0026-895X/97/020343-07$3.00/0

MOLECULAR PHARMACOLOGY, **51:**343–349 (1997).

The Long Cytoplasmic Carboxyl Terminus of the Prostaglandin E_2 Receptor EP_4 Subtype Is Essential for Agonist-Induced Desensitization

MURAT BASTEPE and BARRIE ASHBY

Department of Pharmacology (M.B., B.A.) and Sol Sherry Thrombosis Research Center (B.A.), Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received July 15, 1996; Accepted October 29, 1996

SUMMARY

The 488-amino acid human prostaglandin E_2 receptor EP_4 subtype, which couples to stimulation of adenylyl cyclase, shares the major structural features of G protein-coupled receptors, having seven putative transmembrane domains, an extracellular amino terminus, and a cytoplasmic carboxyl terminus. The latter is composed of 156 amino acids and contains 38 serine and threonine residues, which are potential phosphorylation sites. The carboxyl terminus may be important in receptor function; in some receptors, truncation of the cytoplasmic tail abolishes desensitization. In others, truncation leads to constitutive activity, and in other receptors, truncation has no effect on receptor function. To investigate the role of the long cytoplasmic tail of the EP_4 receptor, we constructed a mutant EP_4 that lacks the last 138 amino acids at the carboxyl terminus, including 36 serine and threonine residues. The truncated EP_4 receptor was stably expressed in Chinese hamster ovary cells at levels comparable to that of the wild-type receptor and exhibited a K_d value for [^{3}H]PGE_2 binding similar to that of the wild-type receptor. PGE_2-mediated adenylyl cyclase activity as a function of PGE_2 concentration was similar in cells expressing the wild-type and truncated EP_4 receptors. Neither the wild-type receptor nor the truncated form showed any constitutive activity. However, the wild-type EP_4 receptor underwent PGE_2-induced desensitization fully within 15–20 min, whereas the truncated EP_4 receptor, lacking 36 of the 38 carboxyl-terminal serines and threonines, displayed a sustained activation. Despite the continuous presence of PGE_2, the rate of cAMP synthesis via stimulation of the truncated receptor remained constant over ≥20 min. These findings suggest that the cytoplasmic tail of EP_4 plays an important role in agonist-induced desensitization.

PGE_2 is involved in many physiological and pathophysiological events (1), and it exerts its actions through binding to at least four distinct EP receptors that are members of the G protein-linked receptor superfamily (2, 3). The EP1 receptor couples to phospholipase C, the EP_2 and EP_4 receptors couple to stimulation of adenylyl cyclase, and the EP_3 receptor couples to inhibition of adenylyl cyclase. All of the receptors have been cloned from human cells. In addition, six isoforms of the human EP_3 receptor have been identified that are identical over the first 359 amino acids but differ in the carboxyl-terminal region, which varies in length from 6 to 65 amino acids after the seventh transmembrane helix (4–8). The EP_3 isoforms also differ in their degree of inhibition (4, 5) and susceptibility to desensitization (9). Truncation of the mouse EP_3 receptor at the splice variant site leads to a fully constitutively active form of the receptor (10), and some of the isoforms show varying degrees of constitutive activity (10, 11).

Many cells express several EP receptor subtypes as well as many of the isoforms of EP_3 (4, 5, 7, 8, 12, 13), which has implications for prostaglandin regulation of cell function. We have shown that coexpression of stimulatory and inhibitory EP receptors leads to a novel form of desensitization of adenylyl cyclase mediated through the EP_3 receptor. Prostaglandins induce time-dependent inhibition with a different concentration dependence from their own activation of adenylyl cyclase (14–17). Colocalization of stimulatory and inhibitory receptors may represent a general mechanism of autocrine or paracrine regulation, buffering against rapid variations in agonist concentration when agonists are produced close to their sites of action.

More typically, desensitization of G protein-linked receptors involves phosphorylation by protein kinases at specific serine and threonine residues in the carboxyl-terminal or third intracellular loop of the receptor, leading to attenuation

This work was supported by National Institutes of Health Grant RO1-HL48114.

ABBREVIATIONS: PGE_2, prostaglandin E_2; HEL, human erythroleukemia; IBMX, 3-isobutyl-1-methylxanthine; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.

of adenylyl cyclase activity over several minutes (18). However, because of the possibility of interference from a colocalized EP_3 receptor and its isoforms, this form of desensitization is not readily observed *in situ*.

For a thorough understanding of the complex system that regulates the PGE_2-mediated cAMP metabolism, components of the system must be investigated individually and as a whole. We describe experiments on desensitization of the cloned EP_4 receptor expressed in CHO cells, which do not express significant levels of other prostaglandin receptors.

EP_4 is a receptor that was recently identified by Coleman *et al*. (3). Its mRNA is abundantly expressed (19–21), suggesting that many actions of PGE_2 initially considered to occur through EP_2 may also be exerted through EP_4. The EP_4 receptor clone was isolated by several groups of researchers, including our group, before the pharmacological identification of EP_4 was made, and the clone was reported as the EP_2 subtype based on the observation that the expressed receptors stimulated adenylyl cyclase (19–22). The lack of binding of the EP_2-selective agonist butaprost was, however, left unexplained in those reports. Identification of EP_4, and, more recently, isolation of a novel cDNA that when expressed has identical characteristics to the pharmacologically defined EP_2 receptor (23) suggested that the previously reported cDNA clones were the EP_4 subtype. This led Nishigaki *et al*. to show that the mouse cDNA previously reported as EP_2 (19) was identical to the pharmacologically defined EP_4 subtype (24). In agreement, we redesignated our cDNA clone from HEL cells as EP_4. Thus, the cloned receptor studied in the current work was the human EP_4 receptor.

