Isoforms of the EP3 Subtype of Human Prostaglandin E_2 Receptor Transduce Both Intracellular Calcium and cAMP Signals‡

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ABSTRACT: The EP3 subtype of prostaglandin E2 receptor transduces diverse physiological responses in mammalian tissues through signaling pathways coupled to heterotrimeric G proteins. Distinct cDNA clones encoding five isoforms of the EP3 receptor were isolated from a human uterus cDNA library. The human EP3 receptor isoforms designated hEP3-I, I′, II, III, and IV are derived from alternative RNA splicing and differ only in the distal sequences of their carboxyl-terminal cytoplasmic tails. The unique cytoplasmic tails consist of 31 amino acids for isoforms I and I′, 29 for II, 6 for III, and 15 for IV. When stably expressed in CHO cell transfectants, all isoforms exhibited similar EP3-specific binding of [^{3}H]-PGE_2 and PGE_2 analogs. The EP3-selective agonist M&B 28767 both decreased the intracellular cAMP concentration ([cAMP]i) and increased the intracellular concentration of calcium ([Ca^{2+}]i) with quantitative differences among different isoforms, but none mediated an increase in [cAMP]i. Pertussis toxin treatment completely blocked the decrease in [cAMP]i, but not the increase in [Ca^{2+}]i evoked by M&B 28767. PGE_2-induced desensitization of [^{3}H]PGE_2 binding by isoforms III and IV was rapid and transient, whereas that by isoform II was slow and persistent. Reverse transcription-PCR amplification of EP3 receptor messages in human kidney and uterine tissue RNA detected expression of all isoforms with different abundancies. The dual signal transduction pathways and distinctive tissue distribution of isoforms of the EP3 receptor are consistent with its mediation of diverse functions of PGE_2.

Prostaglandin E_2 (PGE_2) is a potent mediator of many biologically important functions in the cardiovascular, pulmonary, renal, endocrine, gastrointestinal, neural, reproductive, and immune systems (Coleman et al., 1990). The diverse effects of PGE_2 are both tissue-specific and differentially dependent on biochemical pathways of signal transduction. The complexity of responses to PGE_2 is attributable to its recognition by at least four subtypes of receptors that differ in ligand binding specificity, distribution in tissues, and coupling to pathways of signal transduction. The EP1, EP2, EP3, and EP4 subtypes of PGE_2 receptors were distinguished initially by differences in affinity of binding and potency of effects of a range of natural prostanoids and synthetic agonists and antagonists (Coleman et al., 1990, 1994).

The EP3 subtype of PGE_2 receptor mediates especially diverse actions, including inhibition of water reabsorption in the kidney (Hebert et al., 1993), gastric acid secretion (Chen et al., 1988), neurotransmitter release (Ohia et al., 1990), and contraction of the uterus (Goureau et al., 1992). The recent cloning of cDNAs encoding EP3 receptors of the mouse (Sugimoto et al., 1992), cow (Namba et al., 1993), human (Adam et al., 1994; Yang et al., 1994; Regan et al., 1994), and rabbit (Breyer et al., 1994) has shown that they belong to the seven transmembrane-domain superfamily of receptors that associate with heterotrimeric guanine nucleotide-binding (G) proteins (Dohlman et al., 1991). Several isoforms of the EP3 receptor with different carboxyl-terminal tails appear to be generated by alternative RNA splicing (Namba et al., 1993; Irie et al., 1993; Adam et al., 1994; Breyer et al., 1994; Regan et al., 1994). Some isoforms of mouse and bovine EP3 receptors, when expressed in Chinese hamster ovary (CHO) cells, activated multiple signal transduction pathways by coupling to different G proteins (Namba et al., 1993; Irie et al., 1993). However, detailed studies of signal transduction have not been reported for isoforms of the human EP3 receptor.

We have previously cloned a human kidney EP3 receptor that transduced both decreases in intracellular cAMP concentration ([cAMP]i) and increases in intracellular concentration of free calcium ([Ca^{2+}]i) when transiently expressed in COS-7 cells (Yang et al., 1994). We report here the cloning of additional alternatively spliced human EP3 receptor isoforms and their similar mediation of effects on both [cAMP]i and [Ca^{2+}]i, when stably expressed in CHO cells. None of the natural variants of the human EP3 receptor transduces an increase in [cAMP]i observed in one isoform of the mouse and bovine EP3 receptors. In contrast to the indistinguishable [^{3}H]PGE_2 binding and signal transduction by isoforms of the human EP3 receptor, each mediates agonist-induced desensitization with a distinctive time course.

