Identification of prostaglandin E receptor 'EP2' cloned from mastocytoma cells as EP4 subtype

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Abstract **We previously cloned a cDNA for a mouse PGE receptor positively coupled to adenylate cyclase from mouse mastocytoma cells, and reported it as EP2 subtype of PGE receptor [Honda, A., Sugimoto, Y., Namba, T., Watabe, A., Irie, A., Negishi, M., Narumiya, S. and Ichikawa, A. (1993) J. Biol. Chem. 268, 7759–7762]. However, it is not sensitive to one of the EP2 agonists, butaprost. Recently another subtype of PGE receptor coupled to adenylate cyclase has been identified pharmacologically and named EP4. These findings have led us to examine whether the cloned receptor is the EP4 subtype. AH23848B, a selective EP4 antagonist, not only displaced the [^{3}H]PGE_2 binding to the cloned receptor but antagonized the PGE_2-stimulated cAMP formation in the receptor. In contrast, EP2 specific agonists, butaprost and 19(R)OH-PGE_2 neither bound to the receptor nor stimulated the cAMP formation. These results suggest that this receptor previously reported as 'EP2' subtype is identical to the pharmacologically defined EP4 subtype and not of EP2 subtype.**

Key words: Prostaglandin E; EP4 subtype; Prostanoid receptor

1. Introduction

Prostaglandin (PG) E_2 produces a broad range of biological actions in diverse tissues through its binding to specific receptors on cell membranes [1,2]. PGE receptors were first divided into three subtypes, EP1, EP2 and EP3 [3], on the basis of their relative sensitivities to various agonists and antagonists. The excitatory actions of PGE_2 are mediated by EP1 and EP3 receptors, which are coupled to Ca^{2+} mobilization and the inhibition of adenylate cyclase, respectively [3,4]. On the other hand, inhibitory and relaxant activities of PGE_2 are believed to be mediated by EP2 receptors, coupled to stimulation of adenylate cyclase [3]. We previously isolated a cDNA from mouse mastocytoma cells for a mouse PGE receptor and found that it is positively coupled to adenylate cyclase [5]. The cloned receptor was reported as EP2, because it had properties consistent with those reported for the relaxant EP2 receptor [6], except the lack of binding of one of the EP2 agonist, butaprost [5]. While we isolated and characterized this receptor, Coleman and his colleagues reported a pharmacologically-defined fourth subtype of PGE receptor, the EP4 receptor, which, like the EP2 receptor, is positively coupled to adenylate cyclase, but which differs in the pharmacological activity induced by certain ligands, and they suggested that our cloned receptor may be EP4 subtype [7]. It does not respond to butaprost and is sensitive to AH23848B [7]. We therefore postulated that the previously-cloned 'EP2' receptor may correspond to EP4 reported by Coleman. Here we report that our cloned receptor is identical to the pharmacologically-defined EP4 receptor. We also describe specificity of the receptor for various ligands including EP2 agonists.

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Abbreviations: PG, prostaglandin; CHO, Chinese hamster ovary.

2. Materials and methods

2.1. Materials

AH23848B, butaprost and 1-OH-PGE_1 were generous gifts from Dr. R.A. Coleman of Glaxo Group Research Ltd., Dr. P.J. Gardiner of Bayer UK Ltd. and Dr. D.F. Woodward of Allergan Ltd., respectively. [5,6,8,11,12,14,15-^{3}H]PGE_2 (179 Ci/mmol) was obtained from DuPont-New England Nuclear. PGE_2, 11-deoxy PGE_1, 19(R)OH-PGE_2 and 16,16-dimethyl PGE_2 were purchased from Cayman Chemical (Ann Arbor, MI). ^{125}I-Labeled cAMP assay system was obtained from Amersham Corp.

2.2. Stable expression in CHO cells

cDNA transfection was performed by lipofection [8], essentially as described [9]. Chinese hamster ovary (CHO) cells, deficient in dihydrofolate reductase (dhfr) activity (CHO-dhfr$^-$) [10], were transfected with the cDNA which encodes the cloned receptor [6] inserted into pdKCR-dhfr, a eukaryotic expression vector containing a mouse gene of dihydrofolate reductase as a selection marker [11]. Selection was performed in the α-modification of Eagle's medium, lacking ribonucleosides and deoxyribonucleosides, with 10% dialyzed fetal bovine serum (Cell Culture Laboratory). Clonal cell lines were isolated by single-cell cloning and screened by RNA blotting.

