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A conserved threonine in the second extracellular loop of the human EP_2 and EP_4 receptors is required for ligand binding

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Abstract

G protein coupled receptors for prostaglandins are activated when agonists are bound to a binding pocket formed in part by the seven transmembrane domains. Recent studies have determined that substitution of a conserved threonine in the second extracellular loop of the prostaglandin EP_3 receptor resulted in increased affinity for ligands with a C1 methyl ester moiety. The homologous threonine in the second extracellular loop of the human prostaglandin EP_2 and EP_4 receptors was mutated to alanine. When expressed in COS1 cells, detectable radioligand binding at both of these receptors bearing the threonine to alanine substitution (EP_2T185A; EP_4T168A) was abolished, as well as the receptors' ability to stimulate intracellular [cAMP]. In contrast, EP_2 and EP_4 receptors bearing conservative threonine to serine mutations (EP_2T185S; EP_4T168S) displayed K_d values for [^{3}H]prostaglandin E_2 similar to wild type receptors: 8.8 ± 0.7 nM for EP_2T185S compared to 12.9 ± 1.2 nM for EP_2 wild type; 2.0 ± 0.8 nM for EP_4T168S compared to 0.9 ± 0.3 nM for the EP_4 wild type receptor. The EC_{50} values for cAMP stimulation were 1.3 ± 0.6 nM for EP_2 wild type; 2.7 ± 1.3 nM for EP_2T185S; 1.1 ± 0.3 nM for EP_4 wild type; and 1.4 ± 0.33 nM for EP_4T168S. These studies suggest a critical role for the hydroxyl moiety on these conserved threonine residues at position 168/185 of the second extracellular loop in prostaglandin receptor–ligand interactions.

Keywords: Prostaglandin E_2; G protein coupled receptor; Mutagenesis

1. Introduction

Prostaglandin E_2 elicits a variety of biological effects through interactions with specific cell surface receptors. Molecular cloning has identified four subtypes of prostaglandin E_2 receptor, referred to as EP_1, EP_2, EP_3 and EP_4. These receptors, which belong to the seven transmembrane G protein coupled receptor superfamily, are distinguished by their ligand binding properties as well as their signal transduction pathways (Coleman et al., 1994). The activation of the EP_1 receptor elicits elevation of intracellular calcium, the EP_3 receptor mediates inhibition of cAMP generation, and the EP_2 and EP_4 receptors mediate receptor-evoked increases in intracellular cAMP generation (Funk et al., 1993; Namba et al., 1993; Bastien et al., 1994; Regan et al., 1994).

Extensive mutagenesis studies on G protein coupled receptors for small ligands have suggested that the ligand binding pocket for these receptors is formed by the seven transmembrane helices (Hibert et al., 1991; Probst et al., 1992). Indeed a conserved arginine in transmembrane VII has been identified as a critical determinant of prostanoid receptor–ligand interaction (Negishi et al., 1995; Audoly and Breyer, 1997b). In contrast, the role of the extracellular loop regions in forming the ligand binding pocket of small molecule-binding G protein coupled receptors has not been extensively investigated. Within the prostanoid receptor family, most amino acid conservation occurs in the transmembrane helices, although the N-terminal portion of the second extracellular loop also contains a cluster of conserved residues. Previous mutagenesis studies in this region of the EP_3 receptor, whose conserved second extracellular loop sequence is QWPGTWCF, identified the threonine at position 202 as a critical determinant of ligand selectivity at the C1 carbon (Audoly and Breyer, 1997a). Mutation of this threonine to an alanine increases its affinity dramatically for prostanoid compounds with a C1

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methyl ester, but its affinity is unchanged for compounds with a carboxylate moiety at the C1 position.

To investigate the role of this conserved threonine in the homologous G_s coupled EP_2 and EP_4 receptors, the analogous threonine to alanine substitution was introduced in each of these receptors. In contrast to the loss of selectivity observed in the EP_3 receptor, mutating the conserved threonine in the EP_2 and EP_4 receptors resulted in a complete loss of detectable binding at the receptors. The present studies establish a critical role in ligand binding for a conserved hydroxyl moiety in the second extracellular loop of the human EP_2 and EP_4 receptors.

2. Materials and methods

2.1. Materials

The human EP_2 receptor cDNA was a gift from Dr. Daniel Gil (Allergan). The human EP_4 receptor cDNA was a gift from Dr. Mark Abramovitz (Merck-Frosst, Montreal, Canada). Prostaglandin E_2 and prostaglandin E_2 methyl ester were purchased from Cayman Chemical (Ann Arbor, MI, USA). Misoprostol and misoprostol free acid were gifts from Dr. Paul Collins (Searle). Butaprost was a gift from Dr. M.P.L. Caton (Rhone-Poulenc) and butaprost free acid was a gift from Dr. Jilly Evans (Merck-Frosst). [^{3}H]Prostaglandin E_2 and [^{35}S] protein labeling mix was purchased from DuPont-New England Nuclear (Boston, MA, USA). Lipofectamine and Optimem were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Construction of the hemagglutinin epitope-tagged EP_2 and EP_4 receptors

The 5′ and 3′ untranslated regions of the human EP_2 and EP_4 receptors were removed, and convenient cloning sites were introduced using a polymerase chain reaction strategy. The polymerase chain reaction primers employed amplified the entire coding region of the EP receptors inserting *Eco*RI and *Nde*I restriction sites immediately before the start codon and a *Xho*I site immediately downstream of the stop codon (non-EP receptor sequences are bold; restriction sites are underlined):

