プロスタグランジンE受容体の構造と機能に関する研究

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杉本幸彦

A Study for Structure and Function of the Prostaglandin E Receptor

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INTRODUCTION

Prostanoids comprising various oxygenated metabolites of arachidonic acid such as prostaglandins (PGs) and thromboxane (TX) exert a variety of biological activities for maintenance of local homeostasis in the body (Samuelsson et al., 1978; Moncada et al., 1985). Characterization of the prostanoid receptors has been carried out pharmacologically by comparing the actions and potencies of various types of PGs in different bioassay systems and then by using PG analogues more specific to each action (Halushka et al., 1989). Through these studies it is suggested that each prostanoid has a specific receptor (Fig. 0-1). Among these prostanoids, PGE_2 in particular produces a broad range of biological actions in diverse tissues. These actions of PGE_2 include the contraction or relaxation of vascular and nonvascular muscles, and stimulation or suppression of secretion of the neurotransmitters and hormones (Fig. 0-2).

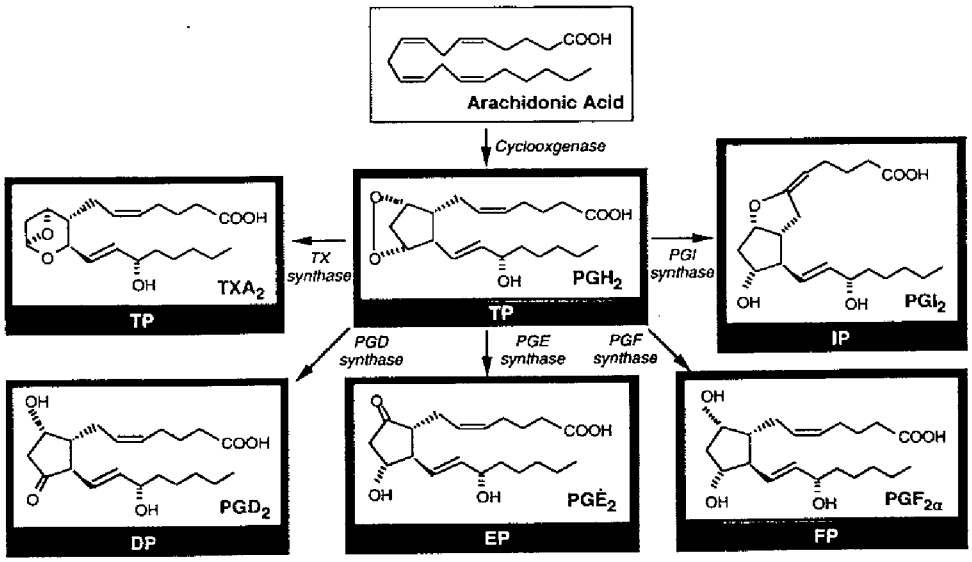


Fig. 0-1. Structures of the prostanoids. Prostanoids are synthesized from arachidonic acid by cyclooxygenase. Prostanoids exert their biological activities through specific cell-surface receptors.

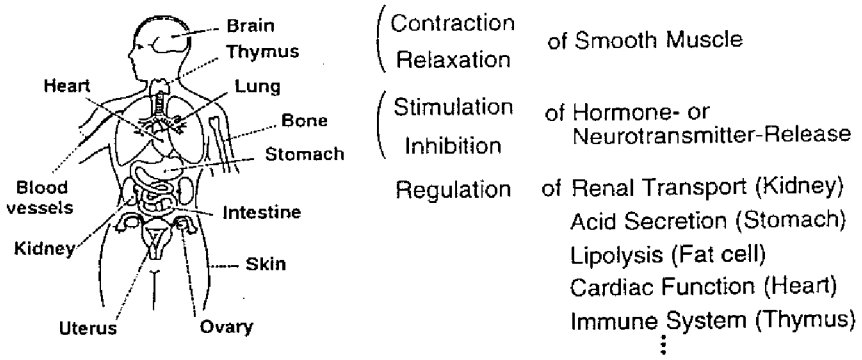


Fig. 0-2. Actions of prostagrandin (PG) E_2. PGE_2 has a wide spectrum of physiological and pharmacological actions in diverse tissues.

PGE receptors are pharmacologically subdivided into three subtypes, EP_1, EP_2 and EP_3 (Coleman et al., 1987; Coleman et al., 1990), and these subtypes are suggested to be different in their signal transduction; they are presumed coupled to stimulation of phospholipase C, stimulation and inhibition of adenylate cyclase, respectively (reviewed by Coleman et al. 1990). Some of pharmacological actions of these subtypes have been characterized (Fig. 0-3). However, contribution of these subtypes to each action of PGE_2 has not yet been established, none of the receptors has been isolated, and their molecular characterization has been carried out only poorly.

Subtypes of Prostaglandin E_2 Receptor

EP_1	IP_3/DG	*Gastrointestinal Smooth Muscle*; Contraction
		Central Neurons; Hyperalgesia
EP_2	cAMP↑	*Trachea*; Relaxation
		Vascular Smooth Muscle; Relaxation
		Chief Cells; Gastric Nonparietal Secretion
		Hypothalamus; Pyrexia
EP_3	cAMP↓	*Adipocytes*; Inhibition of Lipolysis
		Kidney; Inhibition of Sodium and Water Reabsorption
		Parietal Cells; Inhibition of Gastric acid Secretion
		Uterus; Contraction
		Central and Peripheral Neurons; Inhibition of Neurotransmitter Release

Fig. 0-3. Subtypes of PGE receptor. These subtypes are supposed to differ in their signal transduction pathways. Well-characterized biological actions of each subtype are shown.