Hydrophobicity analysis of the deduced amino acid sequence of the EP_4 receptor predicts seven hydrophobic segments followed by a long carboxyl-terminal domain of 156 amino acids, including 38 serine and threonine residues, which are potential phosphorylation sites. Among members of the G protein-coupled receptor family, the β_2-adrenergic receptor has been studied extensively in terms of structure-activity relationship, and its cytoplasmic tail was reported to be important for receptor desensitization (25, 26). Although the existence of such a relationship was postulated for several members of the family, including the platelet-activating factor receptor (27) and the α_{1B}-adrenergic receptor (28), for others, either contradictory findings were reported, such as for the luteinizing hormone receptor (29, 30), or the same domain was assigned to other functions, such as for the angiotensin II receptor (31). This inconsistency among different types of receptors suggests that no common criteria can be set for the entire receptor family in terms of structure-activity relationship concerning desensitization and that each receptor, or receptor group, should be evaluated individually regarding that matter. Therefore, it needs to be determined whether the EP_4 receptor undergoes desensitization and whether its long cytoplasmic tail plays a role in the generation of this response.

The cytoplasmic tail of the EP_4 receptor might also be involved in regulation of isomerization between the inactive and active conformations of the molecule as described by Samama *et al*. for the β_2-adrenergic receptor (32). As for the EP_3 receptor isoforms, the structural difference in the cytoplasmic tail not only results in differential G protein coupling but also determines the extent of agonist-independent constitutive activity (10, 11). Furthermore, a mutant EP_3 receptor truncated at the RNA splicing point retaining the amino acids common to all the isoforms shows full agonist-independent constitutive G_i activity (10). However, the truncated EP_3 receptor shows agonist-dependent G_s activity lower than that of $EP_3\gamma$, the isoform that couples to both G_s and G_i (11).

In the current study, we investigated the EP_4 receptor in terms of its signaling properties. In an attempt to underline the significance of the carboxyl-terminal tail, which forms the distal third of the receptor molecule, we engineered a mutant EP_4 that lacks the last 138 amino acids at its carboxyl terminus. The truncation point was homologous to the splicing site of the EP_3 receptor isoforms. However, although truncation of the EP_3 receptor at the splice site results in constitutive activity, truncation of EP_4 receptor led to no change in activity but abolished agonist-induced desensitization.

Experimental Procedures

Materials. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Kits for RNA extraction, first-strand synthesis, and *Pfu* polymerase were from Stratagene (La Jolla, CA). Sequencing reagents were from United States Biochemical (Cleveland, OH). [α-^{32}P]dCTP, [2,8-^{3}H]adenine, [^{14}C]cAMP, and [^{35}S]dATP were obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H]PGE_2 (182 Ci/mmol) was from Amersham (Arlington Heights, IL). Other chemicals and reagents were from Sigma Chemical (St. Louis, MO). CHO-K1 cells were obtained from American Type Culture Collection (Rockville, MD). The plasmid pUC18 was from Life Technologies (Gaithersburg, MD).

Cloning of the EP_4 receptor cDNA. Total RNA was isolated from mouse kidney by the guanidinium thiocyanate-phenol-chloroform single-step extraction method (33). First-strand cDNA was synthesized from RNA through reverse transcription with a first-strand synthesis kit. Using the mouse first-strand cDNA as template, PCR was performed with *Pfu* polymerase with the use of primers based on the mouse EP_4 sequence. The PCR product, corresponding to nucleotides 211–937 of the mouse receptor, was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) according to the method of Feinberg and Vogelstein (34, 35) by use of a primer labeling kit from Stratagene. Screening of 6×10^5 clones derived from a λgt11 HEL cell cDNA library was performed according to the method of Benton and Davis (36). Hybridization was performed in 10× Denhardt's solution, 5× SSPE (1× = 150 mM NaCl, 10 mM NaH_2PO_4, 1.25 mM EDTA, pH 7.4), 100 μg/ml salmon sperm DNA, 2% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate at 60° for 20 hr. The final wash was carried out with 0.1× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.5% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate at 60° (four washes for 15 min each). Two positive clones were isolated, and the phage DNA was purified with a Magic Lambda Preps kit from Promega (Madison, WI). A single insert of ~3.3 kb was obtained from *Eco*RI digestion of each phage DNA. The inserts were subcloned into pUC18 and sequenced on both strands through use of the dideoxynucleotide chain termination method (37). The cDNA sequences of the clones $phEP_4$–10 and $phEP_4$–11 were identical to each other.

Construction of the truncated EP_4 cDNA. To make the cDNA construct, a fragment of $phEP_4$ extending from the unique *Sac*II site at nucleotide 694 to nucletide 1050, was amplified by PCR. The reverse PCR primer was designed so that the product carried in its 3′ end three different stop codons located in three different reading frames. An *Apa*I site was also tagged on the reverse primer. Forward primer was 5′-CCACC GCG GCC GCC TCG GTT GCC TCC-3′ (underlined bases indicate the unique *Sac*II site), and reverse primer was 5′-GGA GGG CC (CTA)T(TTA)T(TCA) GCG GCA GAA GAG GCA TTT G-3′ (underlined bases indicate the tacked-on *Apa*I site;

bases in parentheses indicate stop codons). The 358-bp PCR product was purified after digestion with *Sac*II and *Apa*I. At the same time, the *Hin*dIII insert of phEP$_4$ was subcloned into pRc/CMV from In-Vitrogen (San Diego, CA); this was named pRc/CMV-hEP$_4$-wt and was used to prepare the cDNA for the truncated receptor. It was digested with *Sac*II and *Apa*I. The 1939-bp insert was discarded, and the 6773-bp linearized plasmid was purified on a 1% low-melting-temperature agarose gel. With T4 DNA ligase (Promega), a ligation reaction was performed between the PCR product with *Sac*II/*Apa*I overhangs and the purified piece of the *Sac*II/*Apa*I digestion products of pRc/CMV-hEP$_4$-wt. The ligation product, named pRc/CMV-hEP$_4$-t350, was sequenced to ensure sequence accuracy.