EXPERIMENTAL PROCEDURES

Materials. A human uterus cDNA library in λgt11 (Clontech Laboratories Inc., Palo Alto, CA), PGE_2 (Upjohn Company, Kalamazoo, MI), [5,6,8,11,12,14,15-^{3}H]PGE_2 (185 Ci/mmol, Amersham Life Sciences, Arlington Heights, IL), M&B 28767 (Rhone-Poulenc Rorer Ltd., Dagenham, Essex), Chinese hamster ovary cells (CHO-K1) and cell culture medium (Cell Culture Facility, UCSF), Geneticin (G-418 sulfate, Gibco Laboratories, Gaithersberg, MD),

‡ The nucleotide sequences reported in this paper have been submitted to the Genbank/EMBL Data Banks with accession numbers L26976, L32660, L32661, and L32662.

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cAMP radioimmunoassay kit (Dupont-NEN, Boston, MA), forskolin, pertussis toxin (PT), and 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical Co., St. Louis, MO), and Fura-2/AM (Calbiochem Inc., La Jolla, CA) were obtained from the designated suppliers.

cDNA Cloning of Isoforms of the EP3 Receptor. One million clones of a human uterus cDNA library were screened under high stringency conditions (Sambrook et al., 1989), using a full-length EP3 receptor cDNA cloned from a human kidney library (Yang et al., 1994) as the hybridization probe. More than 30 positive clones were isolated, subcloned into pBlueScript SK(−) plasmid, restriction-mapped, and partially sequenced. Sequence analysis revealed that these clones fell into five distinct classes that differed between classes only in their 3′-sequences. At least one full-length cDNA clone from each class was sequenced completely on both strands using the dideoxy chain termination method (Sanger et al., 1977).

Genomic Southern Blotting. Human genomic DNA was isolated from a human T lymphoblastoma cell line, T sup-1 (Smith et al., 1984). Replicate 10-μg aliquots of genomic DNA were digested to completion with various restriction enzymes and fractionated on a 0.8% agarose gel. After treatment with 0.1 N HCl and 0.5 N NaOH, the DNA was transferred to Nytran$^+$ nylon membrane (Schleicher & Schuell). The membrane blot was then hybridized with a ^{32}P-labeled 1.1 kb cDNA fragment encoding sequence of the EP3 receptor from translation start codon to Gln359 under high stringency conditions, including a last wash with 0.1× SSC and 0.1% SDS at 62 °C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA of pregnant human uterus (obtained from Dr. R. Kirk Riemer, Obstetrics and Gynecology, UCSF) was isolated by the acid-guanidinium-isothiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). First-strand cDNAs were prepared with oligo-dT primer and Superscript II reverse transcriptase (Gibco-BRL) from uterine RNA and human kidney mRNA (Clontech). First-strand cDNAs and human genomic DNA were used as templates in a 36-cycle PCR reaction with conditions of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The primers were employed in various combinations for amplification of specific isoforms. The upstream primer for all isoforms was 3P1, 5′-TTCTTAATAGCTGTTCGCCTGGC. The downstream primers were as follows: 3P2, 5′-CTAGAATTGAGACTTAGGAAC for isoform I; 9A, 5′-CTGTCCATCATTAGAGCAGCT for isoform II; and 1B9B18A, 5′-AAGAGAGTCATGGAGCTTCCA for isoforms I′, III, and IV (Figure 1A).

Stable Expression of the Cloned EP3 Receptors in CHO Cells. CHO-k1 cells were transfected with the cDNAs encoding EP3 receptor isoforms II, III, or IV in the Geneticin-resistance eukaryotic expression vector pRc/RSV (Invitrogen) using LipofectAmine (Gibco-BRL). Selection for stable transfectants was performed 4 days after transfection in 10% fetal calf serum-supplemented F12 Ham's Nutrient Mix medium containing 500 μg/mL of Geneticin. Geneticin-resistant colonies were isolated after an additional 14 days and further cloned through limiting dilution. Transcription of a specific EP3 receptor isoform mRNA in each CHO cell clone was assessed by RT-PCR. The functional expression of each isoform of the EP3 receptor was evaluated by a binding assay using [^{3}H]PGE$_2$ as

