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Importance of the Extracellular Domain for Prostaglandin EP_2 Receptor Function

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ABSTRACT

The ligand binding pocket of biogenic amine G protein-coupled receptors is embedded in the membrane-spanning regions of these receptors, whereas the extracellular domains of the peptidergic receptors play a key role in the structure and function of this class of receptors. To examine the role of the extracellular sequences in prostaglandin receptor-ligand interaction, chimeras were constructed with the two G_s-coupled E-prostanoid (EP) receptors, replacing each of the extracellular sequences of the human EP_2 receptor with the corresponding human EP_4 receptor residues. Replacement of the third extracellular loop (ECIII) yielded a receptor that binds [^{3}H]prostaglandin E_2 (PGE_2; K_d = 6.3 nM) with similar affinity as the EP_2 wild-type receptor (K_d = 12.9 nM). Similarly, replacement of the nonconserved carboxyl-terminal portion of ECII resulted in a receptor that maintains [^{3}H]PGE_2 binding (K_d = 8.8 nM). In contrast, replacement of the amino terminus, ECI, the entire ECII region, or the residues within the highly conserved motif of the amino-terminal half of ECII yielded chimeras that displayed neither detectable [^{3}H]PGE_2 binding nor receptor-evoked cAMP generation. Immunoprecipitation demonstrated that each chimera is expressed at levels near that of wild-type receptors; however, enzyme-linked immunosorbent assay revealed that inactive chimeras have reduced cell surface expression. Similarly, chimeras that exchange the multiple extracellular loop sequences N/ECI, ECII/ECIII, or all four sequences lacked detectable binding and signal transduction, and although expressed, were not detected on the cell surface. These data suggest that the extracellular sequences of the EP_2 receptor are critical determinants of receptor structure and/or function, unlike other G protein-coupled receptors that bind small molecules.

Prostaglandin E_2 (PGE_2) is a ubiquitous autocoid that exerts a variety of physiological effects through interactions with specific cell-surface receptors. Molecular cloning has identified four subtypes of PGE_2 receptors, referred to as E-prostanoid $(EP)_1$, EP_2, EP_3, and EP_4 (Coleman et al., 1994). These receptors belong to the seven-transmembrane (TM) G protein-coupled receptor (GPCR) superfamily and are classified based on their ligand binding and signal transduction characteristics. 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).

The G_s-coupled EP_2 and EP_4 receptors are distinguished by their ligand selectivity and differential desensitization (Coleman et al., 1994; Nishigaki et al., 1996). Butaprost and AH13205 are selective EP_2 agonists, whereas AH23848B is a weak EP_4-selective antagonist. In addition, [^{3}H]PGE_2 binds to the EP_4 receptor with at least 10-fold higher affinity than the EP_2 receptor. Structurally, the EP_4 receptor has a much longer carboxyl-terminal sequence than the EP_2 receptor and has been shown to undergo short-term agonist induced desensitization, which is absent in the EP_2 receptor (Nishigaki et al., 1996; Bastepe and Ashby, 1997). Northern blot analysis has revealed that the mRNA encoding the EP_4 receptor is highly expressed and widely distributed in the body, whereas the mRNA encoding the EP_2 receptor is expressed at lower levels (Honda et al., 1993; Bastien et al., 1994; Regan et al., 1994).

Extensive mutagenesis studies performed on biogenic amine binding GPCRs suggest that their ligand binding pocket is embedded in the membrane-spanning regions of these receptors (for review, see Savarese and Fraser, 1992). In contrast, studies on calcitonin, vasopressin, and neurokinin peptidergic receptors provide evidence that the extracellular domains are important for the structure and function of this class of peptide binding GPCRs (Fong et al., 1992a; Bergwitz et al., 1996; Howl and Wheatley, 1996). Although prostaglandins are small molecules like the biogenic amines,

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ABBREVIATIONS: PGE_2, prostaglandin E_2; EP, E-prostanoid; TM, transmembrane domain; GPCR, G protein-coupled receptor; HA, hemagglutinin; PCR, polymerase chain reaction; ELISA, enzyme linked immunosorbent assay; EC, extracellular loop.

their receptors share the greatest sequence similarity to a subclass of peptide receptors that includes the vasopressin and gonadotropin-releasing hormone peptidergic receptors (Kolakowski, 1994). Thus, prostanoid receptor-ligand interactions may represent a new paradigm, in which extracellular sequences play a critical role in receptor structure and/or function for these small-molecule-binding receptors. Previous studies have identified residues within the TM regions (Arg in TMVII, Ser in TMVI; Negishi et al., 1995; Huang and Tai, 1996; Audoly and Breyer, 1997b) as well as conserved residues in the second extracellular loop critical for receptor ligand binding and signal transduction (Audoly and Breyer, 1997a; Stillman et al., 1998). The hypothesis of this study is that, as for the related peptidergic receptors, the extracellular regions of the prostanoid receptors are important for receptor structure and function. We explore this hypothesis by creating chimeras replacing EP_2 extracellular regions with the corresponding EP_4 residues. The findings presented here suggest that certain extracellular sequences play a role in receptor structure-function.