Stable expression in CHO-K1 cells. CHO-K1 cells were grown in the nutrient mixture F-12 Ham supplemented with 10% fetal bovine serum at 37° in 5% CO_2. Transfection of either pRc/CMV-hEP$_4$-wt or pRc/CMV-hEP$_4$-t350 was performed with 7.5 μg/ml Lipofectin reagent and 1 μg of DNA according to the method of Felgner *et al*. (38). Selection medium contained 500 μg/ml geneticin sulfate. Single-cell clones were obtained by serial dilution. Positive clones isolated by measuring the PGE$_2$-mediated adenylyl cyclase activity were maintained in culture medium containing 10% fetal bovine serum, 350 μg/ml geneticin sulfate, and 1 mM acetylsalicylic acid (used to inhibit endogenous PGE$_2$ formation).

Determination of adenylyl cyclase activity. Transfected cells grown in six-well culture dishes were labeled for 3 hr with 3 μCi of [^{3}H]adenine (25 Ci/mmol). Medium was replaced with fresh medium containing IBMX for 10 min. For dose-response studies, cells were challenged with 0–30 μM PGE$_2$ for 5 min at 37° in medium containing 2 mM IBMX. For kinetic experiments, cells were challenged with 30 μM forskolin or 10 μM PGE$_2$ in the presence of 2 mM IBMX for various time periods. Reactions were stopped by the replacement of the medium with a stopping solution containing 0.2 M HCl, 0.2% sodium dodecyl sulfate, and 2000 cpm of [^{14}C]cAMP as recovery standard. The supernatant and cells were removed from the dishes and boiled for 15 min, and cAMP, which is expressed as percentage of total adenine nucleotides, was determined according to the two-column method of Salomon (39). The kinetic data for each clonal cell line obtained from PGE$_2$ stimulation were normalized to the corresponding rate of cAMP formation stimulated by 30 μM forskolin. Each data point was multiplied by the ratio of postlag phase rate of forskolin-stimulated cAMP synthesis to the initial rate of cAMP synthesis obtained by PGE$_2$ stimulation.

Binding experiments. Cells stably expressing wild-type or mutant EP$_4$ were lysed by sonication in 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 10 μM leupeptin, and 10 μg/ml soybean trypsin inhibitor. The lysate was centrifuged at 36,000 $\times$ *g* for 20 min at 4°. The pellet was washed once and resuspended in buffer A (50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 10 μM leupeptin). Protein concentrations were determined by the Coomassie Plus protein assay reagent (Pierce Chemical, Rockford, IL) (40). The membrane preparation (0.2–0.8 mg of protein/ml) was incubated with varying concentrations of [^{3}H]PGE$_2$ for 60 min at room temperature in buffer A. In some experiments, the membrane preparation was incubated with 2.5 nM [^{3}H]PGE$_2$ and varying concentrations of nonlabeled agonist. Binding was determined as described previously (41) except the filters were washed with ice-cold 50 mM Tris·HCl, pH 7.5, buffer. To define the nonspecific binding, 1 μM nonlabeled PGE$_2$ was used. The data were analyzed with EBDA software (Biosoft, Cambridge, UK) (42).

Results

We cloned the cDNA for the PGE$_2$ receptor EP$_4$ subtype by probing a λgt11 HEL cell cDNA library with a fragment of mouse EP$_4$ cDNA. The deduced amino acid sequence of our clone (data not shown), although identical to that reported by Bastien *et al.* (21), differs from that reported by An *et al.* (20) in three amino acids: residues 465–467 are Gly-Pro-Ala in our clone but Trp-Ala-Cys in the other. The difference is likely due to sequencing errors. Fig. 1 shows the putative structure of the human EP$_4$ receptor, including its membrane orientation and the deduced amino acid sequence of the cytoplasmic carboxyl terminus. Thirty eight serine and threonine residues are found within this portion. The truncated

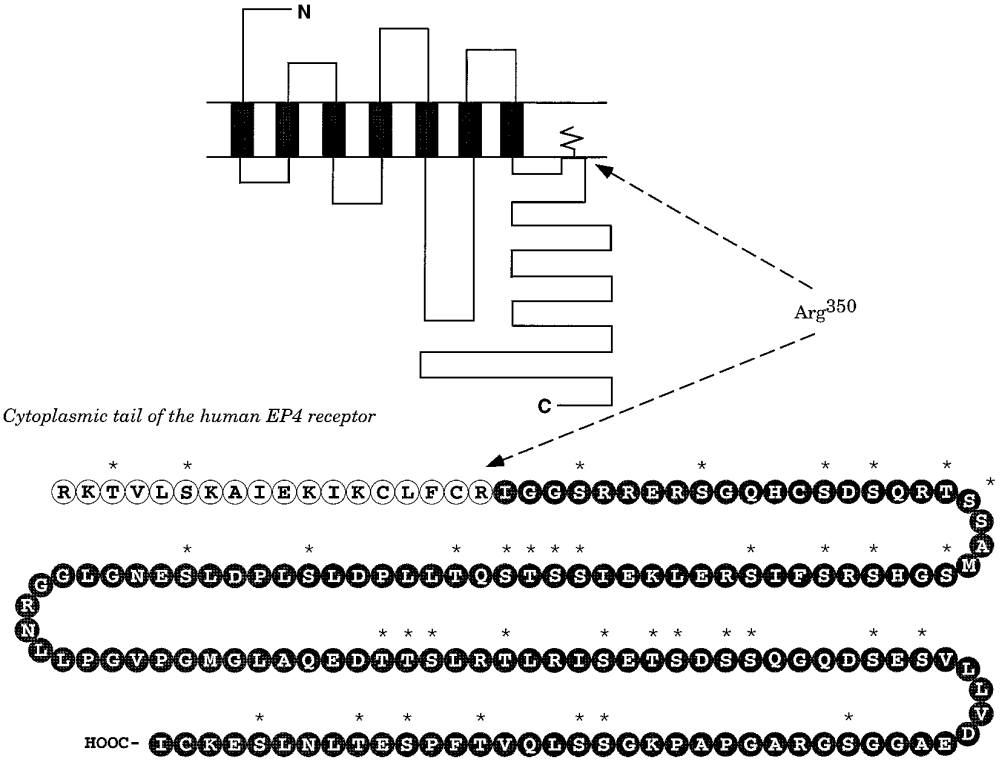

Fig. 1. Membrane orientation of the human EP$_4$ receptor and the deduced amino acid sequence of its cytoplasmic tail. *Encircled letters*, amino acids remaining in the cytoplasmic tail of the truncated mutant; *white letters*, deleted amino acids. *, Potential phosphorylation sites. The *membrane anchorage* depicted in the proximal region of the cytoplasmic terminus marks the putative palmitoylation of either Cys346 or Cys349.