EP2-5: 5′ **GGC GAG AAT TCC ATA** TGG GCA ATG CCT CCA ATG ACT 3′

EP2-3: 5′ **GGC GAC TCG AG**T CAA AGG TCA GCC TGT TTA CT 3′

EP4-5: 5′ **GGC GAG AAT TCC AT**A TGT CCA CTC CCG GGG TCA ATT 3′

EP4-3: 5′ **GGC GAC TCG AG**T TAT ATA CAT TTT TCT GAT AAG TTC AG 3′

The full-length EP open reading frames were amplified at 98°C for 15 s, 54°C for 30 s, and 72°C for 60 s, for 35 cycles, followed by a final extension at 72°C for 10 min. The EP_2 and EP_4 expression plasmids were constructed by ligating a *Hin*dIII–*Nde*I hemagglutinin tag (YPYDVPDYA) containing fragment from pRCCMV77Atag (Audoly and Breyer, 1997b) and the *Nde*I–*Xho*I EP_2 or EP_4 open reading frame fragment into pCDNA3. The sequence of the amplified region in the expression plasmid was verified by dideoxy nucleotide sequencing. These hemagglutinin tagged receptor fusion constructs are referred to as the ‘wild type’ receptor.

2.3. Site directed mutagenesis of receptor cDNAs

Missense mutations were introduced using a polymerase chain reaction method as described previously (Audoly and Breyer, 1997b). Additional silent mutations were included in these primers in order to add or remove diagnostic restrictions sites for screening purposes. For the following oligonucleotides, missense mutations are in bold and silent mutations are underlined.

EP2TA185: 5′ TAC GTC CAA TAT TGC CCT GGT **GCG** TGG TGC 3′

EP2TS185: 5′ TAC GTC CAA TAT TGC CCT GGT **TCG** TGG TGC 3′

EP4TA168: 5′ GGT AGC TCG CGA CTT CAA TAC CCA GAC **GCG** TGG TGC TTC 3′

EP4TS168: 5′ GGT AGC TCG CGA CTT CAA TAC CCA GAC **TCG** TGG TGC TTC 3′

Mutated fragments were amplified at 98°C for 15 s, 54°C for 30 s, and 72°C for 60 s, for 35 cycles, followed by an extension at 72°C for 10 min. The polymerase chain reaction products were digested with internal restriction sites which were unique in both the polymerase chain reaction product as well as the expression vector. The mutagenized cassette was then ligated into the expression vector digested with the same enzymes, reconstituting the full length receptor cDNA bearing the desired mutant. For the EP_2 receptor the *Eco*47III and *Sfi*I sites were used as cloning sites and for the EP_4 receptor the *Pfl*MI and *Sac*II sites were used. The sequence of the polymerase chain reaction amplified region subcloned into the expression plasmid was verified by dideoxy nucleotide sequencing. Two independent clones (for serine mutations) or four independent clones (for alanine) were analyzed for ligand binding studies, and at least two independent clones were analyzed for signal transduction and immunochemical analysis of expression levels.

2.4. EP receptor expression in cell culture

COS1 cells were transiently transfected with pCDNA3 plasmids containing either wild type or mutant EP receptor cDNAs by the lipofectamine method according to the manufacturer’s instructions (Life Science Technologies), using 12 μg plasmid DNA and 45 μl lipofectamine solution. Cells were cultured for 72 h, and 5 mM sodium butyrate was added to culture medium 16 h before lysis.

Total cell membranes were prepared as described previously (Breyer et al., 1994).

2.5. Ligand binding assays

For saturation binding isotherm experiments, 15 μg or 20 μg of membrane protein was incubated with varying [^{3}H]prostaglandin E_2 concentrations, and reactions were stopped by filtration onto glass fiber filters as described previously (Breyer et al., 1994). For competition binding assays, 20 μg membrane protein was incubated with 1 nM of [^{3}H]prostaglandin E_2 and varying concentrations of unlabeled competitors. Reactions were carried out as above.

2.6. Immunoprecipitation

COS1 cells transfected with the EP_2 and EP_4 cDNAs were cultured for 72 h and then grown in cysteine and methionine-free Dulbeccos's Modified Eagle Medium/10% fetal bovine serum for 1 h. Cells were metabolically labeled by adding 0.4 mCi/ml [^{35}S]cysteine and methionine for 3 h. Cells were lysed in Buffer 1 (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM phenylmethylsulfonyl fluoride) and then spun at 100 000 × *g* for 1 h. The supernatant was incubated for 16 h at 4°C with 40 μg/ml anti-hemagglutinin monoclonal antibody (12CA5). Proteins were precipitated by incubation with protein A/agarose for 2 h at 4°C. Samples were centrifuged at 12 000 × *g* for 2 min and washed 6× with Buffer 1. Receptor protein was eluted with polyacrylamide gel electrophoresis sample buffer (4% sodium dodecyl sulfate/0.1 M dithiothreitol) by incubating the samples for 1 h at room temperature. Immunoprecipitated proteins were resolved on a 10% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were visualized by autoradiography.

2.7. Immunofluorescence

COS1 cells were transiently transfected with the expression plasmids encoding the EP_2 or EP_4 wild type or mutant receptors and were distributed onto 24 mm glass coverslips. The cells were washed in phosphate-buffered-saline containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ 72 h after transfection and fixed for 30 min in 4% paraformaldehyde in phosphate-buffered-saline. Cells were then rinsed with phosphate-buffered-saline, incubated in 50 mM NH_4Cl in phosphate-buffered-saline for 15 min, and permeabilized by incubation with 0.2% Triton X-100 in phosphate-buffered-saline for 15 min at 25°C. Cells were rinsed with phosphate-buffered-saline and incubated with 2% BSA/0.1% Triton X-100/phosphate-buffered-saline for 15 min to block, followed by incubation with the primary antibody (12CA5) in the same solution for 1 h at 25°C. Cells were then rinsed in phosphate-buffered-saline and incubated with Cy-3-labeled donkey anti-mouse antibody for 1 h at 25°C. Cells were washed with phosphate-buffered-saline and mounted on slides for analysis by fluorescence microscopy at 570 nm.