Experimental Procedures

Materials. The human EP_2 receptor cDNA was a gift from Dr. Daniel Gil (Allergan, Irvine, CA). The human EP_4 receptor cDNA was a gift from Dr. Mark Abramovitz (Merck-Frosst, Montreal, Canada). PGE_2, PGD_2, and $PGF_{2\alpha}$ were purchased from Cayman Chemical (Ann Arbor, MI). Butaprost free acid was a gift from Dr. Jilly Evans (Merck-Frosst). M&B28767 was a gift from Dr. M. P. L. Caton (Rhone Poulenc Ltd, Dagenham, Essex, United Kingdom). AH13205 was a gift from Dr. Robert Coleman (Glaxo Research Group, Greenford, Middlesex, United Kingdom). [^{3}H]PGE_2 and ^{35}S protein-labeling mix were purchased from DuPont-New England Nuclear (Boston, MA). Lipofectamine and Optimem were purchased from Life Technologies (Grand Island, NY). The 12CA5 monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN).

Construction of the Hemagglutinin Epitope-Tagged EP_2 and EP_4 Receptors. The human EP_2 and EP_4 receptor expression plasmids have been previously described (Stillman et al., 1998). These constructs consist of the EP_2 or EP_4 cDNA-coding region, containing no flanking 5′ or 3′ untranslated regions, in the expression vector pCDNA3 (InVitrogen, San Diego, CA). A DNA sequence coding for a nine-amino acid hemagglutinin (HA) tag (YPYDVPDYA) was fused directly to the start codon. Previous studies indicate that these tagged receptor constructs function identically to the non-tagged EP_2 or EP_4 receptor (Stillman et al., 1998). Throughout this paper, the HA-tagged receptor fusion constructs are referred to as the "wild-type" receptor.

Site-Directed Mutagenesis of Receptor cDNAs. Mutant receptors (Table 1) were constructed with a polymerase chain reaction (PCR) method as described previously (Higuchi, 1989). The following chimeric oligonucleotides were used (EP_4 sequences are in bold; silent mutations to add or remove diagnostic restriction sites for screening purposes are underlined):

$EP_{2/4}$NTs: 5′-**aat tcg tcc gcc tcc ttg agc ccc gac cgg ctg aac** agc cca gcc atc agc tcc gtc-3′; $EP_{2/4}$NTa: 5′- **gct caa gga ggc gga cga att gca ccc ggg agt gga cat** atg acg agg aac tag tgc-3′; $EP_{2/4}$ECIs: 5′-**aag ggc caa tgg ccc ggg ggc cag ccg ctg** tgc acc tac ttc gct ttc gcc-3′; $EP_{2/4}$ECIa: 5′-**gcc ccc ggg cca ttg gcc ctt cat gta cgt** agc cag tac cac tgg gct gat-3′; $EP_{2/4}$ECIIs: 5′-**atc gac tgg acc acc aac gtg acg gcg cac gcc** gct tac ctg cag ctg tac gcc-3′; $EP_{2/4}$ECIIa: 5′-**ggt ggt cca gtc gat gaa gca cca ggt gtc tgg** gta ctg gac gta ctg ccc ata-3′; $EP_{2/4}$ECIIIs: 5′ **agc ttg gag cga gaa gtc agt aaa aat cca** gac ctc caa gct ctt agg ttt-3′; $EP_{2/4}$ECIIIa: 5′-**act gac ttc tcg ctc caa gct tgg ctg** ata tgc aaa aat cgt gaa agg-3′; $EP_{2/4}$ECII-Ns: 5′-ggg cag tac gtc caa tat cct **gat** acc tgg tgc ttc atc-3′; $EP_{2/4}$ECII-Na: 5′-gat gaa gca cca ggt **atc** agg ata ttg gac gta ctg ccc-3′; $EP_{2/4}$ECII-Cs: 5′-**acc acc aac gtg acg gcg cac gcc** gct tac ctg cag ctg tac gcc-3′; $EP_{2/4}$ECII-Ca: 5′-**cgc cgt cac gtt ggt ggt cca gtc** gat gaa gca cca ggt ccc ggg-3′.

Mutagenic fragments were amplified with the human EP_2 receptor as a template, at 98°C for 15 sec, 56°C for 30 sec, and 72°C for 60 sec, for 35 cycles, followed by an extension at 72°C for 10 min. The PCR products were digested with unique internal restriction sites, and the mutagenized fragment was then ligated into the human EP_2 receptor expression vector digested with the same enzymes, reconstituting the full-length receptor cDNA bearing the desired mutant. The following pairs of restriction sites were used as cloning sites for each of the following constructs: $EP_{2/4}$NT, *Hin*dIII/*Bst*EII; $EP_{2/4}$ECI,

TABLE 1

The amino acid sequences exchanged in the extracellular chimera constructs

The EP_2 sequence shown was replaced by the EP_4 sequence shown below it. These extracellular regions are aligned with CLUSTALW sequence alignment (Thompson, 1994), and identical amino acid positions are shown in bold.

