plots of ln ($B_{eq}/B_{eq} - B_t$) are shown (inset to Figure 2). The apparent association rate constant (K_{ob}) was 0.057 ± 0.007

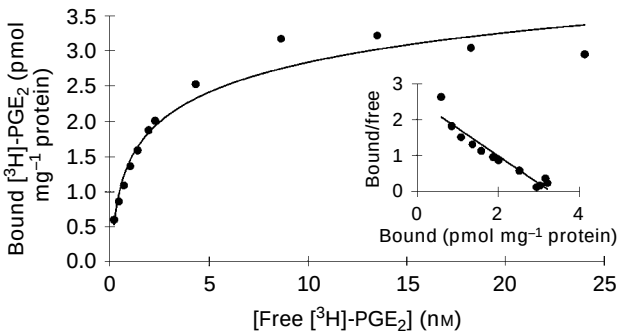

Figure 1 A representative occupancy curve for specific [^{3}H]-PGE_2 binding to EP_4 receptors. Each point represents the mean of triplicate determinations from a single representative experiment. Inset: Scatchard analysis of the data.

min^{-1}. The dissociation of [^{3}H]-PGE$_2$ was initiated by the addition of 5 μM PGE$_2$ (Figure 3). The dissociation was multiphasic and data were best fitted to a double exponential decay equation. The receptor-ligand dissociation comprised of two phases, an initial fast phase which had a dissociation rate (K_{-1}) of 0.25 min^{-1} ($t_{1/2}=3.5$ min) and a second much slower phase, $K'_{-1}=0.04$ min^{-1} ($t_{1/2}=28.2$ min). There was also a portion of specific binding which was apparently irreversible. Since the dissociation time course consisted of more than one phase, the kinetic K_d value was not calculated.

Competition studies

In competition studies the affinities of various prostanoids were determined. These are listed in Table 1. The rank order of potency for naturally occurring prostaglandins in competition for [^{3}H]-PGE$_2$ binding was PGE$_2$=PGE$_1$⋙PGF$_{2\alpha}$=PGI$_2$ (Figure 4a). [^{3}H]-PGE$_2$ binding was also displaced by AH238348B (an EP$_4$ antagonist), butaprost (an EP$_2$ agonist) and to a lesser extent by sulprostone (an EP$_3$ agonist) and AH6809 (an EP$_1$ antagonist) (Figure 4b).

Effects of divalent cations and guanylnucleotides

Competition binding curves to PGE$_2$ were performed in the presence and absence of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$ or $BaCl_2$. Specific binding of 0.4 nM [^{3}H]-PGE$_2$ was increased to over 300% by the addition of divalent cations (see Table 2). In the presence or absence of the divalent cations the Hill coefficients for the inhibition curves of PGE$_2$ were close to unity, and there was a small reduction in the IC$_{50}$ values in the presence of the divalent cations (Table 2).

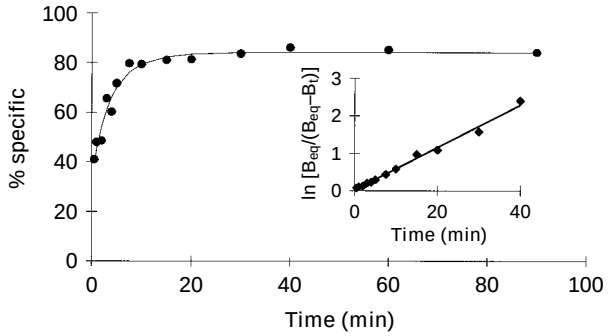

Figure 2 Association time course of [^{3}H]-PGE$_2$ specific binding to EP$_4$ receptors. Data points are the mean±s.e.mean of 3 separate experiments. Inset: plot of ln ($B_{eq}/B_{eq}-B_t$) versus time, where B_{eq}=amount of [^{3}H]-PGE$_2$ specifically bound at equilibrium and