2.8. cAMP measurements

COS1 cells transiently transfected with the EP_2 or EP_4 wild type or mutant encoding expression plasmids were distributed into 24-well plates. The medium was replaced 24 h later with 450 μl of Dulbeccos's Modified Eagle Medium/0.25 mM 3-isobutyl-1-methylxanthine/40 μM indomethacin and incubated further for 1 h at 37°C. Medium containing varying amounts of prostaglandin E_2

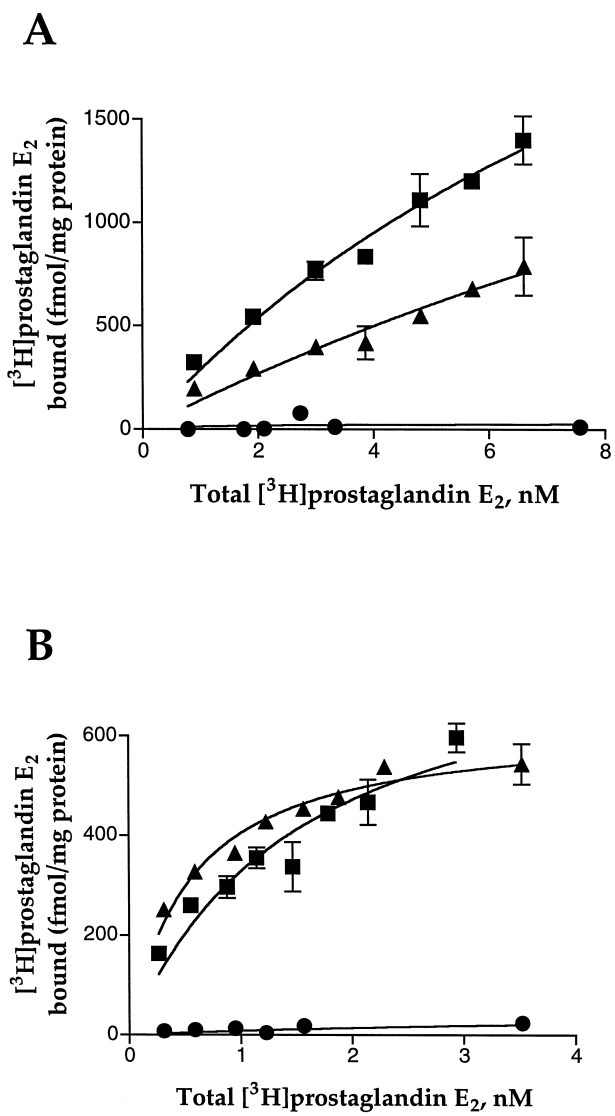

Fig. 1. Saturation binding analysis of the binding of [^{3}H]prostaglandin E_2 by COS1 cell transfectants expressing the human EP_2 (A) or EP_4 (B) receptors. The data shown here are from a single experiment performed in duplicate and are representative of three to four independent experiments. Panel A: (■) EP_2 wild type receptor; (●) EP_2T185A; (▲) EP_2T185S. Panel B: (■) EP_4 wild type receptor; (●) EP_4T168A; (▲) EP_2T168S.

was added to each well and incubated for 5 min. The reactions were stopped by addition of 500 μl of 10% trichloroacetic acid. cAMP measurements of the cell lysates were performed by an enzyme immunoassay, according to manufacturer's instructions (Stratagene).

2.9. Data analysis

All binding assays and cAMP measurements were analyzed using PRISM (GraphPad, San Diego, CA). Statistical analysis was performed using Instat (GraphPad).

3. Results

3.1. Fusion of the hemagglutinin tag to the wild type EP_2 and EP_4 receptors

The EP_2 and EP_4 receptor cDNAs were fused to the hemagglutinin epitope to facilitate detection of receptor protein expression. [^{3}H]prostaglandin E_2 binding was measured in membranes prepared from COS1 cells transiently transfected with wild type or hemagglutinin tagged EP_2 and EP_4 receptor cDNAs. The dissociation constants for hemagglutinin tagged wild type receptors and non-tagged wild type receptors were not statistically significant as determined by an unpaired Student's *t*-test. For the EP_2 receptor the K_d value for [^{3}H]prostaglandin E_2 binding was 12.9 nM for the hemagglutinin tagged receptor vs. 15.7 nM for the non-tagged receptor. These values are necessarily estimates inasmuch as the K_d value obtained approaches the practical limit for measurable affinities by the vacuum filtration method (Limbird, 1996). For the EP_4 receptor, the hemagglutinin tagged receptor had a K_d value of 0.88 nM, and the non-tagged receptor had a value of 0.68 nM.