2.3. Measurement of cAMP formation

The cAMP levels in cells were determined essentially as described previously [9]. CHO cells expressing the receptor cultured in 24-well plates (5×10^5 cells/well) were incubated for 10 min at 37°C in HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES, pH 7.4). Reactions were started by the addition of the test agents along with 100 μM Ro-20-1724. After incubation for 10 min at 37°C, reactions were terminated by the addition of 10% trichloroacetic acid. The cAMP formed was measured by the radioimmunoassay kit. Apparent pA_2 value of AH23848B against PGE_2 was determined by the method of Arunlakshana and Schild [12].

2.4. Membrane preparation and [^{3}H]PGE_2 binding assay

[^{3}H]PGE_2 binding to the membranes of CHO cells expressing the receptor was determined as described previously [13]. CHO cells expressing the receptor were harvested, and then homogenized using a Potter-Elvehjem homogenizer in an ice-cold solution comprising 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 20 μM indomethacin and 0.25 M sucrose. The homogenate was centrifuged at $800 \times g$ for 5 min, the supernatant was further centrifuged at $300,000 \times g$ for 20 min at 4°C, and the pellet was washed once and resuspended in 20 mM HEPES–NaOH (pH 7.4), containing 1 mM EDTA, 10 mM $MgCl_2$ (buffer A), then used for the

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[^{3}H]PGE_2 binding assay. The membrane (40 μg protein) was incubated with 4 nM [^{3}H]PGE_2 (397,000 dpm) at 30°C for 1 h in 100 μl of buffer A, then [^{3}H]PGE_2 binding to the membrane was determined as described previously [13]. Non-specific binding was determined using a 1,000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the non-specific binding from the total binding.

3. Results and discussion

We established the CHO cells stably expressing the previously reported cloned 'EP2' receptor [6]. The number of the expressed receptor is 252 ± 6.3 fmol/mg. PGE_2 in a concentration-dependent manner stimulated cAMP formation in the cells, the value of EC_{50} being 20 nM (Fig. 1A). This concentration–response curve to PGE_2 was shifted one order toward the right in the presence of 30 μM of AH23848B, an EP4 antagonist. Fig. 1B shows the result of the Schild plot analysis obtained by the AH23848B-induced concentration-dependent shift of the response curve to PGE_2. A Schild plot regression line revealed a linear relationship, and the slope was close to unity, indicating that AH23848B behaves as a simple and competitive antagonist of PGE_2 for the receptor. The value of pA_2 is 5.3, and this value is in good agreement with that of AH23848B for EP4 receptor reported previously [7]. Furthermore, AH23848B concentration-dependently inhibited the [^{3}H]PGE_2 binding to the membrane of the receptor-expressing cells, the half-maximal concentration for the inhibition being 30 μM (Fig. 1C). These results demonstrate that the cloned receptor is the EP4 subtype.

We next examined the binding affinities of the receptor for various PG analogues including EP2 agonists by assessing the displacement of [^{3}H]PGE_2 binding to the receptor. As shown in Fig. 2A, 11-deoxy PGE_1, EP2 and EP4 agonist [14], and 16,16-dimethyl PGE_2, a non-selective EP agonist [15], concentration-dependently inhibited the [^{3}H]PGE_2 binding, their half-maximal concentrations for the inhibition being one-order higher than that of PGE_2. In contrast, butaprost and 19(H)OH-PGE_2, specific EP2 agonists, showed very weak inhibition, but 1-OH-PGE_1 had stronger inhibition than the EP2 agonists. We further examined the agonist activities of several PG analogues in the cells. As shown in Fig. 2B, 11-deoxy PGE_1 concentration-dependently stimulated cAMP accumulation with the EC_{50} of 30 nM, which is similar to that of PGE_2. 1-OH-PGE_1 behaved as a partial agonist, but two EP2 agonists, butaprost and 19(R)OH-PGE_2, had no ability to stimulate cAMP accumulation. Thus, the cloned receptor did not respond to the EP2 agonists.