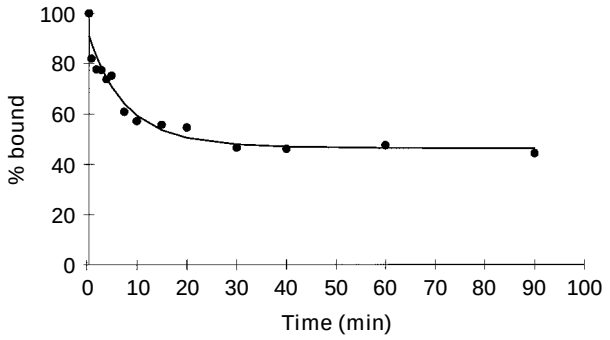

Figure 3 Dissociation time course of [^{3}H]-PGE$_2$ specific binding to EP$_4$ receptors. Membranes were incubated for 45 min before the addition of PGE$_2$ final concentration 5 μM. Each point represents the mean of triplicate determinations from a single representative experiment.

Table 1 Affinity values of prostaglandin ligands for the inhibition of [^{3}H]-PGE$_2$ binding to the cloned human EP$_4$ receptor

Compound	pK_i	n_H
PGE$_2$	8.6±0.1	1.0
PGE$_1$	8.5±0.1	1.0
PGF$_{2\alpha}$	6.1±0.2	0.7
PGI$_2$	6.0±0.2	0.9
Butaprost	5.3±0.3	1.0
Sulprostone	<5	
AH6809	<5	
AH238248	5.4±0.2	0.7

Data are expressed as pK_i values (the negative logarithm of the molar concentration of competing ligand required to inhibit 50% of 0.4 nM [^{3}H]-PGE$_2$ binding), n_H is the Hill coefficient. Values represent the mean±s.e.mean from at least $n=3$ experiments performed in triplicate tubes.

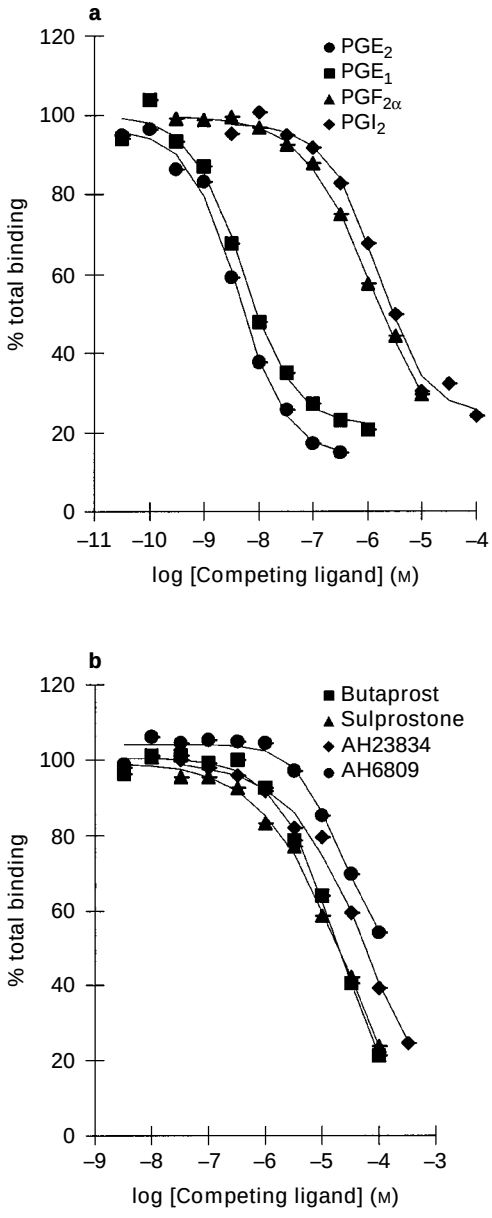

Figure 4 Competition studies of [^{3}H]-PGE$_2$ binding to the cloned human EP$_4$ receptor. [^{3}H]-PGE$_2$ binding assays were performed as described under Methods. (a) The percentage total [^{3}H]-PGE$_2$ binding at various concentrations of competing prostaglandins and prostaglandin analogues was determined. (b) The percentage total [^{3}H]-PGE$_2$ binding at various concentrations of EP-selective receptor agonists and antagonists. Each curve represents the mean of duplicates from a single experiment, representative of at least three separate experiments.