$EP_{2/4}$NT	EP_2	MGNASND**S**QSEDCET**R**QYLPPGE	$EP_{2/4}$NT-ECI
	EP_4	MSTPGVN**S**SASLSPD**R**-LN	
$EP_{2/4}$ECI	EP_2	SYARN**Q**TLVALA**P**ESRA	$EP_{2/4}$ECII-III
	EP_4	TYMKG**Q**WPGGQ-**P**---L	
$EP_{2/4}$ECII	EP_2	**QYCPGTWCFI**RHGR-----**TAY**	$EP_{2/4}$NT-ECIII
	EP_4	**QY-PDTWCFI**DWTTNVTAHA**AY**	
$EP_{2/4}$ECIII	EP_2	MNETSS---RK**E**K---W	
	EP_4	-----QPSLER**E**VSKNP	
$EP_{2/4}$ECII-N	EP_2	**QYCPGTWCFI**	
	EP_4	**QY-PDTWCFI**	
$EP_{2/4}$ECII-C	EP_2	RHGR-----**TAY**	
	EP_4	DWTTNVTAHA**AY**	

*Bst*EII/Eco47III; $EP_{2/4}$ECII, $EP_{2/4}$ECII-N, and $EP_{2/4}$ECII-C, Eco47III/*Sfi*I; $EP_{2/4}$ECIII, *Sfi*I/*Xho*I. The sequences of the PCR-amplified regions subcloned into the expression plasmid were verified by dideoxynucleotide sequencing.

The receptor chimeras exchanging multiple EC regions ($EP_{2/4}$NT-ECI; $EP_{2/4}$ECII-III; $EP_{2/4}$NT-ECIII) were derived from the receptor chimeras described above with unique restriction sites. The $EP_{2/4}$NT-ECI chimera was constructed by ligating a *Ssp*I/*Bst*EII fragment from $EP_{2/4}$NT (encoding the receptor's amino terminus through TMII) with a *Bst*EII/*Ssp*I fragment of $EP_{2/4}$ECI (containing the remaining receptor coding region). The $EP_{2/4}$ECII-III chimera was constructed by ligating a *Ssp*I/*Sfi*I fragment from $EP_{2/4}$ECII (encoding the receptor's amino terminus through TMV) with a *Sfi*I/*Ssp*I fragment of $EP_{2/4}$ECIII (containing the remaining receptor coding region). The $EP_{2/4}$NT-ECIII chimera was constructed by ligating a *Bgl*II/Eco47III fragment from $EP_{2/4}$NT-ECI (encoding the amino terminus through TMIII) with a Eco47III/*Bgl*II fragment from $EP_{2/4}$ECII-III (containing the remaining receptor coding region). Two independent clones of each construct were analyzed in all experiments.

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 Inc., Grand Island, NY) with 12 μg of 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).

Ligand Binding Assays. For saturation binding isotherm experiments, 15 μg or 20 μg of membrane protein was incubated with varying [^{3}H]PGE_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 of membrane protein was incubated with 1 to 2 nM [^{3}H]PGE_2 and varying concentrations of unlabeled competitors.

Immunoprecipitation. COS1 cells transfected with the wild-type or mutant EP_2 cDNAs were cultured for 72 h. Immunoprecipitation was performed as described previously (Stillman et al., 1998) with an anti-HA monoclonal antibody (12CA5). Immunoprecipitated proteins were resolved on a 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by autoradiography.

Cell-Surface Enzyme-Linked Immunosorbent Assay (ELISA). An indirect cellular ELISA, based on a protocol from Schoneberg et al., (1995) was used to quantify the amount of receptors present on the plasma membrane. COS1 cells were transiently transfected with the expression plasmids encoding EP_2 wild-type or mutant receptors and were plated into 96-well plates. After 72 h, the cells were fixed with 4% paraformaldehyde in PBS for 30 min. In some cases, cells were permeabilized with 0.2% Triton X-100 PBS. The cells were then blocked for 30 min with Dulbecco's modified Eagle's medium/10% FBS. The anti-HA monoclonal antibody (12CA5) was diluted 1:100 in culture medium and added to the cells for 2 h at 37°C. After four washes with PBS, the cells were incubated with a donkey antimouse horseradish peroxidase secondary antibody diluted 1:5000 in culture medium. Cells were washed four times in PBS, and then 100 μl of tetramethylbenzidine substrate (Sigma Chemical Co., St. Louis, MO) was added for 20 min. Reactions were stopped by adding 100 μl of 1 M phosphoric acid, and then absorbances at 450 nm were determined on a microtiter plate spectrophotometer.

cAMP Measurements. COS1 cells transiently transfected with expression plasmids encoding the wild-type or mutant EP_2 receptors were distributed into 24-well plates. The medium was replaced 24 h later with 450 μl of Dulbecco's modified Eagle's medium/20 mM HEPES/0.25 mM 3 isobutyl-1-methylxanthine/40 μM indomethacin and incubated for 1 h at 37°C. Medium containing varying amounts of PGE_2 or butaprost free acid was added to each well and incubated for 5 min. The reactions were stopped by the addition of 500 μl of 10% trichloroacetic acid. cAMP measurements of the cell lysates were performed by an enzyme immunoassay kit, according to manufacturer's instructions (Stratagene, La Jolla, CA).