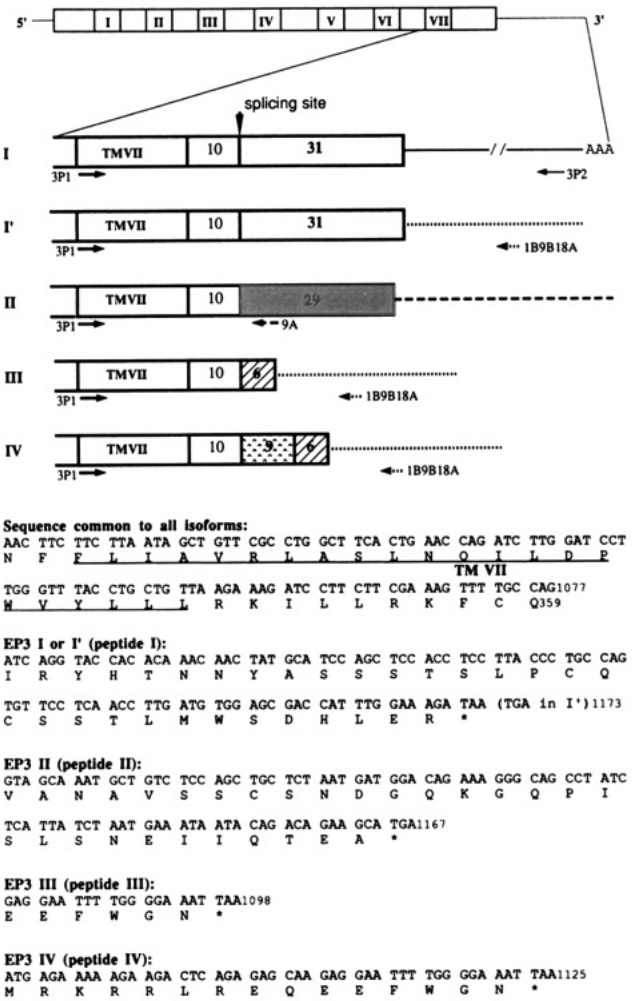

FIGURE 1: (A, top) Schematic representation of five structurally distinct cDNA clones encoding natural isoforms of the human EP3 receptor. *Bars* represent protein coding regions with identical *shading* indicating the same sequences. *Lines* connecting to the bars are 3′-untranslated regions, with the same sequences shown by lines of identical style. *Arrows* indicate the approximate positions of primers used in RT-PCR (Figure 3). (B, bottom) Nucleotide and translated amino acid sequences of the carboxyl termini of human EP3 receptor isoforms I, I′, II, III, and IV. The seventh transmembrane domain predicted by hydropathy analysis is underlined in the sequence common to all isoforms. The termination codons are indicated by *asterisks*. The numbering of nucleotide and amino acid sequences starts from translation initiation codon as in the sequence deposited in the Genbank with accession number L26976.

described (Yang et al., 1994). Binding data were analyzed with the LIGAND computer program.

Quantification of [cAMP]i and [Ca^{2+}]i. CHO cell transfectants expressing each of the isoforms of human EP3 receptor were cultured in 24-well plates to about 80% confluency (approximately 2 × 10^5 cells/well) and pretreated with or without 20 ng/mL of pertussis toxin (PT) for 12 h. The transfectants were then washed twice with 2 mL of Hanks' balanced salt solution (HBSS), preincubated for 10 min at 37 °C in 0.5 mL of HBSS containing 1 mM IBMX, and exposed to 10 pM to 100 nM M&B 28767 in the presence or absence of 1 μM forskolin for 10 min at 37 °C. The reactions were terminated by aspirating the medium and adding 75% ice-cold ethanol. The amounts of cAMP extracted from each well were quantified by radioimmunoassay according to the manufacturer's protocol.

[Ca^{2+}]i was measured as described (Yang et al., 1994). CHO cell transfectants were loaded with 2.5 μM Fura-2/

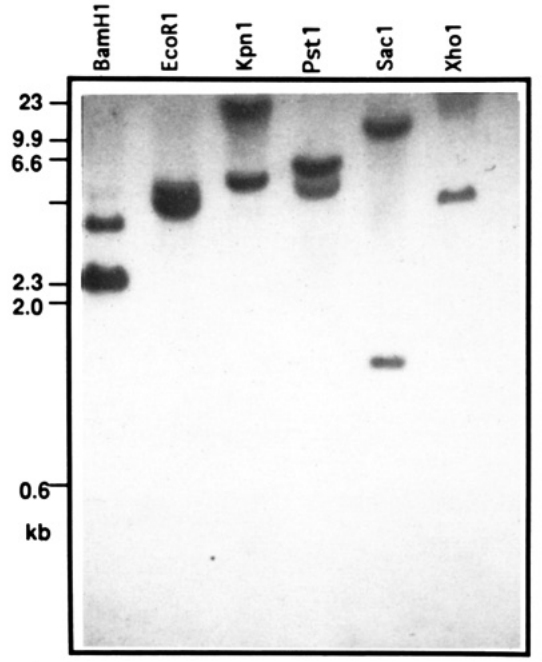

FIGURE 2: Southern blot of human genomic DNA digested with multiple restriction enzymes. Replicate aliquots of 10 μg of genomic DNA isolated from human T lymphoblast cells (T-sup1) were digested with the restriction enzymes denoted above each lane and electrophoresed. After transfer onto a nylon membrane, fragments of DNA encoding the EP3 receptor were detected by hybridization under high stringency conditions with a 1.1 kb cDNA probe containing the entire sequence common to all isoforms.