The effects of guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP) and the two non-hydrolysable analogues guanosine 5′-o-(3-thiotriphosphate) (GTPγS) and 5′-guanylimidodiphosphate (GppNHp) on [^{3}H]-PGE_2 binding were investigated. The order of potency for the inhibition of binding was GTP > GppNHp > GTPγS > GDP. All four guanylnucleotides displaced binding to approximately 40% of specific binding (GTP:36±5%; GDP 40±7%; GTPγS 45±4%; GppNHp 55±5% (Figure 5)). Also, saturation analysis performed in the presence of GTPγS (10 μM) and absence of manganese, produced a 65% decrease in B_{max} with no change in K_d for [^{3}H]-PGE_2 ($n=3$) (Figure 6 and Table 3).

Discussion

In 1994 Coleman and colleagues described the pharmacology of a novel prostaglandin receptor responsible for the relaxation of piglet saphenous vein. The pharmacology of these receptors differed from the other inhibitory prostaglandin receptor subtype, EP_2 in that the selective EP_2 agonists AH13205X and butaprost were virtually inactive. Furthermore two thromboxane receptor antagonists AH22921X and AH23848B were weak antagonists in this preparation whilst being totally inactive against prostanoid effects mediated via EP_1, EP_2 and EP_3 receptors.

The SFV expression system has been used to express a variety of G-protein coupled receptors including neurokinin NK_1 (Lundstrom *et al*., 1994) neurokinin NK_2, dopamine D_3 (Lundstrom *et al*., 1995) and adenosine A_3 receptors (Patel *et al*., 1996). With the SFV expression system, levels of expression of up to 60 pmol mg^{-1} protein of receptor have been obtained. In this study saturation experiments with [^{3}H]-PGE_2 resulted in the calculation of a B_{max} of 3 pmol mg^{-1} protein. This value is quite low for the SFV expression system. However, we have shown that the ligand only labels high affinity states of the receptor (see below for further discussion). It is possible that the true B_{max} is considerably higher but that the remaining receptors are in a low affinity state with respect to agonist binding and are not labelled with this ligand. In the case of the NK_1 receptors the K_d value in SFV-neurokinin-1 infected CHO cells was found to be higher than determined in other systems suggesting that the binding was to a low affinity state of the receptor (Lundstrom *et al*., 1994). The existence of low affinity state receptors in these membranes could be confirmed by using an antagonist ligand, however so far no EP_4 antagonists have been radiolabelled.

In competition binding studies the rank order of potency for the prostaglandins was $PGE_2 = PGE_1 \gg PGF_{2\alpha} = PGI_2$. The rank order and absolute potency values agree with the pharmacological characterization of the prostanoid receptor in

Table 2 The effect of divalent cations (10 mM) on the inhibition binding curves of PGE_2

	pIC_{50}	*n_H*	*% specific binding*
Control	8.0±0.13	0.8±0.1	100
MG^{2+}	8.6±0.03	1.0±0.1	380±26
Mn^{2+}	8.4±0.05	0.8±0.1	428±9
Ca^{2+}	8.6±0.06	1.0±0.1	318±25
Ba^{2+}	8.6±0.05	1.0±0.1	348±22

Data are expressed as pIC_{50} values (the negative logarithm of the concentration of PGE_2 required to inhibit 50% of 0.4 nM [^{3}H]-PGE_2 binding), and as a percentage of the specific [^{3}H]-PGE_2 binding obtained in the absence of divalent cations, n_H is the Hill coefficient. The data are mean±s.e.mean fron $n=3$ experiments performed in triplicate tubes.