Data Analysis. All binding assays and cAMP measurements were analyzed with PRISM software (GraphPad, San Diego, CA). Statistical analysis was performed with Instat software (GraphPad). K_i values were calculated with the method of Cheng and Prusoff (1973).

Results

Exchange of Various EC Sequences Has Differential Effects on Ligand Binding. The putative extracellular sequences of the EP_2 receptor each were replaced by the corresponding EP_4 receptor sequence (Table 1). When transiently

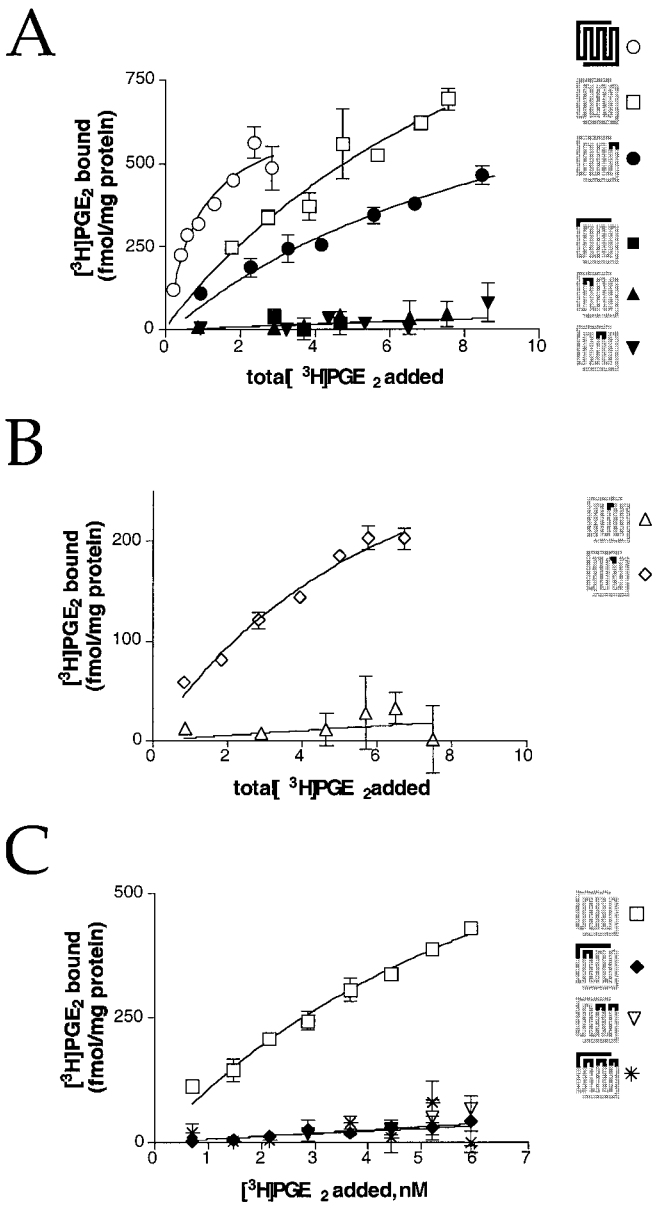

Fig. 1. Saturation isotherms of the binding of [^{3}H]PGE_2 by COS1 cell transfectants expressing the chimeric EP_2 receptors. The data shown here is from a single experiment performed in duplicate and is representative of three to four independent experiments. A: □, EP_2; ○, EP_4; ■, $EP_{2/4}$NT; ▲, $EP_{2/4}$ECI; ▼, $EP_{2/4}$ECII; ●, $EP_{2/4}$ECIII. B: △, $EP_{2/4}$ECII-; ◇, $EP_{2/4}$ECII-C. C: □, EP_2; ◆, $EP_{2/4}$NT-ECI; ▽, $EP_{2/4}$ECII-III; *, $EP_{2/4}$NT-ECIII.

expressed in COS1 cells, the third extracellular loop chimera ($EP_{2/4}$ECIII) demonstrated specific [^{3}H]PGE_2 binding, with a K_d value of 6.0 ± 2.2 nM. Chimeric receptors exchanging the amino terminus ($EP_{2/4}$NT), first extracellular loop ($EP_{2/4}$ECI), and second extracellular loop ($EP_{2/4}$ECII), demonstrated no [^{3}H]PGE_2-specific binding (Fig. 1A).