3.2. Receptor mutagenesis

3.2.1. Mutation of T185 in the EP_2 receptor

Sequence alignments of the cloned EP receptors identified a stretch of conserved amino acid residues in the N-terminal half of the putative second extracellular loop (Audoly and Breyer, 1997a). Previous mutagenesis studies

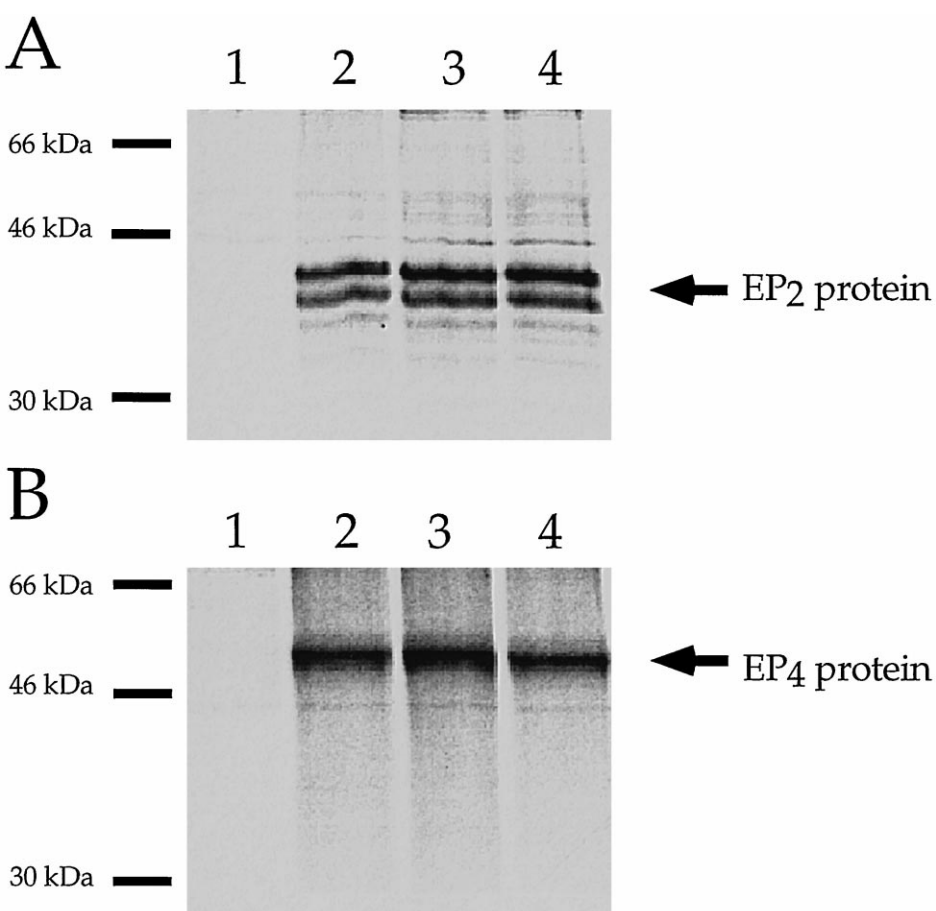

Fig. 2. Immunoprecipitation of EP_2 and EP_4 wild type and mutant receptor constructs. Metabolically labeled wild type and mutant receptors expressed in COS1 cells were immunoprecipitated using a monoclonal antibody (12CA5) recognizing the hemagglutinin epitope fused to the amino terminus of the receptors. This figure is representative of three independent experiments (kDa = kilodaltons). A: (lane 1) vector-only transfected cells; (lane 2) EP_2 wild type; (lane 3) EP_2T185S; (lane 4) EP_2T185A. B: (lane 1) vector only; (lane 2) EP_4 wild type; (lane 3) EP_4T168S; (lane 4) EP_4T168A.

in the rabbit EP_3 receptor demonstrated that substitution of the conserved threonine 202 within this region with an alanine resulted in an increase in affinity of two orders of magnitude for ligands with a C1 methyl ester, while ligands with a C1-carboxylate moiety displayed a modest two-fold increase in ligand affinity (Audoly and Breyer, 1997a). To determine whether this conserved threonine plays a similar role in the structure or function of the human EP_2 and EP_4 receptors, we introduced substitutions at the corresponding positions of those receptors.

Mutation of threonine 185 to alanine (T185A) in the second extracellular loop of the EP_2 receptor resulted in a loss of detectable [^{3}H]prostaglandin E_2 binding (Fig. 1A). To further characterize the importance of this position, the