EP_4 receptor lacks the last 138 amino acids that naturally exist in the wild-type receptor (Fig. 1). Of the 38 serine and threonine residues located in the cytoplasmic tail of the wild-type EP_4, 36 fall within the deleted fragment. The truncated receptor terminates with the Arg350 and has a 13-amino acid cytoplasmic tail containing two cysteines (Cys346 and Cys349) that may be palmitoylated in analogy to Cys322 and Cys323 of rhodopsin and Cys341 of the β_2-adrenergic receptor (43–45) (Fig. 1).

The cDNA for the wild-type EP_4 receptor or the EP_4 receptor with severely truncated cytoplasmic tail was ligated into the eukaryotic expression vector pRc/CMV, which carries a human cytomegalovirus promoter and a neomycin resistance selection marker. pRc/CMV-hEP_4-wt or pRc/CMV-hEP_4-t350 was transfected into CHO-K1 cells. Single-cell cloning was performed by selecting cells resistant to geneticin sulfate through the use of serial dilution, and positive clones were determined with a screen measuring the PGE_2-mediated adenylyl cyclase activity. Clones that displayed a ≥2-fold increase over the basal level of cAMP in response to 0.1 μM PGE_2 were considered to express the transfected receptor. For the wild-type EP_4, 15 of 40 screened clonal cell lines were positive (37.5%), and for the truncated EP_4, 24 of 54 screened clonal cell lines were positive (44%).

We selected several clonal cell lines to study the effects of the cytoplasmic tail truncation. The selected clonal cell lines stably expressing the wild-type EP_4 were hEP_4-wt/CB9 and hEP_4-wt/AG10, and those stably expressing the truncated EP_4 were hEP_4-t350/AA6, hEP_4-t350/AD12, and hEP_4-t350/AE1. Levels of receptor expression in individual clonal cells and the K_d value for PGE_2 were determined by measuring the binding of [^{3}H]PGE_2 to membranes prepared from each selected clone. As seen in Table 1, the wild-type and truncated EP_4 receptors have similar binding affinities for [^{3}H]PGE_2, with K_d values obtained from each clonal cell line of 8.2 ± 0.8–9.2 ± 2.2 nM. The expression level of the wild-type or truncated receptor for each selected clone is also shown in Table 1, and the B_{max} values range from 46.4 ± 14.4 to 263.5 ± 33.4 fmol/mg of protein.

To determine whether the severe cytoplasmic tail truncation led to any functional changes in the G protein/effector coupling properties of the EP_4 receptor, we measured the PGE_2-mediated adenylyl cyclase activity. A concentration-dependent increase in the cAMP level was obtained when each selected clonal cell line was challenged with PGE_2. Fig. 2 shows the PGE_2-mediated cAMP response obtained in the clonal hEP_4-wt/CB9 and hEP_4-t350/AA6 cells. The dose-response curve is typical of those seen in the other selected clonal cells, revealing no significant difference between the dose-response profiles of the wild-type and truncated EP_4 receptors. Notably, saturation of the response could not be obtained regardless of the receptor type, even at PGE_2 concentrations as high as 30 μM. The characteristic natures of the dose-response profiles that we obtained, however, are similar to those reported by others for the expressed wild-type human EP_4 receptor (20).

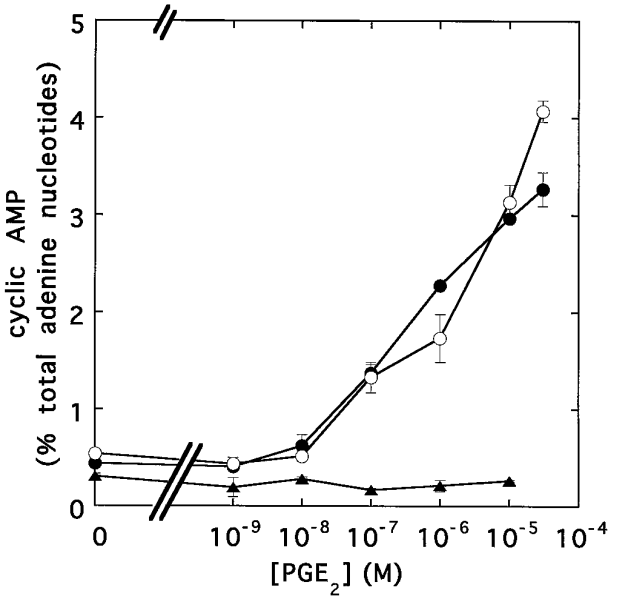

Fig. 2. PGE_2-mediated cAMP formation. Indicated concentrations of PGE_2 was used to stimulate (●) hEP_4-wt/CB9, (○) hEP_4-t350/AA6, or (▲) nontransfected CHO-K1 cells for 5 min in the presence of 2 mM IBMX. Reactions were stopped, and cAMP levels were measured as described in Experimental Procedures. Values are representative of three separate experiments with similar results. Data represent mean ± standard error of duplicate determinations.

TABLE 1

Binding parameters of the wild-type and truncated EP_4 receptors

Total receptor concentrations and the apparent equilibrium binding dissociation constants of the wild-type and truncated EP_4 receptors expressed in CHO-K1 cells are represented as mean ± standard error of two experiments. Binding of varying concentrations of [^{3}H]PGE_2 to the membranes or binding of 2.5 nM [^{3}H]PGE_2 in the presence of varying concentrations of nonlabeled PGE_2 was measured at equilibrium at room temperature, and bound and free fractions were separated by rapid filtration.