The author isolated cDNAs for three subtypes of the mouse PGE receptor, and using these cDNAs, characterized their structural and functional natures (Sugimoto et al. 1992; Honda et al. 1993; Watabe et al. 1993). The author also found their functional cellular distributions by investigating them in the renal nephron as a model system (Sugimoto et al. 1994). Furthermore, the author found the existence of two molecular forms of EP_3 different functionally (Sugimoto et al. 1993). Obtained information in the present study on PGE receptor is surely to facilitate not only understanding of the physiological functions of PGE_2, but also development of more subtype-specific PGE analogues for therapeutic purposes.

The abbreviations used in the text are as follows;

AppNHp, adenyl-5'-yl β,γ-imidodiphosphate

bp, base pair(s)

C-, carboxyl-

cAMP, cyclic 3',5'-adenosine monophosphate

cDNA, complementary DNA

CHO, Chinese hamster ovary

CTP, citidine triphosphate

dCTP, deoxy citidine triphosphate

DHFR, dihydrofolate reductase

DTT, dithiothreitol

EC_{50}, drug concentration to exert 50% of maximal response

EDTA, ethylene diamine tetraacetic acid

G protein, heterotrimeric GTP-binding protein

GTP, guanine triphosphate

GTPase, guanine triphosphate hydrase

GTPγS, guanosine 5'-*O*-(3-thiotriphosphate)

IC_{50}, drug concentration to inhibit response by 50%

MES, 4-morpholineethanesulfic acid

mRNA, messenger RNA

N-, amino-

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PG, prostaglandin

PT, pertussis toxin

SDS, sodium dodecyl sulfate

SSC, standard saline citrate

TM, transmembrane segment

TX, thromboxane

$[Ca^{2+}]i$, intracellular Ca^{2+} concentration

The names of isolated cDNA clones used in the text are as follows;

HPL, the human thromboxane A_2 receptor

MK643, the mouse PGE receptor subtype EP_1

MP412, the mouse PGE receptor subtype EP_2

MP660, the mouse PGE receptor subtype EP_3 ($EP_{3\alpha}$ isoform)

MP653, the mouse PGE receptor subtype EP_3 ($EP_{3\beta}$ isoform)

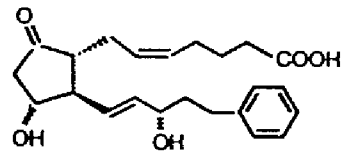
LT3, the mouse thromboxane A_2 receptor (a partial cDNA)

ML64, the mouse PGE receptor subtype EP_3 (a partial cDNA)

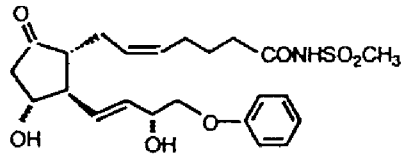
ML42, the mouse PGE receptor subtype EP_1 (a partial cDNA)

The subtype-selective PGE analogues used in the present study are shown in Fig. 0-4.

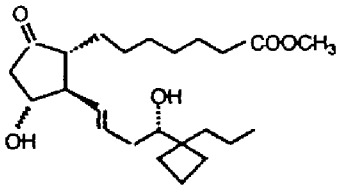
Agonists



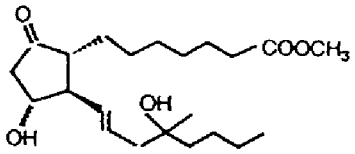
17-phenyl-ω-trinor PGE_2 (EP_1)



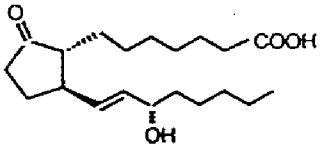
Sulprostone (EP_1, EP_3)



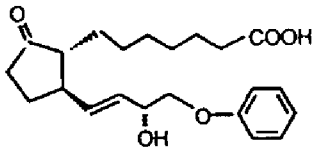
butaprost (EP_2)



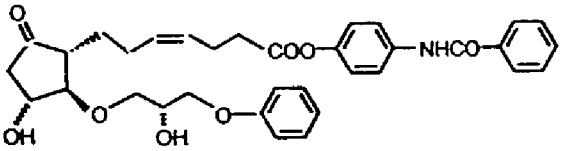
misoprostol (EP_2, EP_3)



11-deoxy-PGE_1 (EP_2, EP_3)

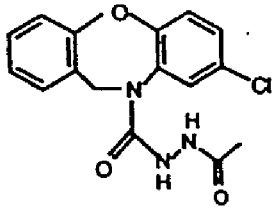


M&B28767 (EP_3)

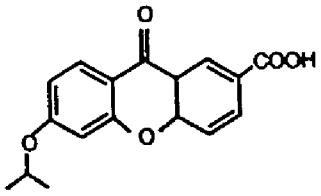


GR63799X (EP_3)

Antagonists



SC-19220 (EP_1)



AH 6809 (EP_1)

Fig. 0-4. Structures of subtype-selective PGE ligands used in the present study.

1. Structure and function of three subtypes of the prostaglandin E receptor

SUMMARY

Functional cDNA clones for three subtypes of mouse PGE receptor were isolated from a mouse cDNA library using polymerase chain reaction based on the sequence of the human TXA_2 receptor and cross-hybridization screening. These receptor together with TX receptor belong to a new family of G protein-coupled receptors.