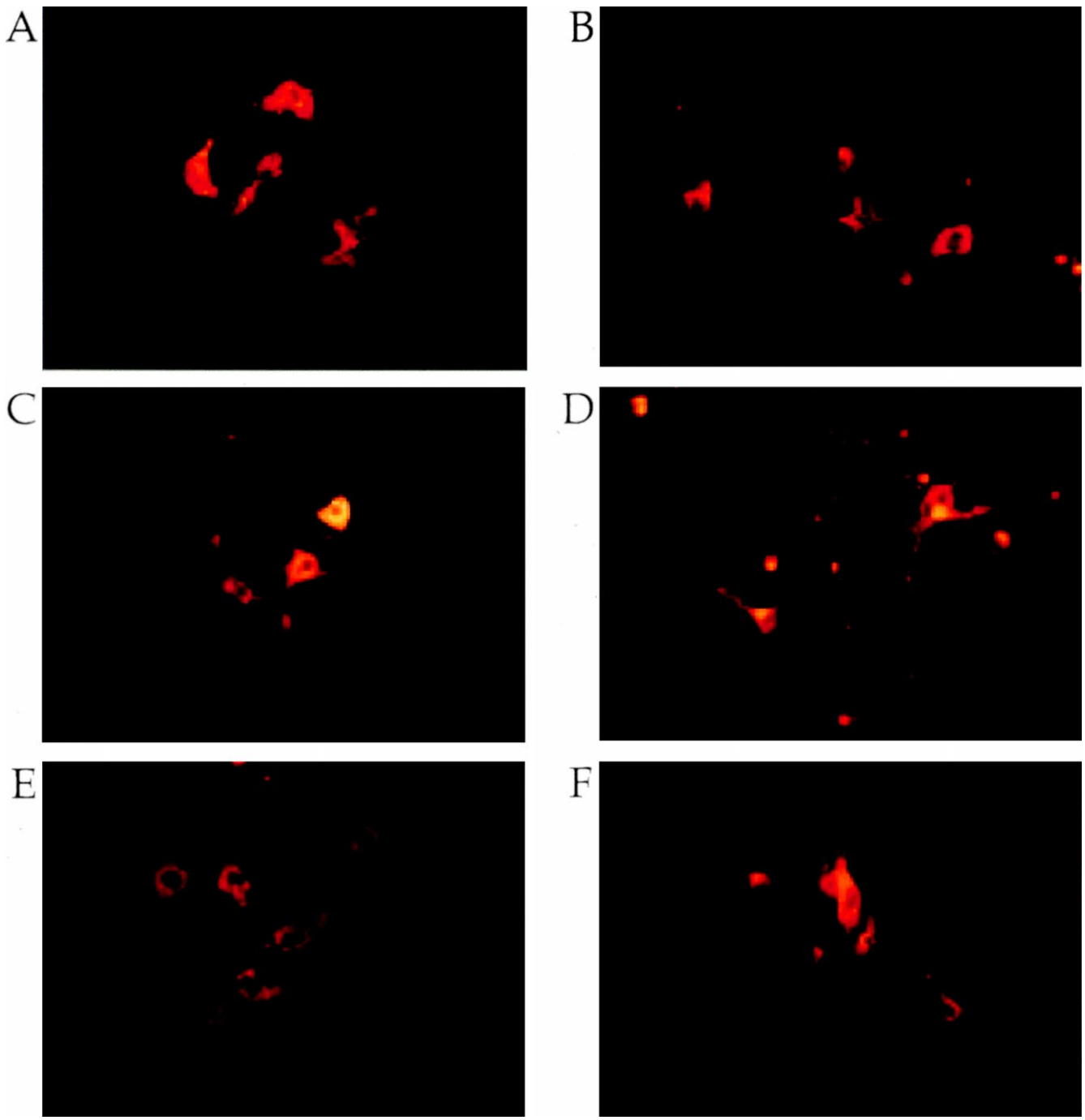

Fig. 3. Immunofluorescence of wild type and mutant EP_2 and EP_4 receptors transfected into COS1 cells. Receptors were detected using the hemagglutinin monoclonal antibody (12CA5). (A) EP_2 wild type; (B) EP_4 wild type; (C) EP_2T185S; (D) EP_4T168S; (E) EP_2T185A; (F) EP_4T168A. No immunofluorescence was detected with the vector-only transfected cells.