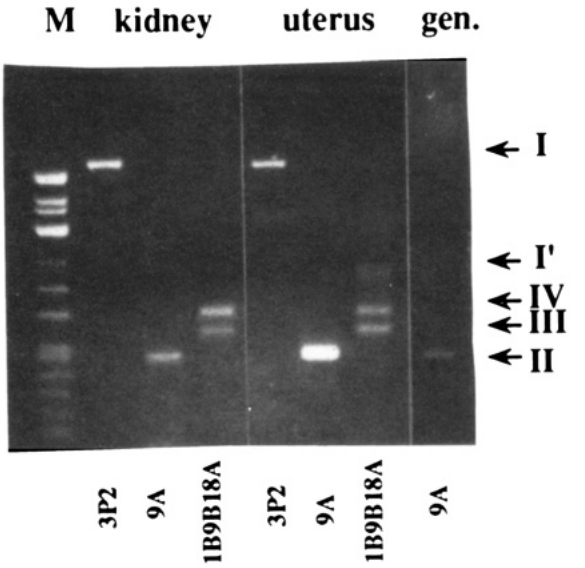

FIGURE 3: PCR amplification of EP3 receptor isoforms in RNA of kidney and uterus, and in genomic DNA. The "*M*" in the left lane is the *Hin*f1 digested ϕX174 serving as a size marker. *Arrows* at the right margin indicate the positions expected for amplified products of each EP3 receptor isoform. 3P1 was used as the upstream primer for all isoforms. Downstream primers were 3P2 for I, 9A for II, and 1B9B18A for I′, III, and IV. The DNA fragments amplified from I, I′, II, III, IV, and genomic DNA were 802, 286, 135, 166, 194, and 135 bp, respectively. The identity of each amplified products as EP3 receptor isoforms was confirmed by DNA sequencing.

AM in HBSS for 30 min at 37 °C in the dark, washed, and resuspended in HBSS containing 1 mM $CaCl_2$. Cuvettes containing 5×10^6 Fura-2-loaded CHO cells in 2 mL were positioned in a Perkin Elmer LS 50B fluorimeter. Fluorescence was recorded before and after addition of 100 nM M&B 28767. The values of $[Ca^{2+}]i$ in nM were calculated from the ratio of the fluorescence intensities at 340 and 380 nm.

Agonist-Induced Desensitization of EP3 Receptor Isoforms. CHO cell transfectants expressing isoforms II, III, and IV of the EP3 receptor were cultured to 95% confluency in 12-well plates. Transfectants in replicate wells were washed once and incubated at 37 °C for 12 h in 1.5 mL of serum-free F12 Ham's medium with addition of 1 μM PGE_2 at 12, 6, 3, 1, and 1/4 h prior to quantification of $[^3H]PGE_2$ binding. Cell layers were washed three times with iced HBSS, detached and disaggregated by pipetting in iced HBSS−10 mM EDTA, and resuspended in HBSS for assessment of specific binding of $[^3H]PGE_2$ at 0 °C (Yang et al., 1994).

RESULTS

More than 30 cDNA clones were isolated from a human uterus cDNA library by hybridization with a cDNA encoding a human kidney EP3 subtype of PGE_2 R (Yang et al., 1994). Each cDNA was assigned to one of five groups based on differences in their 3′-sequences. The structural features of the cDNAs encoding isoforms of the human EP3 receptor, termed I, I′, II, III, and IV, are depicted schematically in Figure 1A. All isoforms have 359 amino acids in common, including the 5′-untranslated sequence and the coding sequences of the first 10 amino acids of the cytoplasmic tail. The sequences after Gln359 diverge to generate different distal carboxyl-terminal amino acid sequences characteristic of each isoform. Isoform I, which is the same as the initially isolated human kidney EP3 receptor (Yang et al., 1994), and isoform I′ have the identical 31 carboxyl-terminal amino acid sequence but differ in 3′-untranslated sequences. Isoform II has a 29 amino acid carboxyl-terminal sequence different from that of isoforms I and I′. Isoform III shares six amino acids at the carboxyl terminus with isoform IV but lacks a nine amino acid segment of isoform IV that is rich in basic residues (Figure 1B). The cytoplasmic tails of isoforms III and IV lack serine or threonine residues that represent potential phosphorylation sites for protein kinases (Dohlman et al., 1991).