	K_d for [^{3}H]PGE_2	B_{max}
	nM	*fmol/mg of protein*
hEP_4-wt/CB9	8.5 ± 0.5	181.3 ± 57.7
hEP_4-wt/AG10	9.2 ± 2.2	48.7 ± 24.5
hEP_4-t350/AA6	8.2 ± 0.8	263.5 ± 33.4
hEP_4-t350/AD12	8.8 ± 0.4	46.4 ± 14.4
hEP_4-t350/AE1	8.8 ± 3.1	108.6 ± 16.3

The basal cAMP level in each clonal cell was also measured to assess any possible alterations in the PGE_2-independent adenylyl cyclase activity. No significant difference existed between the basal levels of cAMP in cells expressing the wild-type versus the truncated EP_4 receptor, with average basal levels of 0.37 ± 0.07% and 0.38 ± 0.06%, respectively. The basal cAMP level in nontransfected CHO cells (0.30 ± 0.03%) also was similar to that in the clonal cells (Fig. 2).

To examine whether the wild-type and the truncated EP_4 receptors undergo agonist-mediated desensitization, we challenged the selected clonal cells with PGE_2 (10 μM) for varying time periods in the presence of 2 mM IBMX, a phosphodiesterase inhibitor, and measured the amount of cAMP accumulated after each stimulation period. In a separate experiment, we challenged the clonal cells with 30 μM forskolin, a direct activator of the adenylyl cyclase, in the same manner. As seen in Fig. 3, *top,* which shows the time course of forskolin-stimulated cAMP levels, the specific activities of the cyclase systems of the clonal cell lines were close to each other, with only minor differences. The data in Fig. 3, *top,* indicate that the rate of forskolin-mediated cAMP formation in each clone was constant throughout the first 30 min, except over the first 4 min, when an apparent lag was ob-

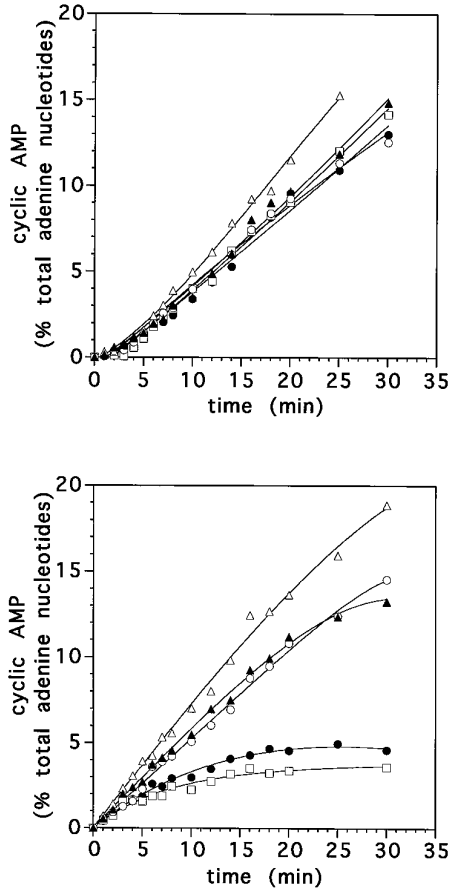

Fig. 3. Time course of cAMP formation in clonal CHO-K1 cells expressing the wild-type or truncated EP_4 receptor. Clonal hEP_4-wt/CB9 (●), hEP_4-wt/AG10 (□), hEP_4-t350/AA6 (○), hEP_4-t350/AD12 (△), or hEP_4-t350/AE1 (▲) cells were stimulated with (*top*) 30 μM forskolin or (*bottom*) 10 μM PGE_2 for indicated time periods in the presence of 2 mM IBMX. Reactions were stopped, and cAMP levels were measured as described in Experimental Procedures. Values are representative of three separate experiments with similar results.

served. This lag, which was consistent throughout the clonal cell lines, is likely a reflection of the time required for forskolin to penetrate the cell membrane. The individual rates of cAMP formation were calculated over the time period after the lag phase. The average rate of synthesis was 0.53 ± 0.01%/min among the clonal cells expressing the wild-type receptor and 0.57 ± 0.4%/min for those expressing the truncated receptor.

Fig. 3, *bottom,* shows the time course of PGE_2-mediated cAMP formation. The data for each clone were normalized to the corresponding rate of cAMP formation stimulated by 30 μM forskolin. The time courses of cAMP formation in Fig. 3, *bottom,* indicate grouping of the clonal cell lines into two distinct kinetic patterns. In clonal hEP_4-wt/CB9 and hEP_4-wt/AG10, which express the wild-type EP_4, a linear increase in the cAMP formation was observed over the first 3 min, indicating a constant rate of PGE_2-mediated activity of adenylyl cyclase. With increasing stimulation time, however, the increase in the cAMP formation curved off and reached a plateau, indicating an incremental decline in the rate of adenylyl cyclase activity (Fig. 3, *bottom*). The rate of cAMP synthesis, although initially an average of 0.38 ± 0.02%/min (before normalization) among the clones that express the wild-type EP_4 receptor, approached zero after ~15 min of agonist stimulation. The decrease in the rate of cAMP synthesis represents desensitization of adenylyl cyclase because the experiment was performed in the presence of IBMX, which inhibits the breakdown of cAMP. The kinetic pattern in cells expressing the truncated receptor was significantly different. In clonal hEP_4-t350/AA6, hEP_4-t350/AD12, and hEP_4-t350/AE1 cells, the increase in the amount of cAMP remained linear over ≥20 min (i.e., the rate of cAMP synthesis, which averaged 0.36 ± 0.04%/min (before normalization) among the three clones, remained constant (Fig. 3, *bottom*). A slight decline in the rate was seen after 20 min of agonist stimulation; however, it was insignificant compared with that observed in cells expressing the wild-type EP_4 receptor. Taken together, the data presented in Fig. 3 indicate a lack of agonist-induced desensitization of the adenylyl cyclase activity in cells expressing the truncated EP_4 receptor.