The mouse EP_3 receptor consists of 365 amino acid residues with an estimated molecular weight of 40,077. [^{3}H]PGE_2 specifically bound to the membrane of mammalian COS cells transfected with the EP_3 cDNA. The binding of EP_3 was displaced with unlabeled PGs in the order of PGE_2 = PGE_1 > iloprost (a prostacyclin agonist) > PGD_2 > $PGF_{2\alpha}$. The EP_3-selective agonists, M&B28767 or GR63799X, potently competed for the [^{3}H]PGE_2 binding, but no competition was found with EP_1- or EP_2-selective ligands. PGE_2 and M&B28767 decreased forskolin-induced cAMP formation in a concentration-dependent manner in Chinese hamster ovary (CHO) cells permanently expressing EP_3. Northern blot analysis demonstrated that the EP_3 mRNA is expressed abundantly in kidney, uterus, and mastocytoma P-815 cells and in a lesser amount in brain, thymus, lung, heart, stomach and spleen.

The mouse EP_2 receptor consists of 513 amino acid residues with an estimated molecular weight of 56,157. [^{3}H]PGE_2 specifically bound to the membrane of COS cells transfected with the EP_2 cDNA. The binding to the membrane was displaced with unlabeled PG in the order of PGE_2 = PGE_1 >> iloprost (a prostacyclin agonist) $\geqq$ $PGF_{2\alpha} \geqq PGD_2$. The binding was also inhibited by misoprostol (an EP_2 and EP_3 agonist), but not by sulprostone (an EP_1 and EP_3 agonist), and SC-19220 (an EP_1 antagonist). PGE_2 markedly increased cAMP level in COS cells transfected with the EP_2

cDNA. The EP_2 mRNA is widely expressed in various tissues, the abundant expression being observed in ileum, thymus and mastocytoma P-815 cells.

The mouse EP_1 receptor consists of 405 amino acid residues with an estimated molecular weight of 42,966. [^{3}H]PGE_2 specifically bound to the membrane of CHO cells stably expressing EP_1. The binding to the membrane was displaced with unlabeled PGs in the order of PGE_2 > iloprost (a prostacyclin analogue) > PGE_1 > $PGF_{2\alpha}$ > U-46619 (a thromboxane A_2 analogue) > PGD_2. The binding was also inhibited by 17-phenyl trinor PGE_2 (an EP_1 agonist), and sulprostone (an EP_1 and EP_3 agonist), but not by 11-deoxy PGE_1 (an EP_2 and EP_3 agonist), and butaprost (an EP_2 agonist). PGE_2 induced a rapid increase in intracellular Ca^{2+} concentration in CHO cells expressing the receptor. The mRNA for EP_1 receptor is expressed abundantly in kidney and in a lessor amount in lung.

RESULTS

1. cDNA cloning strategy for the PGE receptor

It has been reported that the prostanoid receptor in general has cross-reactivity with the other prostanoids in addition to its own prostanoid ligand. For instance, the PGE receptor in various cells and tissues revealed significant binding to iloprost, a prostacyclin agonist, to $PGF_{2\alpha}$, and to PGD_2 in addition to PGE_2 or PGE_1. Recently, Hirata et al. identified the structure of the human TXA_2 receptor (1991), and this receptor is one of the G protein-coupled rhodopsin-type receptors but only limited homology was seen with other members of this type of receptors. From these results, the author supposed that the various prostanoid receptors including the TX receptor construct a novel receptor subfamily of the rhodopsin-type superfamily with homologous structures. In order to isolate cDNA clones encoding these receptors from mouse tissues, the author performed a series of cloning steps as follows; (1) preparation of probe cDNA fragment useful for cross-hybridization (2) isolation of partial cDNA clones by cross-hybridization with the probe (3) searching for an appropriate cell or tissue for cDNA library by Northern hybridization (4) construction of the appropriate cDNA library (5) isolation of a functional cDNA clone from the library. The cloning methods for the mouse PGE receptor is summarized in Fig. 1-1. Detailed conditions of each cloning were described in "*Experimental Procedures*".