The ECII region can be subdivided into an amino-terminal conserved sequence and carboxyl-terminal nonconserved sequence. Each of these regions of the second extracellular loop was separately replaced with the corresponding EP_4 sequence (Table 1). $EP_{2/4}$ECII-N, which exchanges the conserved sequence, lost detectable [^{3}H]PGE_2 binding, whereas $EP_{2/4}$ECII-C bound [^{3}H]PGE_2 with a similar affinity to the wild-type EP_2 receptor with a K_d value of 8.8 ± 2.6 nM (Fig. 1b). The K_d values of each of the chimeras that were able to bind PGE_2 are intermediate to values obtained for the EP_2 and EP_4 wild-type receptors, which are 0.88 nM and 12.8 nM, respectively.

All Chimeric Receptors are Expressed in COS-1 Cells at Wild-Type Levels. To determine whether chimeric receptors were expressed at levels similar to the wild-type EP_2 receptors, each of the receptor proteins was immunoprecipitated from COS-1 cells with a monoclonal antibody to the HA tag. Proteins were detected near the predicted molecular mass for each of the receptor constructs (Fig. 2). The presence of multiple bands clustered near this size may be caused by variable post-translational modification, such as glycosylation or phosphorylation of the receptors.

ECII-C and ECIII Chimeras Successfully Traffic to the Cell Surface. The ability of the chimeric receptors to traffic to the cell surface may affect receptor function. Cell surface expression of chimeric receptors was assayed by a whole-cell ELISA technique (Schoneberg et al., 1995) with the 12CA5 anti-HA tag monoclonal antibody. The extracellular location of the amino-terminal HA tag renders it inaccessible to antibody unless the receptor is expressed on the cell surface. As a control for total receptor expression for a particular chimeric construct, cells were permeabilized with Triton X-100. The receptor constructs that were able to bind PGE_2 (wild-type, $EP_{2/4}$ECIII, $EP_{2/4}$ECII-C) were each detected on the cell surface at levels similar to that observed for the wild-type receptor. Of the chimeras with no detectable PGE_2 binding, $EP_{2/4}$ECI was detected on the surface at 25% of wild-type levels, whereas expression of the $EP_{2/4}$NT, $EP_{2/4}$ECII, and $EP_{2/4}$ECII-N chimeras was not detectable at all. ELISA performed in the presence of Triton X-100 detergent demonstrated that each of the receptor proteins was expressed at similar levels (Fig. 3) in agreement with immunoprecipitation experiments.

Receptor Chimeras Possess Specificity of the EP_2 Receptor. The ligand-binding selectivity of the chimeras that displayed [^{3}H]PGE_2 binding was examined in competition binding assays with the naturally occurring prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, butaprost free acid (EP_2 selective), AH13205 (EP_2 selective), and M&B28767 ($EP_{3/4}$ selective) compounds. Each chimeric receptor possessed ligand selec-

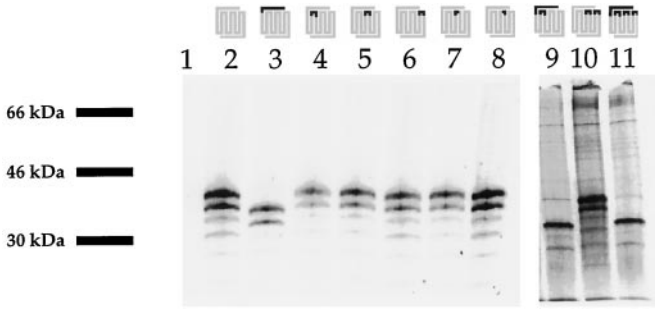

Fig. 2. Immunoprecipitation of EP_2 wild-type and chimeric receptor constructs. Metabolically labeled wild-type and mutant receptors expressed in COS1 cells were immunoprecipitated with a monoclonal antibody (12CA5) recognizing the HA epitope fused to the amino terminus of the receptors. This figure is representative of three independent experiments. Lane 1, vector-only transfected cells; lane 2, EP_2 wild-type; lane 3, $EP_{2/4}$NT; lane 4, $EP_{2/4}$ECI; lane 5, $EP_{2/4}$ECII; lane 6, $EP_{2/4}$ECIII; lane 7, $EP_{2/4}$ECII-N; lane 8, $EP_{2/4}$ECII-C; lane 9, $EP_{2/4}$NT-ECI; lane 10, $EP_{2/4}$ECII-III; lane 11, $EP_{2/4}$NT-ECIII.