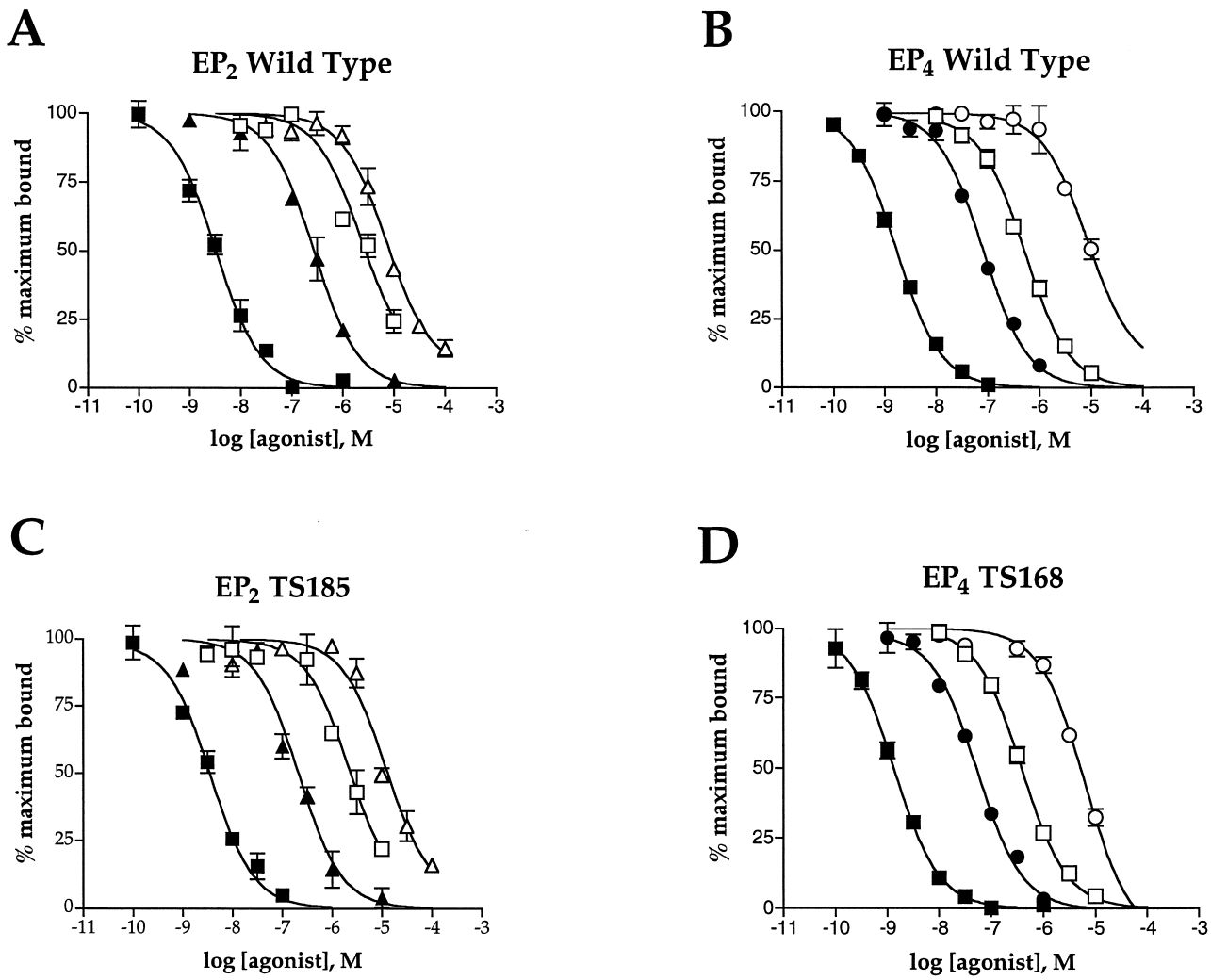

Fig. 4. Competition binding profiles of wild type and threonine to serine mutant receptors expressed in COS1 cells. Membranes were incubated with 1 nM [^{3}H]prostaglandin E_2 and varying concentrations of competitors. The data shown are from a single experiment performed in triplicate and are representative of three experiments. (Panel A) EP_2 wild type receptor; (Panel B) EP_4 wild type receptor; (Panel C) EP_2 TS185 receptor; (Panel D) EP_4 TS168 receptor. (■) prostaglandin E_2; (□) prostaglandin E_2 methyl ester; (▲) butaprost free acid; (Δ) butaprost; (●) misoprostol free acid; (○) misoprostol.

threonine was mutated to serine, a conservative substitution which preserves the hydroxyl moiety on the amino acid side chain. Saturation binding experiments with this T185S mutant indicated that its K_d value for [^{3}H]prostaglandin E_2 binding is 8.7 ± 0.7 nM, which is similar to the K_d value of 12.9 nM obtained for the wild type receptor.

Table 1
Competition for [^{3}H]prostaglandin E_2 specific binding to COS1 cell membranes expressing wild type and mutant EP receptors by various agonists

Agonist	EP_2 wt	EP_2T185S
Prostaglandin E_2	$3.20 \pm 0.82 \times 10^{-8}$ M	$1.54 \pm 0.02 \times 10^{-8}$ M
Prostaglandin E_2 methyl ester	$9.13 \pm 3.34 \times 10^{-6}$ M	$7.49 \pm 1.07 \times 10^{-6}$ M
Butaprost free acid	$1.21 \pm 0.41 \times 10^{-6}$ M	$1.01 \pm 0.06 \times 10^{-6}$ M
Butaprost	$3.98 \pm 1.35 \times 10^{-5}$ M	$5.25 \pm 1.58 \times 10^{-5}$ M
Agonist	**EP_4 wt**	**EP_4T168S**
Prostaglandin E_2	$1.25 \pm 0.3 \times 10^{-9}$ M	$2.19 \pm 0.65 \times 10^{-9}$ M
Prostaglandin E_2 methyl ester	$2.42 \pm 0.03 \times 10^{-7}$ M	$2.39 \pm 0.74 \times 10^{-7}$ M
Misoprostol free acid	$2.52 \pm 0.61 \times 10^{-8}$ M	$4.86 \pm 1.11 \times 10^{-8}$ M
Misoprostol	$3.42 \pm 0.4 \times 10^{-6}$ M	$5.30 \pm 1.8 \times 10^{-6}$ M

K_i values from competition binding isotherms (shown on Fig. 4) of EP_2 wild type and EP_2T185S receptors and EP_4 wild type and EP_4T168S receptors. K_i values were averaged from three independent experiments (±standard error) performed in triplicate.

3.2.2. Mutation of T168 in the EP_4 receptor

The corresponding conserved threonine in the second extracellular loop of the EP_4 receptor was also mutated. While the wild type EP_4 receptor K_d value for [^{3}H]prostaglandin E_2 was 0.9 ± 0.2 nM, construction of the EP_4T168A mutant resulted in a loss of detectable [^{3}H]prostaglandin E_2 binding (Fig. 1B). When this threonine was mutated to a serine (T168S), high affinity binding was observed, with a K_d value for [^{3}H]prostaglandin E_2 of 2.0 ± 0.8 nM.

3.3. Immunoprecipitation of epitope-tagged receptors

Immunoprecipitation of each of the EP_2 and EP_4 receptor variants using the antibody against the hemagglutinin fusion detected protein products near the expected molecular mass ($EP_2 = 39.4$ kDa; $EP_4 = 52$ kDa). Proteins were detected at similar levels for each wild type and mutant receptor protein (Fig. 2). The presence of multiple bands clustered near the expected size may be due to post-translational modification such as glycosylation or phosphorylation of the receptor.

3.4. Immunofluorescence

Immunohistochemical detection of COS1 cells expressing wild type and mutant demonstrated a similar profile of receptor expression (Fig. 3). These studies were performed in the presence of detergent permitting detection of plasma membrane as well as intracellular protein. Taken together with the immunoprecipitation data, these studies provide strong evidence that the wild type and each mutant EP_2 and EP_4 receptor construct are expressed at similar levels and have a similar pattern of distribution in the COS1 cells.