Isoform I is the human homolog of the mouse EP3α (Irie et al., 1993), and isoform II is the counterpart of the mouse EP3γ (Irie et al., 1993) and bovine EP3D (Namba et al., 1993). A homolog of isoform III has been isolated from rabbit kidney (Breyer et al., 1994), while isoforms I′ and IV have only been identified in humans. Recently, three isoforms of the EP3 receptor were cloned from human kidney and uterus cDNA libraries (Adam et al., 1994) and a human small intestine cDNA library (Regan et al., 1994) that correspond in sequences to isoforms I, II, and III (Figure 1).

The mRNAs of different isoforms of the EP3 receptor probably result from alternative splicing of a precursor RNA encoded by a single gene. The alignment of nucleotide sequences downstream from Gln359 showed that isoforms III and IV share the same sequence with isoform I′, but with 119 and 92 bp deletions, respectively. The nucleotide sequence of the putative 5′-splicing junction flanking Gln359, as deduced from the PCR-amplified genomic DNA (Figure 3, right lane), complies with the expected consensus sequence having AG upstream and GT downstream of the splicing site. A genomic Southern blot analysis (Figure 2), using a

Table 1: Characteristics of Binding of [^{3}H]PGE_2 by CHO Cell Transfectants Expressing Isoforms of the Human EP3 Receptor[a]

transfectant	isoform	B_{max} (fmol/ 10^6 cell)	K_d (PGE_2) (nM)	IC_{50} (M&B28767) (nM)
9A-2	II	321 ± 58	6.03 ± 1.04	2.3
9B-3	III	35.7 ± 7.3	4.90 ± 0.80	2.0
1B-6	IV	127 ± 6.8	2.39 ± 0.49	3.4

[a] Values shown are mean ± SE of triplicate determinations.

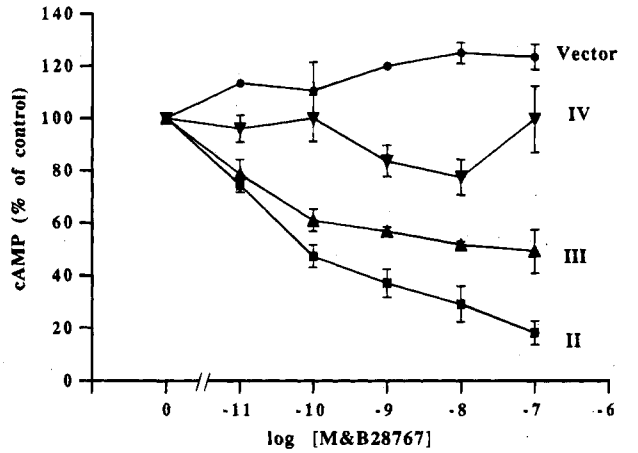

FIGURE 4: Effects of M&B 28767 on forskolin-induced increases in [cAMP]i in CHO cell-transfectants expressing natural isoforms of the EP3 receptor. Each point represents the mean ± SE of triplicate samples. The results shown are from one study that is representative of three or more experiments. The mean values of [cAMP]i before and after forskolin treatment of the cells were 0.99 and 17.1 pmol/well for vector alone, 0.62 and 6.66 pmol/well for II, 0.78 and 7.30 pmol/well for III, and 0.74 and 12.4 pmol/well for IV.

probe containing the entire common cDNA sequence, showed a simple hybridization pattern consistent with a single gene encoding the EP3 receptor. Assuming complete digestion, two hybridization fragments were expected for restriction enzymes *Bam*H1 and *Kpn*1, for which recognition sites are present in the cDNA probe sequence. Since there are no *Eco*R1, *Pst*1, and *Sac*1 sites in the cDNA sequence, the double hybridization bands in these digestions suggested the presence of intron(s) within the cDNA probe sequence of the gene.

To assess the expression and relative abundance of various EP3 receptor isoforms in human tissues, first-strand cDNAs from human kidney and uterus tissues were amplified by PCR using different primer combinations that are specific for each isoform. Almost all isoforms are expressed in both kidney and uterus, although with different relative abundancies (Figure 3). The kidney and uterus express similar amounts of isoforms I, III, and IV, and the uterus expresses more abundant isoform II in this particular RNA preparation.