Table 1
Comparison of binding affinities and cAMP accumulation activities of various PGE analogues of the EP4 and EP2 receptors

	EP4		EP2***	
	K_i*	EC_{50}**	K_i*	EC_{50}**
PGE_2	1.0	1.0	1.0	1.0
16, 16-Dimethyl PGE_2	7.3		2.4	
11-Deoxy PGE_1	7.3	0.75	1.9	
1-OH-PGE_1	360	17.0¶	>200	38
Butaprost	>1200	>500	9.5	>34
19(R)OH-PGE_2	850	>500	30	

*,**The K_i values of binding displacement and EC_{50} values of cAMP accumulation for PGE analogues are shown as equipotent molar ratios relative to PGE_2. The K_i and EC_{50} values for PGE_2 of EP4 and EP2 are 8.0 ± 0.9 and 53 ± 14 nM (K_i), and 20 ± 3 and 43 ± 6 nM (EC_{50}), respectively. Values shown are the means for triplicate determination. ¶Full agonist potency was not observed (partial agonist). ***Regan et al. (1994) Mol. Pharmacol. 46, 213–220.

Recently, a human PGE receptor subtype has been cloned, which has characteristics of the pharmacologically defined EP2 subtype [16]. We compared the K_i values of binding displacement and EC_{50} values of cAMP accumulation for PGE analogues used here between our cloned EP4 receptor and the recently reported human EP2 receptor. As shown in Table 1, 11-deoxy PGE_1 is the good agonist for both EP2 and EP4 receptors. Butaprost and 19(R)OH-PGE_2 are selective EP2 agonists. The EP4 receptor and EP2 receptor differ in several ligand affinity.

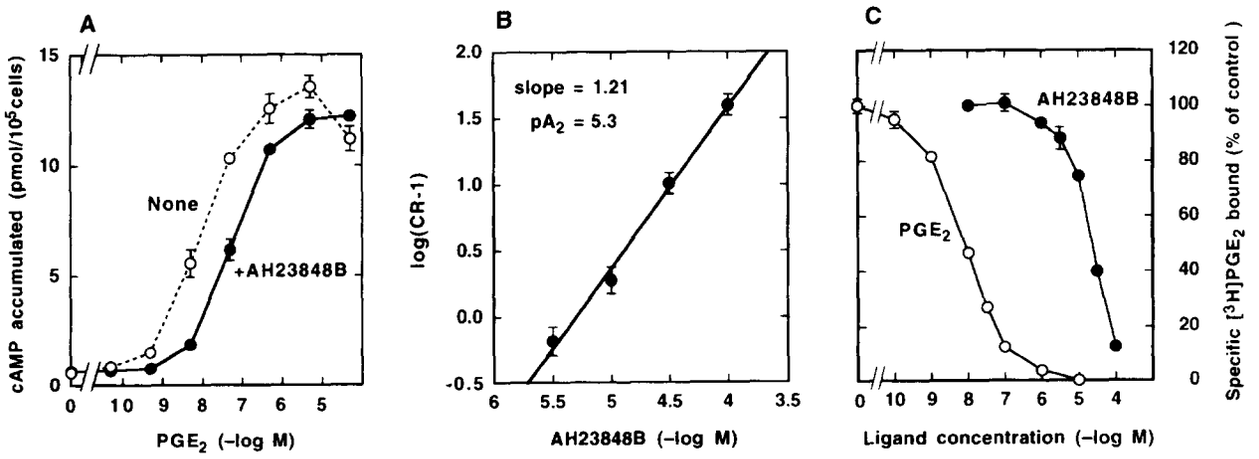

Fig. 1. Effects of AH23848B on PGE_2-induced cAMP formation and PGE_2 binding in the cloned PGE receptor-expressing CHO cells. (A) Concentration–response curve. After the cells had been preincubated for 30 min in the absence (○) and presence (●) of AH23848B (30 mM), they were incubated for 10 min at 37°C with the indicated concentrations of PGE_2, and the cAMP formed was determined as described in section 2. (B) Schild plot for the antagonism by AH23848B against cAMP response to PGE_2. The concentration–response curves to PGE_2 were obtained after the preincubation with various concentrations of AH23848B. (C) Displacement of [^{3}H]PGE_2 binding to the receptor. Unlabeled AH23848B (●) or PGE_2 (E) was added to the binding assay mixture at indicated concentrations, and specific [^{3}H]PGE_2 binding was determined as described in section2. The results shown are the means ± S.E.M. for triplicate determination.