3.5. Ligand selectivity of the EP_2T185S and EP_4T168S mutants

Previous mutagenesis studies with the threonine 202 in the EP_3 receptor resulted in proteins with altered ligand binding selectivity for methyl ester compounds. To compare the binding selectivity of the wild type vs. the EP_2T185S and EP_4T168S receptors, the affinity for two sets of free acid/methyl ester compounds for both the EP_2 and EP_4 constructs was assessed. No differences in selectivity for the mutant receptors were detected for any of the compounds tested (Fig. 4). For the EP_2 wild type and T185S receptors, the agonist order of potency is prostaglandin E_2 > butaprost free acid > prostaglandin E_2 methyl ester > butaprost with similar K_i values (see Table 1). The EP_4 wild type and T168S receptors demonstrated identical orders of potency: prostaglandin E_2 > misoprostol free acid > prostaglandin E_2 methyl ester > misoprostol with similar K_i values (see Table 1; Fig. 4).

3.6. Signal transduction studies

3.6.1. cAMP stimulation by EP_2 wild type and mutant receptors

To characterize the mutant receptor proteins' signal transduction properties, the ability of each receptor to elicit receptor-evoked increases in intracellular cAMP levels was assayed. Receptor cDNAs were expressed in COS1 cells

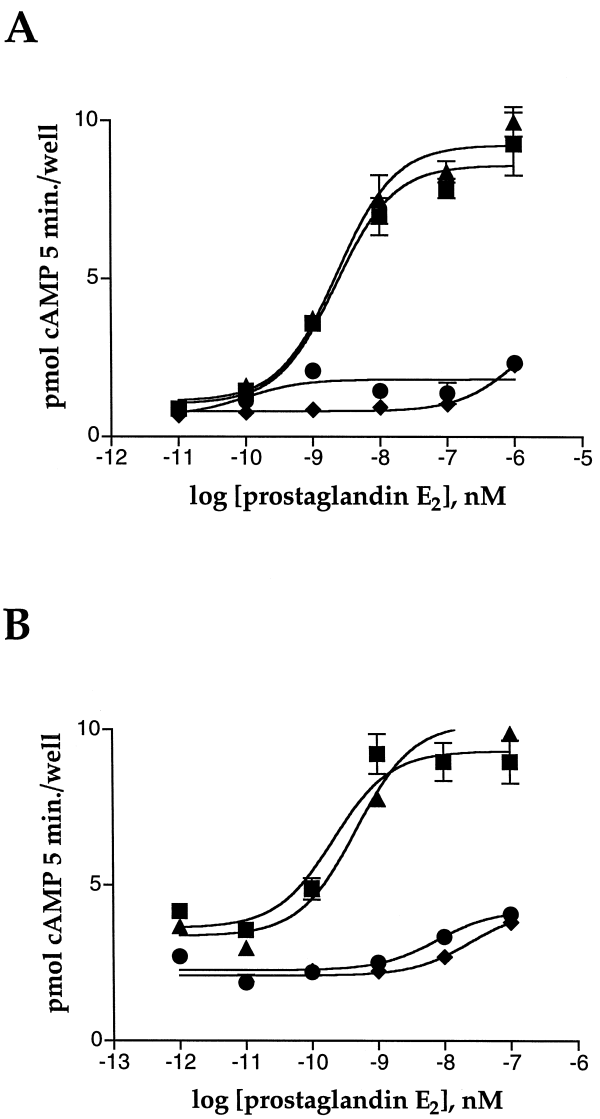

Fig. 5. Prostaglandin E_2 mediated stimulation of intracellular cAMP production in COS1 cells transfected with EP_2 (A) and EP_4 (B) wild type and mutant receptors. Cells were incubated with various concentrations of prostaglandin E_2 in the presence of 3-isobutyl-1-methylxanthine for 5 min. The reactions were stopped with 10% trichloroacetic acid, and cell lysates were analyzed for cAMP content by a cAMP enzyme immunoassay kit. Data shown are from a single experiment performed in duplicate and are representative of three independent experiments. Panel A: (■) EP_2 wild type receptor; (●) EP_2T185A; (▲) EP_2T185S; (♦) vector only. Panel B: (■) EP_4 wild type receptor; (●) EP_4T168A; (▲) EP_2T168S; (♦) vector-only.

and stimulated with prostaglandin E_2. Both the wild type and T185S mutants demonstrated receptor-evoked stimulation of intracellular cAMP with EC_{50} values of 1.3 ± 0.6 nM for wild type and 2.7 ± 1.3 nM for EP_2T185S. Receptors bearing threonine to alanine substitutions demonstrated no receptor-evoked stimulation of cAMP (Fig. 5A).

3.6.2. cAMP stimulation by EP_4 wild type and mutant receptors

Similarly, the EP_4 wild type and EP_4T168S receptors displayed receptor evoked increases in cAMP with EC_{50} values for cAMP stimulation (1.1 ± 0.3 nM for wild type vs. 1.4 ± 0.3 nM for EP_4T168S), while the EP_4T168A mutant did not increase cAMP levels above that of the control (Fig. 5B).

4. Discussion

The present study reveals the importance of a key threonine in the second extracellular loop region in the structure and/or function of the EP_2 and EP_4 receptors. Taken together with previous studies on the role of this loop in the EP_3 receptor (Audoly and Breyer, 1997a), these data suggest that the conserved stretch of residues in the N-terminal half of the loop is important for receptor–ligand interaction across the prostanoid receptor family. This provides evidence supporting the hypothesis that determinants of receptor structure and/or function do not lie exclusively in the transmembrane helices for these prostanoid receptors. While mutation of threonine 202 in the second extracellular loop of the EP_3 receptor produced proteins with increased ligand affinity, the conserved threonine residue, and more specifically the hydroxyl moiety on its side chain, in the loop of the EP_2 and EP_4 receptors was shown to be essential for ligand binding as demonstrated by the phenotypes observed for the threonine to serine and threonine to alanine mutations. Differences observed in mutating the conserved threonine of the EP_2 and EP_4 receptors described here, as compared with previous studies for the EP_3 receptor, suggest that the threonine plays distinct roles in these G_s receptors vs. the G_i coupled EP_3 prostaglandin receptor. To our knowledge this is the first description of mutations of non-cysteine residues in prostanoid receptors that disrupt ligand binding which do not lie in a transmembrane domain. Among the prostanoid receptors, the human thromboxane receptor and prostacyclin receptor contain a serine at this corresponding conserved position, while the remaining prostanoid receptors contain a threonine.