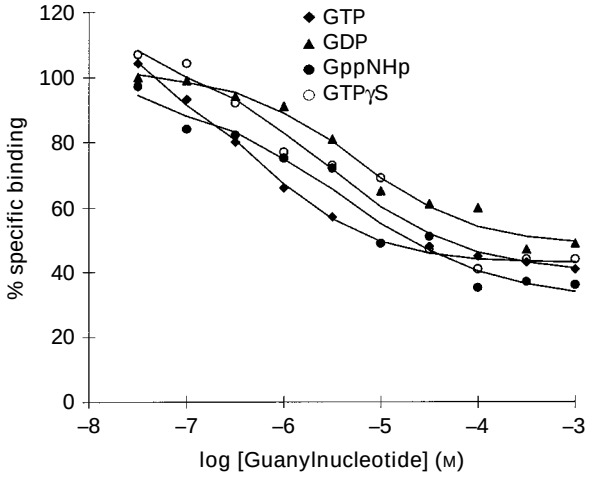

Figure 5 The effect of guanylnucleotides on the specific binding of [^{3}H]-PGE_2 defined by the addition of unlabelled PGE_2 (1 mM). Binding assays were performed as described in Methods. Each point represents the mean of triplicate determinations from a single representative experiment.

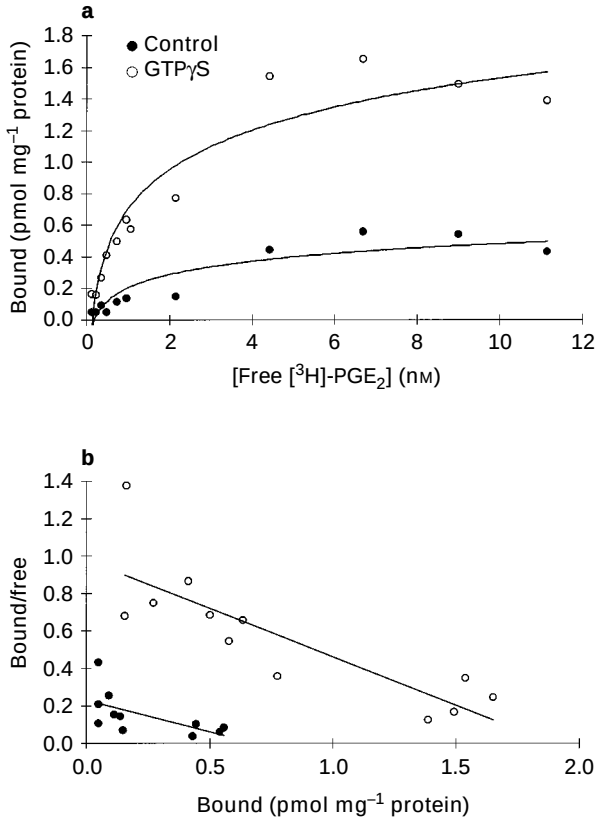

Figure 6 Saturation binding experiments in the absence (control) and presence of GTPγS (10 mM). (a) A representative occupancy curve for specific [^{3}H]-PGE_2 binding to EP_4 receptors. (b) Scatchard analysis of the specific binding shown in (a).

Table 3 The effect of GTPγS (10 μM) on saturation binding

	−GTPγS, +$MnCl_2$ (1 mM)	*+GTPγS (10 μM), −$MnCl_2$*
K_d (nM)	1.4±0.4	1.5±0.6
B_{max} (pmol mg^{-1} protein)	2.3±0.4	0.8±0.1

The data are mean±s.e.mean from $n=3$ experiments performed in triplicate tubes.

piglet saphenous vein (Coleman *et al*., 1994) and with what has previously been obtained for the cloned mouse EP_4 receptor stably expressed in CHO cells (Katsuyama *et al*., 1995), and for the human EP_4 receptor transiently expressed in COS cells (Bastien *et al*., 1994). Furthermore the affinity of the EP_4 antagonist AH238348 measured in this study ($pK_i = 5.4$) is identical with the functional antagonist potency in the pig saphenous vein ($pA_2 = 5.4$).

The G-protein coupling of the receptors in this system was examined by investigating the action of guanylnucleotides and divalent cations on the binding of [^{3}H]-PGE_2. In this study an approximate decrease of 40% of [^{3}H]-PGE_2 specific binding sites was observed in experiments in which dose-dependent inhibition of [^{3}H]-PGE_2 binding by a variety of guanylnucleotides was measured. This suggests that these receptors were G-protein coupled. For most G protein coupled receptors the addition of guanylnucleotides results in a shift in the equilibrium between agonist high and low affinity states. In the present study the radioligand only labels a single high affinity state of the receptor and, hence, the addition of guanylnucleotides is seen as a reduction in the apparent B_{max} since binding to the low affinity state will be lost. A similar effect of guanylnucleotides has been obtained for [^{3}H]-N$^{\alpha}$-methylhistamine binding to histamine H_3 receptors (Clark & Hill, 1995).