Discussion

The human PGE_2 receptor EP_4 subtype is a 488-amino acid membrane protein with a significantly long cytoplasmic tail that forms almost one third of the entire molecule. We cloned the wild-type EP_4 receptor from HEL cells and subsequently constructed a cDNA encoding a mutant that lacks part of the carboxyl terminus. We preserved Cys346 and Cys349 in the carboxyl terminus of the truncated EP_4 receptor because a conserved cysteine residue located among the first 15 amino acids of cytoplasmic tails of many G protein-coupled receptors has been implicated in high affinity ligand binding and/or G protein coupling (45, 46). We truncated the EP_4 receptor at a site homologous to the splice variant site of the EP_3 receptor, which in the case of the EP_3 receptor led to fully constitutive activity (10).

The binding studies indicate that the deleted portion of the cytoplasmic tail of EP_4 does not seem to be crucial for receptor expression or ligand binding. This suggests that the truncated receptor was processed correctly in terms of post-translational modification, receptor folding, and membrane trafficking. The dose-response studies reveal that the receptor/G protein coupling also is not altered by the truncation of the carboxyl terminus, suggesting that the regions of the molecule involved in G protein coupling are not present within the truncated segment.

In the dose-response experiments, we were unable to saturate the response defined by the increase in the level of cAMP in response to PGE_2, despite high agonist concentrations. Therefore, an EC_{50} value (concentration of agonist yielding half-maximal activity) could not be extrapolated for either the wild-type or the truncated receptor. The rightward shift in the dose-response curve might be due to poor efficiency of the receptor/G protein coupling. The same kind of relationship with a lack of saturation in the cAMP response was reported previously for the cloned human EP_4 receptor expressed transiently (20). Similar dose-response patterns were also reported for the cloned mouse EP_4 receptor expressed either transiently (19) or stably (24).

Unlike the truncated EP_3 receptor, truncation of the EP_4 receptor did not result in constitutive activity as judged by the similarity of the basal (agonist-independent) levels of cAMP in the clonal and nontransfected cells. Lack of transformation to a constitutively active state was further sup-

ported by the finding that the affinity of the truncated receptor for PGE_2 did not change. Studies involving constitutively active mutant receptor activity have demonstrated that the mutants have significantly higher affinity for agonists (32). Therefore, it seems that the deleted portion of EP_4 does not play a role in receptor isomerization between inactive and active states.

We studied the effects of truncation on agonist-induced desensitization by using several clonal cell lines expressing either the wild-type or truncated receptor. Our method of measuring desensitization involved measurement of cAMP formation as a function of stimulation time. This allowed us to directly observe the change in the actual rate of PGE_2-mediated adenylyl cyclase activation. As a control, we also measured the time-course of forskolin-stimulated cyclase activities. We used the rate of cAMP synthesis measured on forskolin stimulation of individual clonal cell lines and normalized the kinetic data obtained from PGE_2 stimulation to take into account the differences in receptor expression levels. The normalization of the data certainly did not alter the kinetic patterns seen when raw data were plotted (data not shown).

This type of desensitization experiment provides reliable data because the experimental conditions allow the system to operate without any interference, such as washing away of the agonist after a certain pretreatment period. In settings in which intact cells are studied, such manipulations can be misleading because it is often hard to completely wash off the agonist or bring down the concentration of the second messenger to normal basal levels before initiation of the stimulation. Lipophilic ligands in particular, such as prostaglandins, are very difficult to remove from the environment, making it almost impossible to achieve accurate concentrations at the receptor site for post-treatment stimulation. We validated the method used in the current study through detailed studies of prostaglandin receptor desensitization in intact cells (16, 17).

The intrinsic adenylyl cyclase activities of individual clonal cells measured by forskolin stimulation displayed minor variations. This was not unexpected because we have observed such differences among clonal cell lines derived from the same cell type. The difference is due likely to cell-to-cell variations that either exist naturally or were acquired on transfection.

The rate of cAMP formation in response to PGE_2 did not change over $\geq$20 min in clonal cells expressing the truncated EP_4 receptor. After 20 min, however, a slight decline was observed in the clones hEP_4-t350/AD12 and hEP_4-t350/AE1 and, to a lesser extent, in the clone hEP_4-t350/AA6. This may be due to the onset of other desensitization mechanisms, such as receptor sequestration, which involves removal of the receptor molecules from the cell surface into cellular compartments where ligand interaction is no longer possible (18). The clonal difference in the extent of this late-onset rate change may be a reflection of the variation in the receptor expression levels. The clone with the highest expression level, hEP_4-t350/AA6, is the least affected, whereas the one with the lowest expression level, hEP_4-t350/AD12, is the most affected of the three. The change in the rate of synthesis can also be attributed to the removal of inhibition of cAMP phosphodiesterase. IBMX is a competitive inhibitor, and its inhibitory effect can be overcome by increasing concentrations of the substrate, which is cAMP.

By examining agonist-induced desensitization patterns of the wild-type and truncated EP_4 receptors, we showed that the EP_4 receptor-mediated cAMP response is attenuated by continuous agonist exposure and that the cytoplasmic carboxyl terminus has a significant role in this adaptation. In general, desensitization of G protein-coupled receptors involves uncoupling of receptor/G protein complex, sequestration of receptors, and, in the long term, receptor down-regulation (18). Studies with β-adrenergic receptors have indicated the role of phosphorylation by serine/threonine kinases in the uncoupling of receptor from G protein. In the case of β_2-adrenergic receptor desensitization, β-adrenergic receptor kinase phosphorylates multiple sites in the cytoplasmic tail of the agonist-occupied receptor (47). Our truncated EP_4 receptor is missing 36 serines and threonines in its carboxyl terminus. By analogy to the β-adrenergic receptors, these amino acid residues may be phosphorylated in the wild-type on receptor/agonist interaction by β-adrenergic receptor kinase or other members of the G protein-coupled receptor kinase family (48). Proof of this hypothesis requires further investigation involving site-directed mutagenesis methods and direct demonstration of agonist-induced receptor phosphorylation by using specific receptor antibodies. Although such work is currently under way in our laboratory, the results presented in this article clearly demonstrate the requirement of the cytoplasmic tail in desensitization of EP_4, and help focus further endeavors to this particular region.