We have previously demonstrated that isoform I of the EP3 receptor, when expressed in COS-7 cells, transduced suppression of [cAMP]i and an increase in [Ca^{2+}]i in response to PGE_2 (Yang et al., 1994). To study the signal transduction properties of the novel isoforms identified here, we established CHO cell lines that each permanently express isoforms II, III, and IV. Binding studies using [^{3}H]PGE_2 (Table 1) showed that all isoforms bind to PGE_2 with high affinity, as determined by K_d values of 2.4−6.0 nM. Each isoform is of the expected EP3 subtype (Coleman et al.,

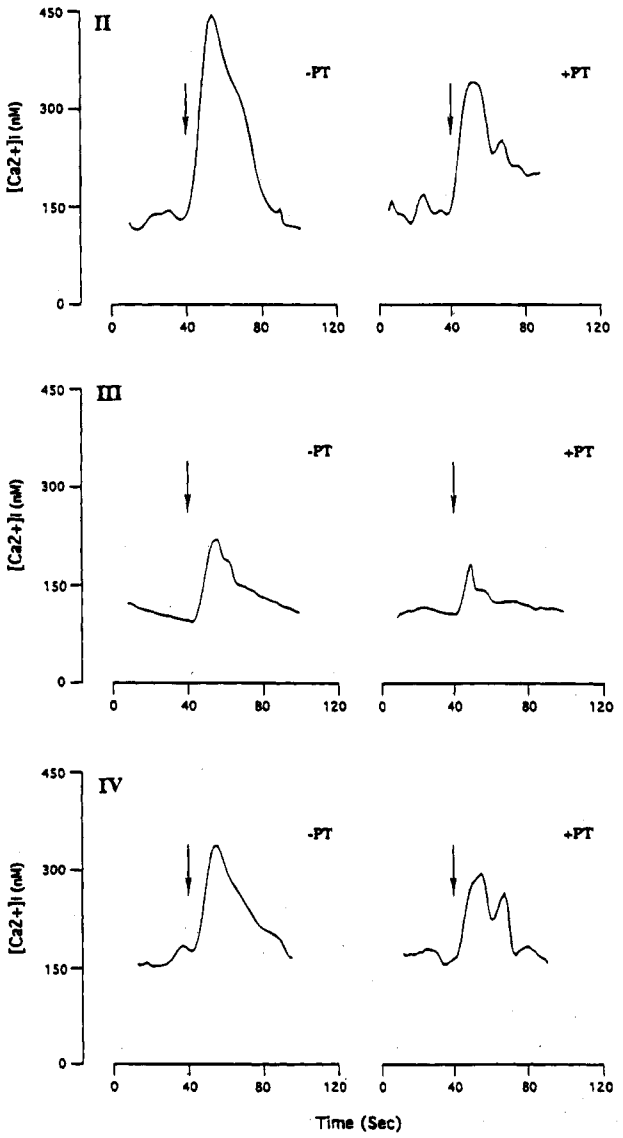

FIGURE 5: Effect of pertussis toxin on M&B 28767-induced elevation of [Ca^{2+}]i in CHO cell transfectants expressing EP3 isoforms. Fluorescence was recorded before and after addition of M&B 28767 to a final concentration of 100 nM (indicated by the *arrows*). No change in [Ca^{2+}]i was observed in control CHO cells.

1990), since their binding to [^{3}H]PGE_2 was competitively inhibited by EP3-selective agonist M&B 28767 (IC_{50} of 2.0−3.4 nM) but not by the EP1-selective antagonist SC19220 or EP2-selective agonist butaprost (IC_{50} > 1 μM). Control CHO cells transfected with the expression vector pRc/RSV alone did not bind [^{3}H]PGE_2 specifically.

The capacity of novel isoforms of the EP3 receptor to alter increases in [cAMP]i elicited by forskolin stimulation of adenylyl cyclase was examined in the same lines of stably transfected CHO cells. M&B 28767 significantly suppressed forskolin-induced increases in [cAMP]i in CHO cells expressing isoforms II and III in the range of 0.1−100 nM (Figure 4). In CHO cells expressing isoform IV, the lesser but significant suppressive effect on [cAMP]i was restricted to M&B 28767 concentrations of 1 and 10 nM ($p < 0.05$ versus control group by Student t test). When the cells were pretreated with pertussis toxin, 100 nM M&B 28767 failed to suppress forskolin-induced increases of [cAMP]i in all isoforms (data not shown). At concentrations of 10 pM to 1 μM, M&B 28767 did not increase [cAMP]i in CHO cells