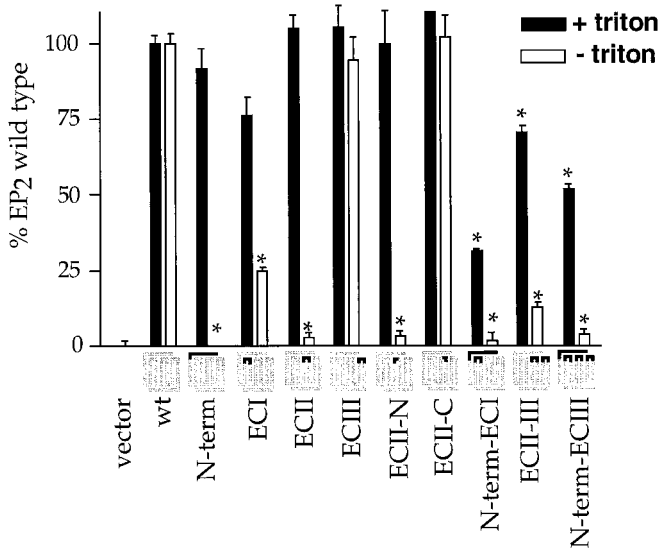

Fig. 3. Cell surface ELISA of wild-type and chimeric EP_2 receptors transfected into COS1 cells. In the absence of Triton X-100 (A), only those receptors expressed on the cell surface were detected, whereas in the presence of 0.2% Triton X-100 (B), surface and internal receptors were detected. Absorbance values are displayed as a percentage of the A_{450} value observed for the wild-type EP_2 receptor, with each data point in triplicate. For EP_2 wild-type, 100% A_{450} = 0.733 (+ triton) and 0.402 (− triton). Data were averaged from three independent experiments on each receptor construct (*$p < .05$). N-term, amino terminus.

TABLE 2

K_i values for prostanoid ligands at wild type and chimeric receptors

Values are in nM; K_i values were calculated from IC_{50} values.

	EP_4	EP_2	$EP_{2/4}$ECII-C	$EP_{2/4}$ECIII
PGE_2	1.2 ± 0.30	32 ± 8	12 ± 1	12 ± 1
$PGF_{2\alpha}$	750 ± 110	6000 ± 1100	7200 ± 1100	3600 ± 720
PGD_2	1100 ± 130	10000 ± 3200	4800 ± 1300	4100 ± 670
Butaprost free acid	Not detected	1200 ± 400	1200 ± 200	550 ± 110
AH13205	Not detected	1000 ± 140	510 ± 150	320 ± 85
M&B28767	32 ± 7.5	5100 ± 1400	3300 ± 820	1500 ± 410

tivity indistinguishable from that of the wild-type EP_2 receptor for all drugs tested, with none of the K_i values for the chimeric receptors statistically different from the EP_2 wild-type values (Table 2).

Only Chimeras Expressed on the Cell Surface Regulate cAMP Production. The ability of PGE_2 and the EP_2-selective agonist butaprost free-acid to elicit receptor-evoked increases in intracellular cAMP levels was examined (Fig. 4; Table 3). Although the EP2/4ECIII and EP2/4ECII-C chimeras were able to stimulate cAMP production, the EP2/4NT, EP2/4ECI, EP2/4ECII, and EP2/4ECII-N chimeras demonstrated no receptor-evoked stimulation of cAMP above the vector-only transfected control cells (Fig. 4).