In previous work, we have shown that prostaglandin regulation of adenylyl cyclase may involve colocalized stimulatory (EP_2, EP_4) and inhibitory (EP_3) receptors, which gives rise to a form of desensitization. The contribution of colocalized receptors to desensitization can be assessed because we can prepare forms of the receptors that do not undergo desensitization by phosphorylation-dependent mechanisms. We have expressed a truncated form of EP_4 that does not desensitize and certain isoforms of EP_3 do not desensitize.

References

1. Campbell, W. B., and P. V. Halushka. Lipid-derived autocoids, in *Pharmacological Basis of Therapeutics* (A. G. Gilman, J. G. Hardman, L. L. Limbird, P. B. Molinoff, and R. W. Ruddon, eds.). McGraw-Hill, Health Professions Division, New York, 601–616 (1996).
2. Coleman, R. A., I. Kennedy, P. P. A. Humphrey, K. Bunce, and P. Lumley. Neurotransmitter, autocoid receptors, and prostanoid receptors, in *Comprehensive Medicinal Chemistry* (C. Hansch, P. G. Sammes, J. B. Taylor, J. C. Emmett, P. D. Kennewell, and C. A. Ramsden, eds.). Vol. 3. Pergamon Press, Oxford, 643–714 (1990).
3. Coleman, R. A., S. P. Grix, S. A. Head, J. B. Louttit, A. Mallett, and R. L. Sheldrick. A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* **47:**151–168 (1994).
4. Regan, J. W., T. J. Bailey, J. E. Donello, K. L. Pierce, D. J. Pepperl, D. Zhang, C. E. Fairbairn, A. M. Bogardus, D. F. Woodward, and D. W. Gil. Molecular cloning and expression of human EP_3 receptors: evidence of three variants with differing carboxyl termini. *Br. J. Pharmacol.* **112:**377–385 (1994).
5. An, S., J. Yang, S. W. So, L. Zeng, and E. J. Goetzl. Isoforms of the EP_3 subtype of human prostaglandin E_2 receptor transduce both intracellular calcium and cAMP signals. *Biochemistry* **33:**14496–14502 (1994).
6. Adam, M., Y. Boie, T. H. Rushmore, G. Muller, L. Bastien, K. T. McKee, K. M. Metters, and M. Abramovitz. Cloning and expression of three isoforms of the human EP_3 prostanoid receptor. *FEBS Lett.* **338:**170–174 (1994).
7. Schmid, A., K. H. Thierauch, W. D. Schleuning, and H. Dinter. Splice variants of the human EP_3 receptor for prostaglandin E_2. *Eur. J. Biochem.* **228:**23–30 (1995).
8. Kotani, M., I. Tanaka, Y. Ogawa, T. Usui, K. Mori, A. Ichikawa, S. Narumiya, and K. Nakao. Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative

messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol.* **48:**869–879 (1995).
9. Negishi, M., Y. Sugimoto, A. Irie, S. Narumiya, and A. Ichikawa. Two isoforms of prostaglandin E receptor EP_3 subtype: different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *J. Biol. Chem.* **268:**9517–9521 (1993).
10. Hasegawa, H., M. Negishi, and A. Ichikawa. Two isoforms of the prostaglandin E receptor EP_3 subtype different in agonist-independent constitutive activity. *J. Biol. Chem.* **271:**1857–1860 (1996).
11. Negishi, M., H. Hasegawa, and A. Ichikawa. Prostaglandin E receptor $EP_3\gamma$ isoform, with mostly full constitutive Gi activity and agonist-dependent Gs activity. *FEBS Lett.* **386:**165–168 (1996).
12. Irie, A., Y. Sugimoto, T. Namba, A. Harazono, A. Honda, A. Watabe, M. Negishi, S. Narumiya, and A. Ichikawa. Third isoform of the prostaglandin-E-receptor EP_3 subtype with different C-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *Eur. J. Biochem.* **217:**313–318 (1993).
13. Sugimoto, Y., M. Negishi, Y. Hayashi, T. Namba, A. Honda, A. Watabe, M. Hirata, S. Narumiya, and A. Ichikawa. Two isoforms of the EP_3 receptor with different carboxyl-terminal domains: identical ligand binding properties and different coupling properties with Gi proteins. *J. Biol. Chem.* **268:**2712–2718 (1993).
14. Ashby, B. Kinetic evidence indicating separate stimulatory and inhibitory prostaglandin receptors on platelet membranes. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11:**291–300 (1986).
15. Ashby, B. Cyclic AMP turnover in response to prostaglandins in intact platelets: evidence for separate stimulatory and inhibitory prostaglandin receptors. *Second Messengers Phosphoproteins* **12:**45–57 (1988).
16. Ashby, B. Model of prostaglandin-regulated cyclic AMP metabolism in intact platelets: examination of time-dependent effects on adenylate cyclase and phosphodiesterase activities. *Mol. Pharmacol.* **36:**866–873 (1989).
17. Ashby, B. Novel mechanism of heterologous desensitization of adenylate cyclase: prostaglandins bind with different affinities to both stimulatory and inhibitory receptors on platelets. *Mol. Pharmacol.* **38:**46–53 (1990).
18. Lohse, M. J. Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta* **1179:**171–188 (1993).
19. Honda, A., Y. Sugimoto, T. Namba, A. Watabe, A. Irie, M. Negishi, S. Narumiya, and A. Ichikawa. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_2 subtype. *J. Biol. Chem.* **268:**7759–7762 (1993).
20. An, S., J. Yang, M. Xia, and E. J. Goetzl. Cloning and expression of the EP_2 subtype of human receptors for prostaglandin E_2. *Biochem. Biophys. Res. Commun.* **197:**263–270 (1993).
21. Bastien, L., N. Sawyer, R. Grygorczyk, K. M. Metters, and M. Adam. Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. *J. Biol. Chem.* **269:**11873–11877 (1994).
22. Bastepe, M., G. F. Mao, S. P. Kunapuli, J. K. deRiel, and B. Ashby. Cloning of a cDNA for the prostaglandin E receptor EP_2 subtype from human erythroleukemia cells (abstract). *Can. J. Physiol. Pharmacol.* **72** (Suppl. 1)**:**542 (1994).
23. Regan, J. W., T. J. Bailey, D. J. Pepperl, K. L. Pierce, A. M. Bogardus, J. E. Donello, K. M. Kedzie, D. F. Woodward, and D. W. Gil. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2 subtype. *Mol. Pharmacol.* **46:**213–220 (1994).
24. Nishigaki, N., M. Negishi, A. Honda, Y. Sugimoto, T. Namba, S. Narumiya, and A. Ichikawa. Identification of prostaglandin E receptor 'EP_2' cloned from mastocytoma cells as EP_4 subtype. *FEBS Lett.* **364:**339–341 (1995).
25. Roth, N. S., P. T. Campbell, M. G. Caron, R. J. Lefkowitz, and M. J. Lohse. Comparative rates of desensitization of β-adrenergic receptors by the β-adrenergic receptor kinase and the cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **88:**6201–6204 (1991).
26. Hausdorff, W. P., P. T. Campbell, J. Ostrowski, S. S. Yu, M. G. Caron, and R. J. Lefkowitz. A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc. Natl. Acad. Sci. USA* **88:**2979–2983 (1991).
27. Takano, T., Z. Honda, C. Sakanaka, T. Izumi, K. Kameyama, K. Haga, T. K. Haga, K. Kurokawa, and T. Shimizu. Role of cytoplasmic tail phosphorylation sites of platelet-activating factor receptor in agonist-induced desensitization. *J. Biol. Chem.* **269:**22453–22458 (1994).
28. Lattion, A. L., D. Diviani, and S. Cotecchia. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the α1B-adrenergic receptor. *J. Biol. Chem.* **269:**22887–22893 (1994).
29. Sanchez-Yague, J., M. C. Rodriguez, D. L. Segaloff, and M. Ascoli. Truncation of the cytoplasmic tail of the lutropin/choriogonadotropin receptor prevents agonist-induced uncoupling. *J. Biol. Chem.* **267:**7217–7220 (1992).
30. Zhu, X., T. Gudermann, M. Birnbaumer, and L. Birnbaumer. A luteinizing hormone receptor with a severely truncated cytoplasmic tail (LHR-ct628) desensitizes to the same degree as the full-length receptor. *J. Biol. Chem.* **268:**1723–1728 (1993).
31. Ohyama, K., Y. Yamano, S. Chaki, T. Kondo, and T. Inagami. Domains for G-protein coupling in angiotensin II receptor type I: studies by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **189:**677–683 (1992).
32. Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz. A mutation-induced activated state of the β2-adrenergic receptor: extending the ternary complex model. *J. Biol. Chem.* **268:**4625–4636 (1993).
33. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159 (1987).
34. Feinberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:**6–13 (1983).
35. Feinberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity (addendum). *Anal. Biochem.* **137:**266–267 (1984).
36. Benton, W. D., and R. W. Davis. Screening λgt recombinant clones by hybridization to single plaques in situ. *Science (Washington D. C.)* **196:** 180–182 (1977).
37. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:**5463–5467 (1977).
38. Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, and M. Danielsen. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84:** 7413–7417 (1987).
39. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10:**35–55 (1979).
40. Sedmak, J. J., and S. E. Grossberg. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* **79:**544–552 (1977).
41. Kunapuli, S. P., G. Fen Mao, M. Bastepe, L. Y. Liu-Chen, S. Li, P. P. Cheung, and B. Ashby. Cloning and expression of a prostaglandin E receptor EP_3 subtype from human erythroleukaemia cells. *Biochem. J.* **298:**263–267 (1994).
42. McPherson, G. A. A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.* **17:**107–113 (1983).
43. O'Brien, P. J., and M. Zatz. Acylation of bovine rhodopsin by [^{3}H]palmitic acid. *J. Biol. Chem.* **259:**5054–5057 (1984).
44. Ovchinnikov, Y. A., N. G. Abdulaev, and A. S. Bogachuk. Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitoylated. *FEBS Lett.* **230:**1–5 (1988).
45. O'Dowd, B. F., M. Hnatowich, M. G. Caron, R. J. Lefkowitz, and M. Bouvier. Palmitoylation of the human β2-adrenergic receptor: mutation of Cys^{341} in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J. Biol. Chem.* **264:**7564–7569 (1989).
46. O'Dowd, B. F., M. Hnatowich, J. W. Regan, W. M. Leader, M. G. Caron, and R. J. Lefkowitz. Site-directed mutagenesis of the cytoplasmic domains of the human β2-adrenergic receptor: localization of regions involved in G protein-receptor coupling. *J. Biol. Chem.* **263:**15985–15992 (1988).
47. Benovic, J. L., R. H. Strasser, M. G. Caron, and R. J. Lefkowitz. *Beta*-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. USA* **83:**2797–2801 (1986).
48. Inglese, J., N. J. Freedman, W. J. Koch, and R. J. Lefkowitz. Structure and mechanism of the G protein-coupled receptor kinases. *J. Biol. Chem.* **268:**23735–23738 (1993).

Send reprint requests to: Barrie Ashby, Ph.D., Department of Pharmacology, Temple University School of Medicine, 4320 N. Broad Street, Philadelphia, PA 19140.