These results indicate that there may be a portion of high affinity agonist binding which is insensitive to guanylnucleotides. This may be due to the existence of a population of receptor G-protein complexes which are inaccessible to guanylnucleotides, or this may reflect the rate of turnover of the receptor/G protein complex, such that there is always a proportion of the receptors in a G-protein coupled state even in the presence of guanylnucleotides.

An alternative explanation is given by the ternary complex model of Lefkowitz *et al*. (1993): $R \leftrightharpoons R^* \leftrightharpoons R^*G$ where R and R* represent inactive and active states of the receptor with respect to its ability to interact with G proteins and transduce a signal. R and R* have low and high affinities, respectively, for agonist binding. R*G represents the G-protein-coupled form of the receptor. This can exist in the presence and absence of agonist. In the absence of agonist the existence of R*G is seen as constitutive activity of the receptor. Binding of ligands to the receptor affects the equilibrium between R and R*, for example agonists act to increase the proportion of receptors in the R* state and therefore result in an increase in receptor signalling through G proteins. In this study the agonist ligand is presumed to bind to R* and R*G, both of which have a similar affinity for the agonist. The addition of guanylnucleotides results in the conversion of receptors in the inactive, unlabelled R state. Binding to R* is still observed in the presence of guanylnucleotides.

Divalent cations act by stabilizing R*G and hence have the opposite effect to guanylnucleotides. In the presence of divalent cations the equilibrium will be shifted towards the right and therefore the proportion of receptors in the R* form will increase. The addition of divalent cations increased the amount of specific binding of [^{3}H]-PGE_2 and appeared to cause a small decrease in the IC_{50} for displacement with cold PGE_2, under both sets of conditions the Hill coefficients were close to unity suggesting that binding was to a single affinity state of the receptor. It should be noted that the amount of binding detected at the low ligand concentration (0.4 nM) in the absence of divalent cations was low and the competition curves for PGE_2 and subsequent IC_{50} values were less reproducible than in the presence of cations.

In order to investigate the effect of guanylnucleotides and divalent cations on the B_{max} and K_d of [^{3}H]-PGE_2 binding in more detail saturation studies were conducted under conditions in which the equilibrium shown above would be to the left, i.e. towards R (plus GTPγS and minus divalent cations) or under conditions in which the equilibrium would be to the right ie towards R*G (in the presence of 10 mM $MnCl_2$). The presence of GTPγS and the removal of manganese ions reduced the B_{max} by 65%. However, the K_d value was unchanged. These results are consistent with ligand labelling the R* and R*G forms of the receptor with an equal and high affinity but not labelling the R form.

In kinetic experiments a monophasic association was observed. However, the dissociation of [^{3}H]-PGE_2 was clearly multiphasic. Since both the saturation and association experiments suggested binding to a single site, this was an unexpected result. Biphasic dissociations have been observed previously for agonist ligands at G-protein linked receptors under conditions which give single site saturation binding isotherms, for example [^{3}H]-senktide binding to NK_3 receptors (Guard *et al*., 1990). It is not understood how the behaviour seen in the dissociation experiments relates to the different states of the receptor described above. Also unexpected was the small portion of binding which appeared in the dissociation experiments to be irreversible. In competition studies all ligands were able to displace binding to the same level as cold PGE_2. This difference may be due to the long time course of the dissociation experiments and perhaps represents some longer term change in the receptor-G protein complex which is not seen in the shorter time-span used in the competition studies, or when the competing ligand is included from the start of the incubation.

In summary, the use of the Semliki Forest virus expression system has allowed us to generate large quantities of the human EP_4 receptor, which has enabled a detailed characterization of the pharmacology and G protein coupling of the receptor. The assay may allow identification of selective tools for this receptor which are necessary if we are to elucidate the physiological or pathophysiological role of this receptor.

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