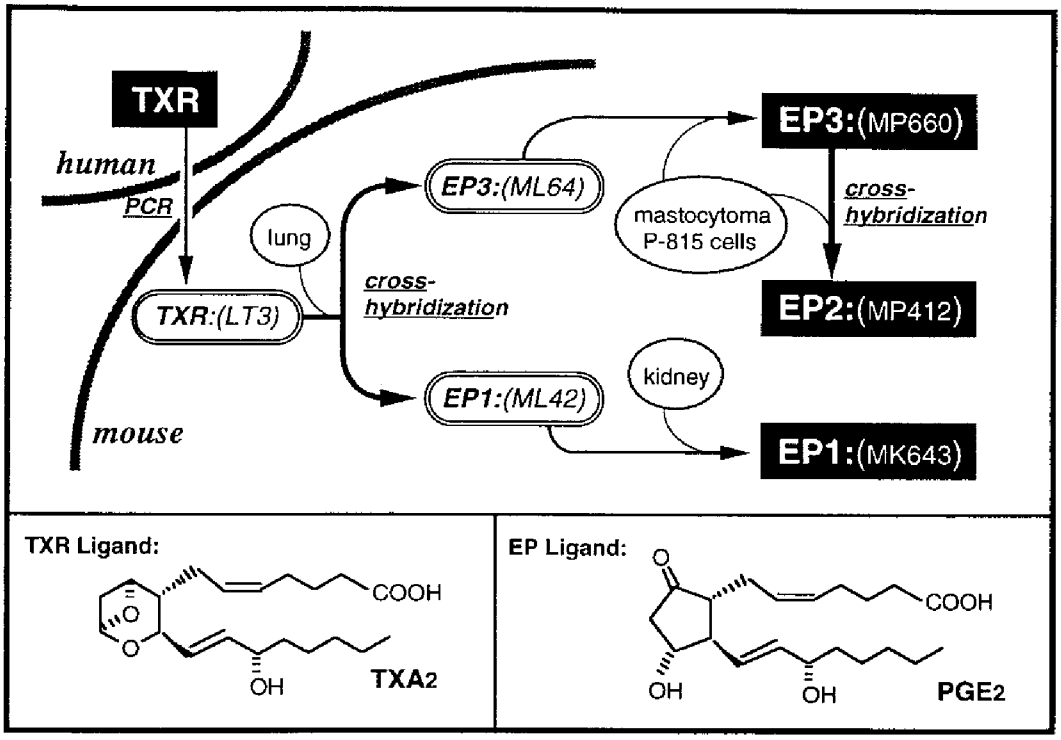


Fig. 1-1. Cloning strategy for the mouse PGE receptor cDNAs. LT3 (a partial cDNA fragment of the mouse TX receptor) was used as a probe in cross-hybridization for isolation of EP_3 and EP_1 cDNAs. A partial cDNA fragment of the mouse EP_3 cDNA was used in isolation of EP_2 cDNA. Detailed conditions for molecular cloning for three PGE receptors are described under *"Experimental Procedures"*.

2. Structures of the mouse PGE receptors.

Fig. 1-2a-c shows nucleotide and deduced amino acid sequences of MP660, a functional cDNA for the mouse EP_3 receptor, MP412 for the EP_2 receptor, and MK643 for the EP_1 receptor. The EP_3 polypeptide consists of 365 amino acid residues with an estimated molecular weight of 40,077. The EP_2 polypeptide consists of 513 amino acid residues with an estimated molecular weight of 56,157. The EP_1 polypeptide consists of 405 amino acid residues with an estimated molecular weight of 42,966. The hydropathicity profiles determined by Kyte and Doolittle method (1982) and the sequence homology analyses indicated that all these receptor proteins possess seven hydrophobic segments and share significant sequence similarity with other members of G protein-coupled receptors (O'Dowd et al. 1989a), especially with the human TX receptor (Hirata et al. 1991). Similar to other G protein-coupled receptors (Hubbard and Ivatt 1981; Lefkowitz and Caron 1988), the EP_1 receptor has two potential *N*-glycosylation sites at the extracellular amino- (N-) terminal region, and the EP_2 and EP_3 receptor have the sites at the N-terminal and the second extracellular loop regions. Every EP receptor has multiple serine and threonine residues at the cytoplasmic loops or the carboxyl- (C-) terminal tail as possible phosphorylation sites. Among three receptors, the EP_2 receptor possesses a relatively longer third intracellular loop (59 amino acid residues) than the EP_3 receptor (19 residues). This receptor also possesses a long C-terminal tail (153 amino acids), as do the β_2-adrenergic (Dixon et al. 1986) and dopamine-D_1 (Dearry et al. 1990) receptors.