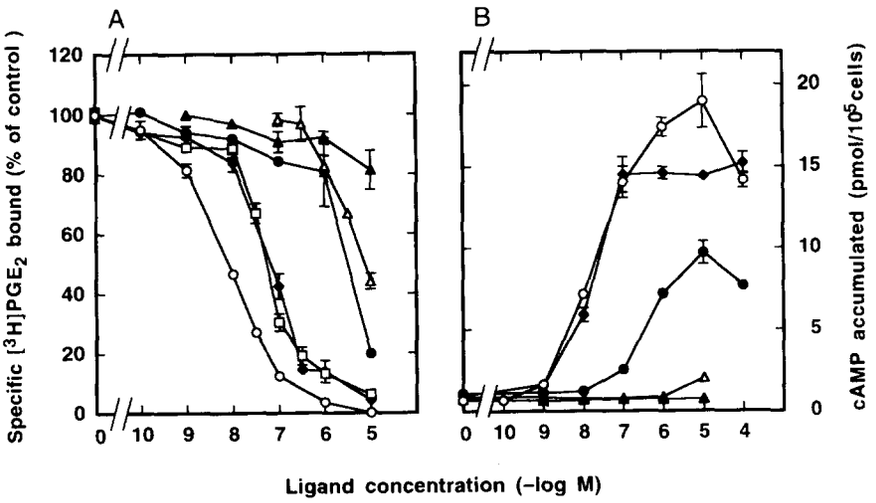

Fig. 2. Effects of PGE analogues on $[^3H]PGE_2$ binding and cAMP formation. (A) Displacement of $[^3H]PGE_2$ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations and specific $[^3H]PGE_2$ binding was determined as described in section 2. (B) cAMP formation. CHO cells were incubated with the indicated concentrations of PGs along with 100 μM Ro-20–1724, and the cAMP formed was determined as described in section 2. The results shown are the means ± S.E.M. for triplicate determination. ○, PGE_2; ●, 1-OH-PGE_1; ▲, butaprost; △, 19(R)OH-PGE_2; ◆, 11-deoxy PGE_1; □, 16,16-dimethyl PGE_2.

We previously revealed that the mRNA for the cloned 'EP2' receptor is highly expressed in ileum and thymus, followed by lung, spleen, heart and uterus [5]. Whereas pharmacological actions of EP4 receptors so far reported are limited to several smooth muscles including piglet saphenous vein [7] and rabbit ductus arteriosus [17], the study of mRNA expression showed that the EP4 receptor is widely distributed in the body and may mediate various PGE_2 actions through intracellular cAMP increase in many tissues and cells. Thymocytes exhibit specific PGE binding activity, and PGE_2 inhibits the function and proliferation of T cells by increasing cAMP levels [18]. High expression of the EP4 receptor in thymus suggest that this inhibitory actions of PGE_2 are mediated by the EP4 receptor. The EP4 receptor may play an important role in immune system. We also demonstrated that the EP4 receptor is expressed in kidney [19]. As based on in situ hybridization study, the EP4 receptor is localized to the mesangial cells of glomeruli. In cultured mesangial cells, PGE_2 is known to elicit cAMP formation and attenuate the contractility induced by various vasoconstrictors [20,21]. This relaxant action of PGE_2 may be mediated by the EP4 receptor, and the receptor may be involved in regulation of glomerular filtration. The EP4 receptor may regulate a variety of cellular functions in the body.

In summary, we report here that the PGE receptor cloned from mastocytoma cells is identical to pharmacologically defined EP4 receptor. This work clearly demonstrates difference in ligand specificity between the EP4 and EP2 receptors and will be useful for understanding diverse physiological roles of PGE_2 through PGE receptor subtypes.

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