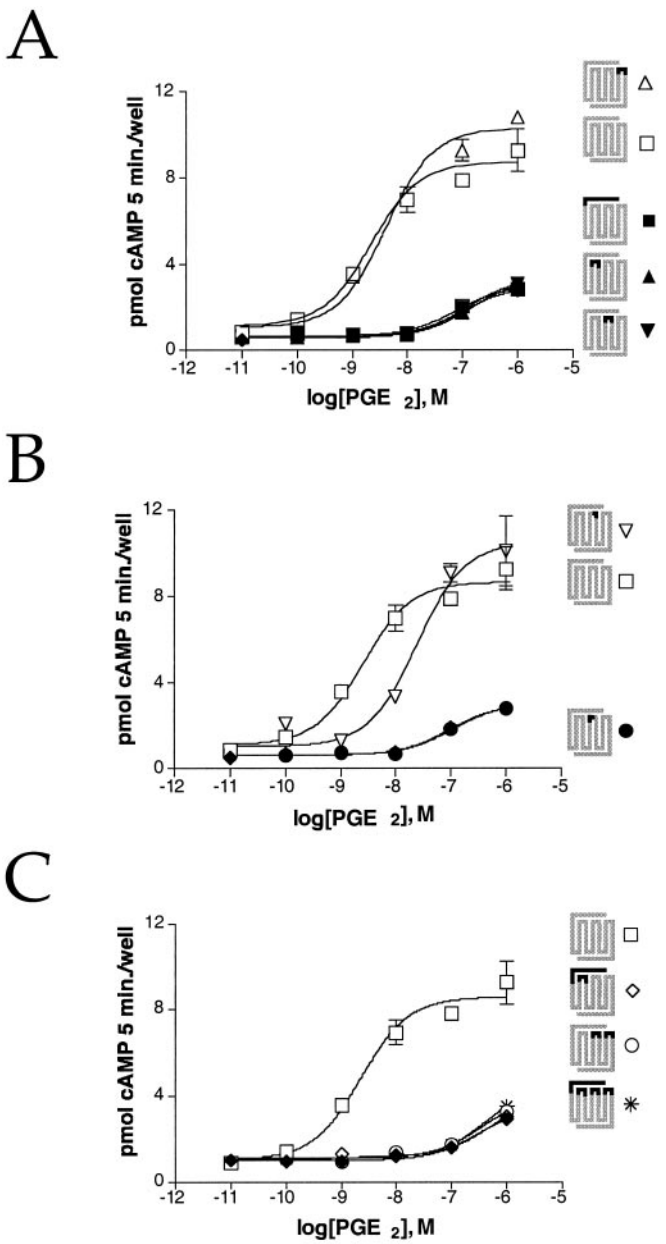

Fig. 4. Receptor-evoked stimulation of intracellular cAMP production in COS1 cells transfected with wild-type and chimeric EP_2 receptors. Cells were incubated with various concentrations of PGE_2, were lysed, and cell lysates were analyzed for cAMP content. Data shown is from a single experiment performed in duplicate and are representative of three independent experiments. A, ♦, vector only; □, EP_2 wild-type; ■, $EP_{2/4}$NT; ▲, $EP_{2/4}$ECI; ▼, $EP_{2/4}$ECII; △, $EP_{2/4}$ECIII. B, ♦, vector only; [sqlo], EP_2 wild-type; ●, $EP_{2/4}$ECII-N; ▼, $EP_{2/4}$ECII-C. C, ♦, vector only; □, EP_2 wild-type; ◇, $EP_{2/4}$NT-ECI; ○, $EP_{2/4}$ECII-III; *, $EP_{2/4}$NT-ECIII. In B and C, the data from A for EP_2 wild-type (□) is replotted for comparison.

Chimeras with Multiple EC Exchanges Do Not Rescue Receptor Function. The loss of function of the chimeric receptors might be because of incompatibility of the EP_4 receptor EC sequences with the EC sequences of the of the EP_2 receptor. To investigate this possibility, multiple EC sequences of the EP_2 receptor were replaced with the corresponding EP_4 sequences. Three additional chimeric receptors were constructed that had either the first two EC sequences exchanged ($EP_{2/4}$NT-ECI), the last two EC sequences exchanged ($EP_{2/4}$ECII-ECIII), or all four EC sequences of the EP2 receptor replaced with the corresponding EP_4 sequences, ($EP_{2/4}$NT-ECIII; Table 1). Neither ligand binding nor signal transduction could be detected with any of these constructs (Figs. 1C and 4C). As with the other nonfunctional chimeras, although receptor expression could be detected by immunoprecipitation (Fig. 2), receptor expression could not be detected on the cell surface (Fig. 3).

Discussion

A phylogenetic tree of the GPCR superfamily has revealed that the prostanoid receptor family is most homologous to a subfamily of peptide receptors that includes the vasopressin receptors (Fig. 5; Kolakowski, 1994). In light of this observed homology, it is of interest that the EC sequences of the EP receptors are critical determinants of receptor structure/function, as has been observed for the peptidergic receptors. Replacement of the amino terminus, ECI, or the conserved sequence motif of ECII of the EP_2 protein resulted in receptors that were unable to bind [^{3}H]PGE_2 or stimulate intracellular cAMP generation. Moreover, replacement of multiple EP_2 EC sequences with the corresponding EP_4 sequences did not rescue receptor function, which suggests that the loss function is not simply because of incompatibility among the EP receptor loops. One possible explanation for the observed phenotypes is that EC sequences form part of the ligand binding surface and, as observed for the peptidergic receptors, mutation of these putative contact surfaces contributes to the loss of ligand binding and signal transduction. However, we found that each of the nonfunctional receptors could not be detected on the plasma membrane as determined by ELISA. It is possible that each of the chimeric receptors is unfolded and this precludes their trafficking to the plasma membrane. Alternatively, these chimeras could represent correctly folded receptors with specific trafficking defects, and this expression in an inappropriate cellular compartment allows neither ligand binding nor participation in ligand-elicited signal transduction. The $EP_{2/4}$ECI chimera was expressed on the cell-surface at 25% of the level of the EP_2 wild-type receptor, although [^{3}H]PGE_2 binding was not ob-

TABLE 3

EC_{50} values for cAMP stimulation of COS1 cells transfected with wild-type or mutant EP receptors

	PGE_2	Butaprost FA
EP_4	1.0 ± 0.14	Not detected
EP_2	1.4 ± 0.31	140 ± 48
$EP_{2/4}$ECII-C	45.0 ± 14	5400 ± 290
$EP_{2/4}$ECIII	6.8 ± 1.6	220 ± 75

served. Detection of $EP_{2/4}$ECI on the cell surface suggests that, at least for the $EP_{2/4}$ECI chimera, altered receptor trafficking alone does not cause the loss of binding and receptor-evoked signaling.