a

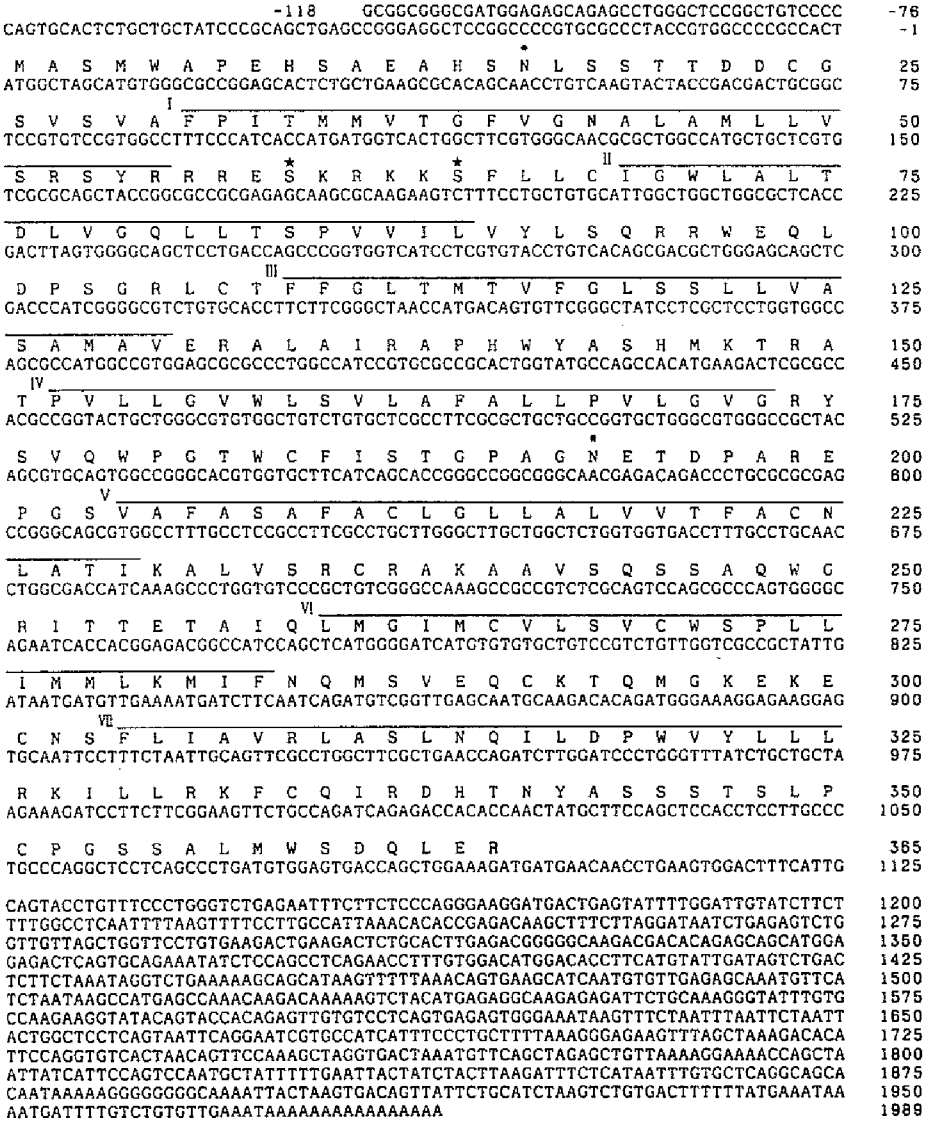
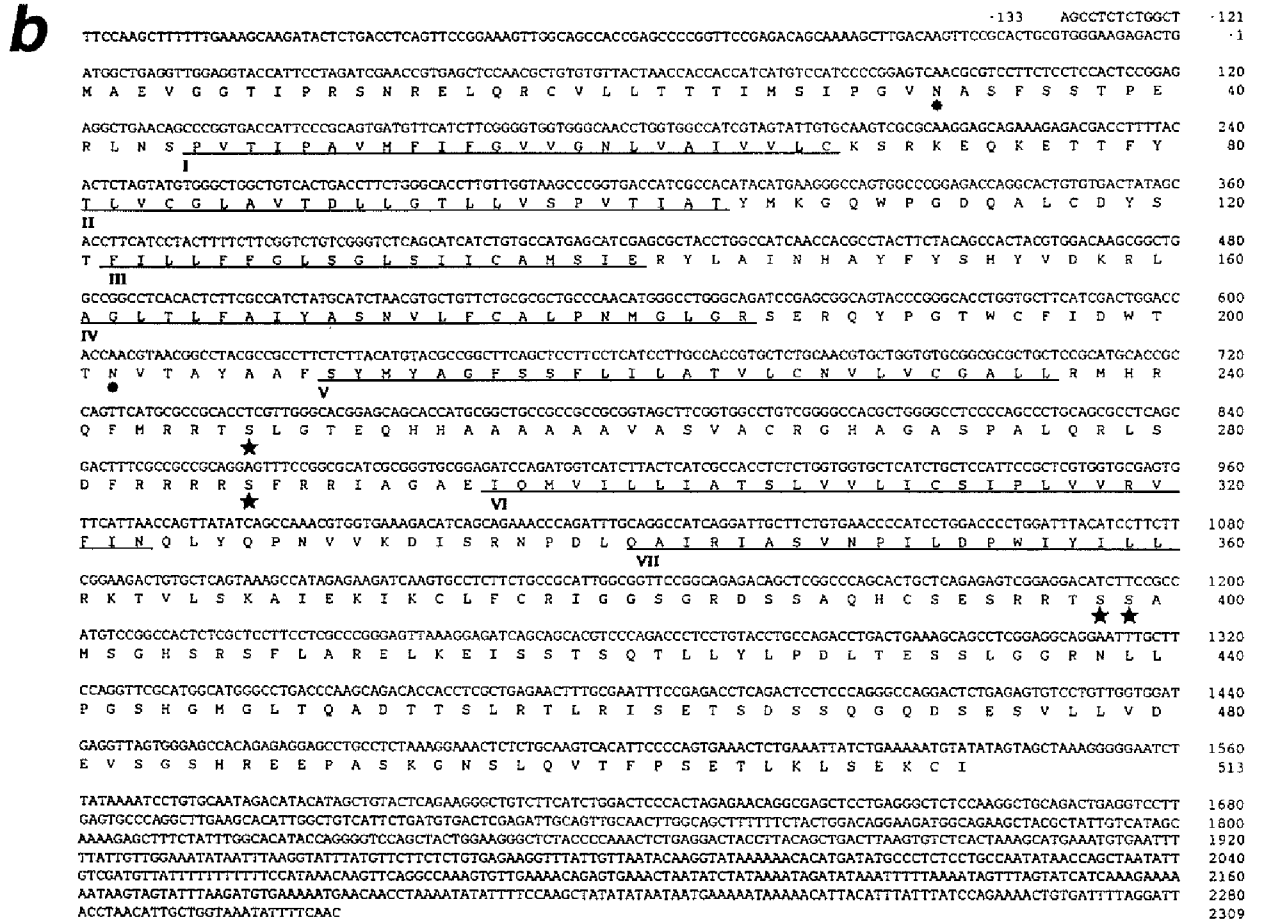


Fig. 1-2. Nucleotide and deduced amino acid sequences of EP_3- (a), EP_2- (b) and EP_1-cDNA (c). The deduced amino acid sequence is shown above or below the nucleotide sequence using single letter code. Positions of the putative transmembrane segments I - VII are indicated by overlines or underlines. The termini of each segment are tentatively assigned on the basis of a hydropathicity profile and comparison with other G protein-coupled receptors. a and b, *Asterisks*, potential N-glycosylation sites in the extracellular regions. *Stars*, potential phosphorylation sites by cAMP-dependent protein kinase. c, *Crosses*, potential *N*-glycosylation sites in the extracellular regions; *Asterisks and parallel crosses*, potential phosphorylation sites by protein kinase A and protein kinase C, respectively.