Table 2: Time Course of PGE_2-Induced Desensitization of Binding of $[^3H]PGE_2$ by CHO Cell Transfectants Expressing EP3R Isoforms[a]

isoform	residual specific binding of $[^3H]PGE_2$ (% of control)				
	1/4 h	1 h	3 h	6 h	12 h
II	80.4 ± 2.4*[b]	68.4 ± 1.2**	55.9 ± 5.7*	50.1 ± 7.3*	51.5 ± 6.1*
III	51.5 ± 7.6**	76.6 ± 4.3**	91.7 ± 0.7*	88.7 ± 4.4	96.7 ± 3.3
IV	70.9 ± 4.9**	67.7 ± 6.0**	80.5 ± 4.7*	97.9 ± 2.3	106 ± 3.3

[a] The control transfectants preincubated for 12 h at 37 °C without PGE_2 had specific $[^3H]PGE_2$ binding of 28.1 ± 0.7 fmol/10^5 cells, 3.42 ± 1.0 fmol/10^5 cells, and 11.3 ± 0.7 fmol/10^5 cells for isoforms II, III, and IV, respectively, that were taken as 100%. Values shown are the mean ± SE of triplicate determinations. [b] *, $p < 0.05$; **, $p < 0.025$ by Student paired t-test when compared to the respective controls.

expressing any of the isoforms beyond the background changes observed in untransfected CHO cells and CHO cells transfected with vector alone (data not shown).

Isoform I of the EP3 receptor had been shown to transduce a modest elevation in $[Ca^{2+}]i$ in COS-7 cells in response to PGE_2 (Yang et al., 1994). In CHO cells permanently expressing isoform II, III, and IV, but not in the control CHO cells, an increase in $[Ca^{2+}]i$ was consistently observed after addition of 10−1000 nM M&B 28767. The response of $[Ca^{2+}]i$ was greatest for isoform II and less prominent for isoforms III and IV (Figure 5). PT pretreatment did not abolish the M&B 28767-evoked increases in $[Ca^{2+}]i$. Similar results were obtained in at least one other line of CHO cells transfected with each of the novel isoforms. Intracellular stores of calcium appear to be the principal source of the increase in $[Ca^{2+}]i$, as removal of extracellular calcium failed to reduce the increase in $[Ca^{2+}]i$ elicited by M&B 28767 in any of the transfectants. That 100 nM M&B 28767 rapidly stimulated transient increases in the level of inositol 1,4,5-trisphosphate as measured by a radioreceptor assay (NEN-Dupont, data not shown) supported this assumption.

These results suggest that each isoform of the human EP3 receptor identified is capable of coupling to at least two different signal transduction pathways, when expressed in CHO cells. The PT-sensitive G_i protein probably is responsible for mediating the inhibitory effect of these receptors on adenylyl cyclase. However, elevation of $[Ca^{2+}]i$ seems to be mediated, at least partially, via a PT-insensitive G protein. None of the isoforms mediated an increase in [cAMP]i, in contrast to the results of studies of mouse and bovine EP3 receptors.

To delineate functional differences among the human EP3 receptor isoforms, we examined their responses to agonist-induced desensitization. In one pilot experiment, a 30-min preincubation of CHO cells expressing each isoform with 1 μM PGE_2 resulted in similar decreases of 30−40% in $[^3H]$-PGE_2 binding and similar reduction in the elevation of $[Ca^{2+}]i$ evoked by 100 nM M&B 28767 (data not shown). In contrast, each isoform showed a distinctive time course of desensitization over a longer period of time (Table 2). Maximal loss of specific $[^3H]PGE_2$ binding reached 50% by 3 h for isoform II, which persisted up to 12 h, whereas maximal desensitization was achieved more rapidly for isoforms III and IV, which reverted to control level by 3 h.

DISCUSSION

The diversity of tissue expression and biological functions of the product of a single mammalian gene may be enhanced greatly by alternative RNA splicing that generates structurally distinct protein isoforms differing in regulation of expression and activities. Distinct isoforms encoded by alternatively spliced mRNA have been identified for several G protein-coupled receptors, including subsets of those that bind dopamine (Giros et al., 1989; Monsma et al., 1989), glutamate (Tanabe et al., 1992), pituitary adenylyl cyclase-activating polypeptide (Spengler et al., 1993), and monocyte chemoattractant peptide 1 (Charo et al., 1994). Multiple isoforms of the EP3 subtype of PGE_2 receptor with different carboxyl-terminal tails have been detected in several species (Namba et al., 1993; Irie et al., 1993; Adam et al., 1994; Breyer et al., 1994; Regan et al., 1994). We identified here five alternatively spliced variants of human EP3 receptor that differ only in the distal sequences of their carboxyl-terminal tails (Figure 1). All EP3 receptor isoforms are expressed in the human kidney and uterus (Figure 3), as well as in skin mast cells (data not shown), with different abundancies. Whether certain isoforms are expressed in a cell-specific manner within the same tissue or under different physiological conditions remains to be determined.