As with the rabbit EP_3 (Audoly and Breyer, 1997b) and EP_4 receptors (Breyer et al., 1996), the human EP_2 receptor demonstrated a marked preference for ligands with a free acid at the C1 carbon with approximately 30- to 250-fold preference for butaprost free acid and prostaglandin E_2 respectively, as compared with the methyl ester derivatives. This suggests that the preference for a free acid at the C1 position will be found across the family of prostanoid receptors.

The proposed three-dimensional models of receptor–ligand interactions for small ligand G protein coupled receptors have not considered the contribution of the extracellular loop regions to ligand binding. In the thromboxane receptor model (Yamamoto et al., 1993), the authors propose residues that interact with the ligand, all of which lie in the transmembrane helices. This model, as well as those for other well-studied G protein coupled receptors which bind small molecules, such as the adrenergic and dopaminergic receptors (IJzerman et al., 1992; MaloneyHuss and Lybrand, 1992; Hutchins, 1994), do not include the loops when deriving the energy-minimized receptor structure. The involvement of the second extracellular loop in ligand binding of the EP_3 receptor (Audoly and Breyer, 1997a) as well as the EP_2 and EP_4 receptors described here, suggest that the prostanoid receptors may belong to a group distinct from the small molecule binding receptors (e.g., the biogenic amine receptors), where evidence of second extracellular loop involvement is limited to critical disulfide linkages as determined by site directed mutagenesis and deletion analysis studies (Dixon et al., 1987; Dohlman et al., 1990). It is noteworthy however that antibodies directed against the second extracellular loop of the α_1 adrenoceptor can activate that receptor (Fu et al., 1994), suggesting that there may be a contribution of the second extracellular loop to ligand binding by biogenic amine receptors.

There are at least three possible mechanisms that might explain the role of the implicated hydroxyl moiety in the second loop of the EP_2 and EP_4 receptors in ligand binding. The most straightforward explanation is that the hydroxyl moiety is directly interacting with receptor ligands. In this case, the second extracellular loop region could play a role as a selectivity filter for ligands. One model for G protein coupled receptor specificity suggests that transmembrane residues near the extracellular surface function as a selectivity filter to restrict ligand access (Turner et al., 1996); this analogy could be extended to residues in the extracellular loop regions. Alternatively, the hydroxyl residue might be hydrogen bonding with another residue in the receptor, by analogy with exofacial loops in pore formation of ion channels (MacKinnon, 1995). A third possibility is that the loop is vital for proper receptor structure by contributing to the overall conformation of the receptor, yet does not directly interact with the transmembrane domains. Some G protein coupled receptors have been shown to be activated by extracellular loop mutations or, as noted above, antibodies developed against extracellular loops (for review see Schwartz and Rosenkilde, 1996). These studies provide evidence that the extracellular loop regions can affect receptors' ability to interconvert between the inactive (R) and active (R^*) states, suggesting

that the second extracellular loop does indeed play a role in receptor conformation.

Recent mutagenesis studies of other G protein coupled receptors have revealed the importance of the second extracellular loop in ligand binding. Olah et al. (1994) recently showed that substitution of the distal eleven amino acids of the second extracellular loop in the adenosine A_3 receptor resulted in drastic changes in affinity for adenosine receptor agonists and antagonists. In addition, adenosine A_2 receptors containing single point mutations in glutamate residues in the second extracellular loop were unable to bind radioligands (Kim et al., 1996). Substitution of the second extracellular loop residues in the peptide-binding somatostatin type 5 receptor also prevented receptors from binding radioligands, while substitution of the first or third extracellular loops only had minor effects on ligand binding affinities (Greenwood et al., 1997).

Each of the prostanoid, adenosine, and somatostatin receptors mentioned contain at least one cysteine residue within the region of the second extracellular loop shown to be important for function. It is possible that these cysteine residues could form disulfide bridges to bring the second extracellular loop in physical proximity to the ligand binding pocket. It should be noted however that mutagenesis of the conserved cysteine 204 in the second extracellular loop of the EP_3 receptor had no effect on receptor function or ligand binding (Audoly and Breyer, 1997a). Although studies on the prostanoid receptors have been limited, protein folding studies on the adenosine receptor have revealed that its second extracellular loop is flexible enough to form β-turns and possibly fold back into the receptor transmembrane region (Van Galen et al., 1994).

Relatively little is known regarding the structural determinants of EP receptor–ligand interactions. Several groups have previously identified the importance of an arginine residue in transmembrane VII that is conserved throughout the prostanoid receptor family (Audoly and Breyer, 1997b; Negishi et al., 1995). The present studies reveal that the second extracellular loop of the EP_2 and EP_4 receptors also contributes critical structure to the receptor that is vital for ligand binding. Mutation of this motif has had effects on three EP receptors examined thus far (EP_2, EP_3, EP_4), and it will be important to extend these findings across the prostanoid receptor family. This study provides further evidence that receptor loop regions need to be included in three-dimensional receptor models in order to establish a more complete picture of receptor–ligand interactions.

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