b
-133 AGCCTCTCTGGCT -121
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ATGGCTGAGGTTGGAGGTACCATTCCTAGATCGAACCGTGAGCTCCAACGCTGTGTGTTACTAACCACCACCATCATGTCCATCCCCGGAGTCAACGCGTCCTTCTCCTCCACTCCGGAG 120
M A E V G G T I P R S N R E L Q R C V L L T T T I M S I P G V N A S F S S T P E 40
AGGCTGAACAGCCCGGTGACCATTCCCGCAGTGATGTTCATCTTCGGGGTGGTGGGCAACCTGGTGGCCATCGTAGTATTGTGCAAGTCGCGCAAGGAGCAGAAAGAGACGACCTTTTAC 240
R L N S P V T I P A V M F I F G V V G N L V A I V V L C K S R K E Q K E T T F Y 80
I
ACTCTAGTATGTGGGCTGGCTGTCACTGACCTTCTGGGCACCTTGTTGGTAAGCCCGGTGACCATCGCCACATACATGAAGGGCCAGTGGCCCGGAGACCAGGCACTGTGTGACTATAGC 360
T L V C G L A V T D L L G T L L V S P V T I A T Y M K G Q W P G D Q A L C D Y S 120
II
ACCTTCATCCTACTTTTCTTCGGTCTGTCGGGTCTCAGCATCATCTGTGCCATGAGCATCGAGCGCTACCTGGCCATCAACCACGCCTACTTCTACAGCCACTACGTGGACAAGCGGCTG 480
T F I L L F F G L S G L S I I C A M S I E R Y L A I N H A Y F Y S H Y V D K R L 160
III
GCCGGCCTCACACTCTTCGCCATCTATGCATCTAACGTGCTGTTCTGCGCGCTGCCCAACATGGGCCTGGGCAGATCCGAGCGGCAGTACCCGGGCACCTGGTGCTTCATCGACTGGACC 600
A G L T L F A I Y A S N V L F C A L P N M G L G R S E R Q Y P G T W C F I D W T 200
IV
ACCAACGTAACGGCCTACGCCGCCTTCTCTTACATGTACGCCGGCTTCAGCTCCTTCCTCATCCTTGCCACCGTGCTCTGCAACGTGCTGGTGTGCGGCGCGCTGCTCCGCATGCACCGC 720
T N V T A Y A A F S Y M Y A G F S S F L I L A T V L C N V L V C G A L L R M H R 240
V
CAGTTCATGCGCCGCACCTCGTTGGGCACGGAGCAGCACCATGCGGCTGCCGCCGCCGCGGTAGCTTCGGTGGCCTGTCGGGGCCACGCTGGGGCCTCCCCAGCCCTGCAGCGCCTCAGC 840
Q F M R R T S L G T E Q H H A A A A A A V A S V A C R G H A G A S P A L Q R L S 280
GACTTTCGCCGCCGCAGGAGTTTCCGGCGCATCGCGGGTGCGGAGATCCAGATGGTCATCTTACTCATCGCCACCTCTCTGGTGGTGCTCATCTGCTCCATTCCGCTCGTGGTGCGAGTG 960
D F R R R R S F R R I A G A E I Q M V I L L I A T S L V V L I C S I P L V V R V 320
VI
TTCATTAACCAGTTATATCAGCCAAACGTGGTGAAAGACATCAGCAGAAACCCAGATTTGCAGGCCATCAGGATTGCTTCTGTGAACCCCATCCTGGACCCCTGGATTTACATCCTTCTT 1080
F I N Q L Y Q P N V V K D I S R N P D L Q A I R I A S V N P I L D P W I Y I L L 360
VII
CGGAAGACTGTGCTCAGTAAAGCCATAGAGAAGATCAAGTGCCTCTTCTGCCGCATTGGCGGTTCCGGCAGAGACAGCTCGGCCCAGCACTGCTCAGAGAGTCGGAGGACATCTTCCGCC 1200
R K T V L S K A I E K I K C L F C R I G G S G R D S S A Q H C S E S R R T S S A 400
ATGTCCGGCCACTCTCGCTCCTTCCTCGCCCGGGAGTTAAAGGAGATCAGCAGCACGTCCCAGACCCTCCTGTACCTGCCAGACCTGACTGAAAGCAGCCTCGGAGGCAGGAATTTGCTT 1320
M S G H S R S F L A R E L K E I S S T S Q T L L Y L P D L T E S S L G G R N L L 440
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P G S H G M G L T Q A D T T S L R T L R I S E T S D S S Q G Q D S E S V L L V D 480
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E V S G S H R E E P A S K G N S L Q V T F P S E T L K L S E K C I 513
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ACCTAACATTGCTGGTAAATATTTTCAAC 2309
c
-71 GCAGCAGGAGCCAAGTTCCAGGCCATGGGGACCCTGCATCCTGAGCAGCACTGGCCCTCTTGGCCACTGAT -1
ATGAGCCCCTGCGGGCTTAACCTGAGCCTAGCGGATGAGGCAGCAACGTGCGCAACACCCAGGCTCCCCAATACATCTGTGGTGCTGCCAACAGGCGATAATGGCACATCACCAGCGCTG 120
M S P C G L N L S L A D E A A T C A T P R L P N T S V V L P T G D N G T S P A L 40
I
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P I F S H T L G A V S N V L A L A L L A Q V A G R M R R R R S A A T F L L F V A 80
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S L L A I D L A G H V I P G A L V L R L Y T A G R A P A G G A C H F L G G C M V 120
II III
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F F G L C P L L L G C G M A V E R C V G V T Q P L I H A A R V S V A R A R L A L 160
IV
GCCGTGCTGGCTGCCATGGCTTTGGCAGTGGCGCTGCTGCCACTGGTACACGTGGGTCGCTACGAGTTACAGTACCCTGGCACCTGGTGTTTTATTAGCCTTGGGCCTCGTGGAGGTTGG 600
A V L A A M A L A V A L L P L V H V G R Y E L Q Y P G T W C F I S L G P R G G W 200
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R Q A L L A G L F A G L G L A A L L A A L V C N T L S G L A L L R A R W R R R R R 240
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S R R F R K T A G P D D R R R W G S R G P R L A S A S S A S S I T S A T A T L R 280
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S S R G G G S A R R V H A H D V E M V G Q L V G I M V V S C I C W S P L L V L V 320
VI
GTGTTGGCCATCGGGGGCTGGAACTCTAACTCCCTGCAGCGGCCACTCTTTCTGGCTGTACGCCTCGCATCGTGGAACCAGATCCTGGACCCATGGGTGTACATCCTGCTGCGCCAGGCC 1080
V L A I G G W N S N S L Q R P L F L A V R L A S W N Q I L D P W V Y I L L R Q A 360
VII
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GGTTTCAGTCACCTCTGAATGTGCCCAGAAGCTAAGTCAAA 1241
G F S H L 405