In the absence of structural data, it cannot be determined if the EC domains are directly involved in prostanoid binding. Previous characterization of EC mutations have not assessed, in parallel, surface expression of mutant receptors. It had been shown that mutation of residues within a conserved motif of ECII of prostaglandin E_2 receptors results in receptors with altered EP_3 receptor ligand selectivity (Audoly and Breyer, 1997a) or loss of receptor function for the EP_2 and EP_4 receptors (Stillman et al., 1998). Similarly, other point mutations in ECI or ECII of the human EP_2 receptor (Kedzie et al., 1998) or the related thromboxane receptor (D'Angelo et al., 1996) resulted in a loss of ligand binding and/or signal transduction. Taken together with the current study, these data demonstrate that the amino terminus, ECI, and ECII sequences are critical determinants of prostaglandin receptor structure and/or function. It is possible that the role of the conserved motif across the prostanoid receptor family in the EC sequences is to ensure proper receptor folding, and the phenotypes of EC mutants reported in previous studies are caused by improper folding and/or trafficking.

Overall, the phenotypes observed for the $EP_{2/4}$ chimeric receptors are in sharp contrast to those observed for other small-ligand GPCRs. Mutation of the extracellular domains of GPCRs for small ligands have generally not had effects on ligand binding or receptor structure. Dixon et al. (1987) demonstrated that deletion of the extracellular domains of the β-adrenergic receptor had no effect on receptor function. Studies with chimeric receptors of small-ligand GPCR subtypes have not revealed binding determinants in the extracellular domains (Frielle et al., 1988; Kobilka et al., 1988; Robinson and Caron, 1996). Replacement of the extracellular domains of the β_2-adrenergic receptor with the corresponding α_{1a}-adrenergic receptor sequences had no effect on the ligand binding selectivity of this receptor (Zhao et al., 1998). Much of the mutagenesis and biochemical evidence obtained so far suggest that the ligand binding pocket of these small molecule-binding GPCRs is embedded within the transmembrane-spanning helices (Savarese and Fraser, 1992). Moreover, these data suggest that the extracellular sequences of the biogenic amine receptors are not critical determinants of receptor folding or trafficking as observed in the present study for the prostanoid receptors. In contrast, studies of peptide GPCRs have supported a role for the extracellular domains in ligand binding and receptor structure. The vasopressin V_{1a} and V_2 receptors are 20% identical with the EP_2 receptor. Howl et al. demonstrated that the amino terminus, ECI, and ECII of the V_{1a} receptor participate in the formation of the ligand binding pocket (Howl and Wheatley, 1996), and in the V_2 receptor, a point mutation in ECII eliminated vasopressin binding and signal transduction (Pan et al., 1994). Mutagenesis studies have revealed principal binding site determinants in the EC domains of other peptidergic GPCRs such as the angiotensin (Hjorth et al., 1994), thrombin (Gerszten et al., 1994), and neurokinin receptors (Fong et al., 1992a,b; Yokota et al., 1992; Huang et al., 1994).

Based on the accumulation of mutagenesis data, it is now evident that the GPCR superfamily contains receptor subgroups possessing distinct motifs of receptor-ligand interactions. For example, ligands can bind to the TM core (e.g., small ligand receptors), to both the TM core and the EC (peptide receptors), or to only the amino-terminal domain (metabotropic glutamate receptors; Ji et al., 1998). Prostaglandin receptors are currently classified with the receptors for small ligands, which include the biogenic amine and nucleotide receptors (Ji et al., 1998). Although data presented here do not demonstrate direct interaction of the EC sequences with the prostaglandin ligand, taken together with the prostanoid receptor phylogeny, they suggest that these receptors share structural requirements in the EC sequences similar to the peptidergic GPCRs.

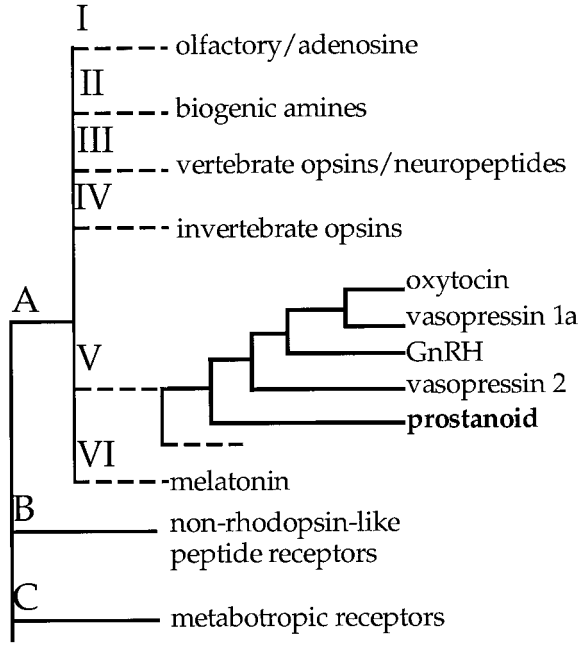

Fig. 5. A partial phylogenetic tree of the GPCR superfamily. Prostaglandin receptors lie in a sub-branch of peptide receptors, Group V, whereas the prototypical small-ligand GPCRs are in Group II (modified from Table 2 of Kolakowski, 1994).

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