Some G protein-coupled receptors can activate multiple second messenger systems by coupling to two or more G proteins (Milligan, 1993). The capacity of the receptors to interact with a set of G proteins has been demonstrated by using several experimental approaches, such as reconstitution of purified receptors and G proteins in lipid vesicles (Asano et al., 1984; Florio & Steinweis, 1985; Cerione et al., 1986; Ashkenazi et al., 1987), overexpression of receptors in heterologous cells (Cotecchia et al., 1990; Chabre et al., 1992; Hung et al., 1992; Eason et al., 1992), use of precipitating or neutralizing antibodies against G proteins (Okuma & Reisine, 1992; Aragay et al., 1992; Law et al., 1993), and introduction of antisense RNA against G proteins (Goetzl et al., 1994). Previous studies demonstrated that EP3 receptors couple to inhibition of adenylyl cyclase via G_i protein to exert characteristic physiological actions (Coleman et al., 1990; Breyer et al., 1992). Recently, certain isoforms of bovine and mouse EP3 receptors have been shown to activate multiple second messenger pathways by coupling to different G proteins when overexpressed in CHO cells (Namba et al., 1993; Irie et al., 1993). We now demonstrate that isoforms of the human EP3 receptor transduce both suppression of [cAMP]i and elevation of $[Ca^{2+}]i$ (Figures 4 and 5). These two pathways appear to be mediated by different G proteins, as PT completely blocked one pathway but not the other (Figures 4 and 5). The same conclusion has been reached for bovine EP3D receptor (Namba et al., 1993) and mouse EP3γ (Irie et al., 1993). However, our findings for human EP3 receptors contrast with those of bovine and mouse EP3 receptors in one major respect. Human isoform II did not transduce increase in [cAMP]i beyond the background increase observed in control CHO cells, indicating that it does not couple to G_s protein productively, as for its mouse and bovine counterparts in response to greater than 100 nM M&B 28767 (Namba et

al., 1993; Irie et al., 1993). This background increase in [cAMP]i is likely due to activation of endogenous G_s-coupled EP2 receptor in the untransfected CHO cells by high concentration of M&B 28767.

Although more stringent receptor−G protein interaction may occur *in vivo*, results of *in vitro* biochemical studies using cell lines and natural tissues suggested that EP3 receptors couple to signal transduction pathways other than G_i-mediated inhibition of adenylyl cyclase, which is consistent with their diverse physiological functions. It has been shown that EP3 receptors stimulate phosphoinositide metabolism and increase $[Ca^{2+}]i$ in mast cells (Nishigaki et al., 1993). PGE_2 receptors in bovine adrenal medulla, which appeared to be of the EP3 subtype, are negatively coupled to adenylyl cyclase via G_i and to phosphoinositide metabolism via a PT-insensitive G protein (Negishi et al., 1989). In the uterus, EP3 receptors evoke contraction of smooth muscle, which is presumed to be attributable to an elevation of $[Ca^{2+}]i$ (Goureau et al., 1992; Senior et al., 1993). Diverse isoforms of human EP3 receptors with multifunctional signaling potentials may provide the molecular basis for transduction of a broad range of physiological responses to PGE_2 in these tissues.

The carboxyl-terminal tails of the G protein-coupled receptors may serve as targets of protein serine/threonine kinases that have been implicated in the agonist-induced desensitization process (Dohlman et al., 1991). In this regard, it is interesting that human EP3 isoforms I/I′ and II have multiple serine/threonine residues in their carboxyl-termini, whereas isoforms III and IV have none. The time courses of desensitization of specific binding of $[^3H]PGE_2$ by preincubation with 1 μM PGE_2 for $^1/_4$−12 h showed distinctive features for each of the CHO cell lines expressing different EP3 receptor isoforms (Table 2). That isoform II desensitized slowly and persistently, whereas isoforms III and IV desensitized more rapidly and transiently, may reflect differences in phosphorylation of their cytoplasmic tails. Previous studies also have demonstrated substantial differences in PGE_2-induced desensitization between the mouse EP3α and EP3β isoforms (Negishi et al., 1993).

In summary, we have identified five alternatively spliced variants of the human EP3 receptor that differ only in their distal carboxyl-terminal sequences. When stably expressed in CHO cells, these isoforms of EP3 receptor are capable of coupling both to suppression of [cAMP]i and elevation of $[Ca^{2+}]i$, which are presumed to be mediated by different G proteins. The distinctive patterns of tissue distribution and biochemical responses transduced by the EP3 receptor isoforms may provide part of the molecular basis for the diverse functional roles of PGE_2 *in vivo*.

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