3. Binding characters of the three PGE receptors.

The cloned mouse EP_3 receptor was expressed in COS-1 cells and its membranes were subjected to binding assays using $[^3H]PGE_2$. Scatchard analysis of this binding yielded a dissociation constant (Kd) of 2.9 nM. The average density of binding sites in three experiments was 770 fmol/mg of protein of the transfected COS cell membranes. Specificity of this binding is shown in Fig. 1-3a. The binding of $[^3H]PGE_2$ was inhibited by unlabeled PGs in the order of $PGE_2 = PGE_1 >$ iloprost, a PGI_2 analogue > $PGF_{2\alpha} > PGD_2$. We further characterized the specificity of this $[^3H]PGE_2$ binding using ligands specific for PGE receptor subtypes. As shown in Fig. 1-3b, among various PGE analogues, only EP_3-specific agonists, GR63799X and M&B28767, specifically competed for the $[^3H]PGE_2$ binding with equal potency, and they were more potent than PGE_2 itself. On the other hand, no competition was found at all with either SC-19220 (an EP_1-specific antagonist), or butaprost (an EP_2-specific agonist).

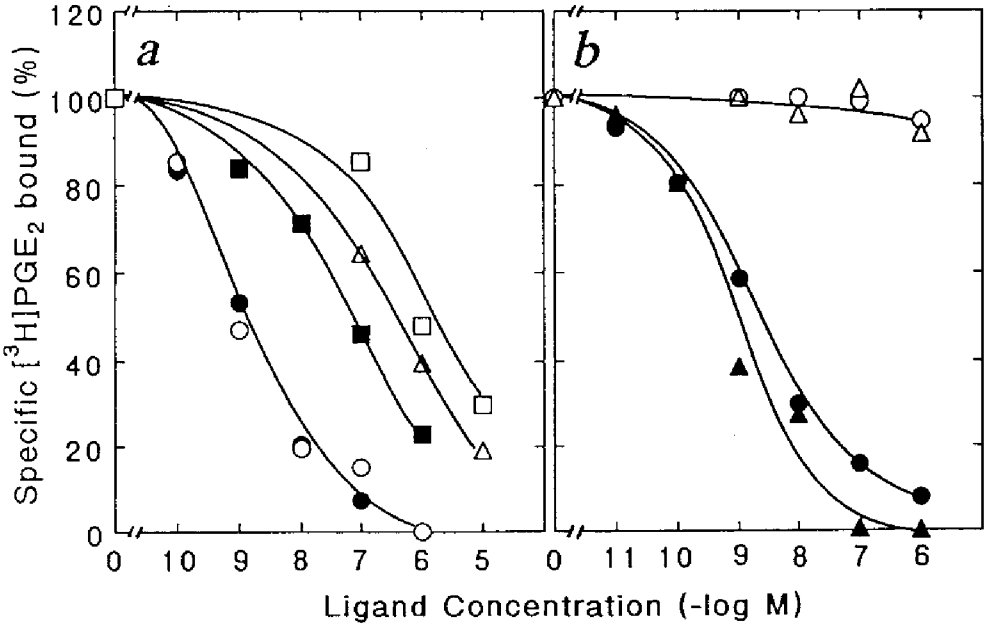

Fig. 1-3. Binding of $[^3H]PGE_2$ to EP_3 in the MP660-transfected COS-1 cell membranes. *a.* Displacement of $[^3H]PGE_2$ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations and $[^3H]PGE_2$ binding was determined. PGE_2 (○), PGE_1 (●), iloprost (■), $PGF_{2\alpha}$ (△) and PGD_2 (□). *b.* Displacement of $[^3H]PGE_2$ binding by subtype-selective PGE analogues. Ligands used are M&B28767 (▲), GR63799X (●), butaprost (△) and SC-19220 (○).

When the EP_2 receptor was expressed in COS-1 cells, $[^3H]PGE_2$ specifically bound to the membrane of the transfected cells. Scatchard analysis of this binding yielded a dissociation constant of 11.2 nM and the maximal binding of 946 fmol/mg. Specific $[^3H]PGE_2$ binding to the membrane of untransfected cells was almost negligible (data not shown). Fig. 1-4a shows the binding specificity of the EP_2 receptor. Specific $[^3H]PGE_2$ binding was inhibited by unlabeled PG in the order of $PGE_2 = PGE_1 >>$ iloprost, a stable PGI_2 analogue $\geqq PGF_{2\alpha} \geqq PGD_2$. Among these PGs, the EP_2 receptor shows higher specificity for PGE_1 and PGE_2 than EP_3 receptor. As shown in Fig. 1-4b, the binding of EP_2 receptor was inhibited by misoprostol (an EP_2 and EP_3 agonist) and more weakly by M&B28767 (an EP_3 agonist). On the other hand, sulprostone (an EP_1 and EP_3 agonist), SC-19220 (an EP_1 antagonist), and butaprost (an EP_2 agonist), did not inhibit it.

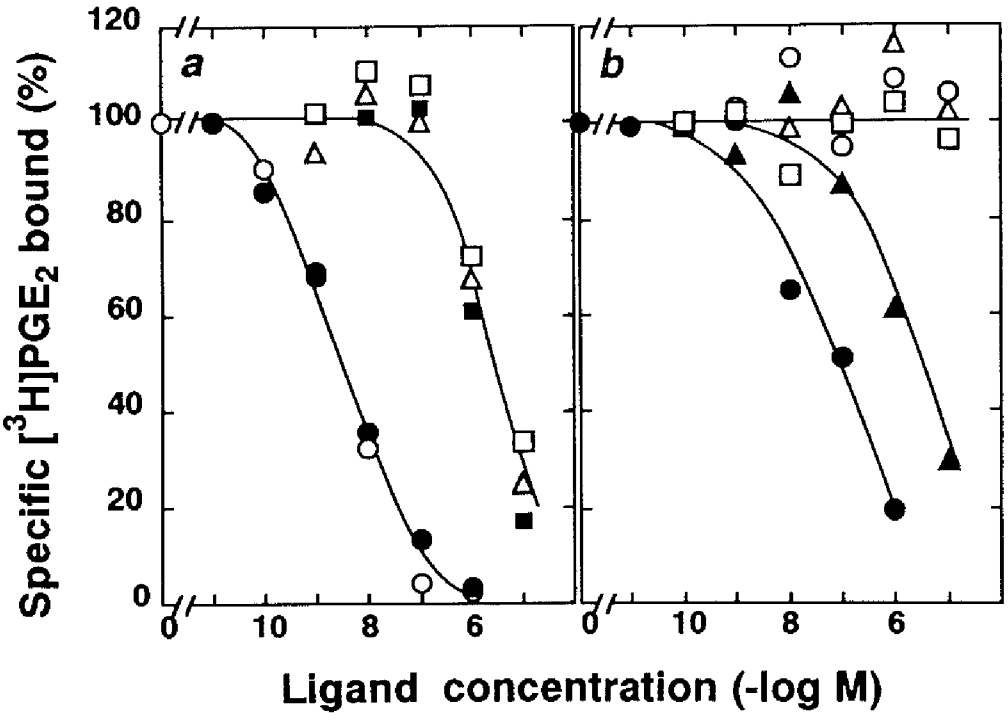

Fig. 1-4. Binding of $[^3H]PGE_2$ to EP_2 in the MP412-transfected COS-1 cell membrane. a, displacement of $[^3H]PGE_2$ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations, and specific $[^3H]PGE_2$ binding was determined. ○ , PGE_2; ● , PGE_1; ■ , iloprost; △ , $PGF_{2\alpha}$, and □ , PGD_2. b, displacement of $[^3H]PGE_2$ binding by agonist or antagonist for PGE receptor subtypes. ● , misoprostol; ▲ , M&B28767; □ , sulprostone; △ , butaprost and ○ , SC-19220.

A CHO cell clone showing stable expression of the EP_1 receptor, and the binding activities of this receptor was investigated by using $[^3H]PGE_2$. $[^3H]PGE_2$ specifically bound to the membrane of the transfected cells, while only negligible binding was found in the membrane of the mock-transfected cells. Scatchard analysis of this binding of EP_1 receptor yielded a dissociation constant of 21 nM. Fig. 1-5A shows the binding specificity of EP_1 receptor. Specific $[^3H]PGE_2$ binding was inhibited by unlabeled PGs in the order of PGE_2 > iloprost (a stable prostacyclin analogue) > PGE_1 > $PGF_{2\alpha}$ > U-46619 (a stable TXA_2 analogue) > PGD_2. In contrast to EP_2 and EP_3 receptors, this receptor shows higher affinity to PGE_2 than PGE_1. As shown in Fig. 1-5B, the $[^3H]PGE_2$ binding of EP1 receptor was inhibited by 17-phenyl-18,19,20-trinor PGE_2 (an EP_1 agonist), and by sulprostone (an EP_1 and EP_3 agonist), while the inhibition by M&B28767, by 11-deoxy PGE_1 (an EP_2 and EP_3 agonist), and by butaprost, was weak or negligible. On the other hand, AH6809 (an EP_1 antagonist) showed only weak inhibition of the binding.

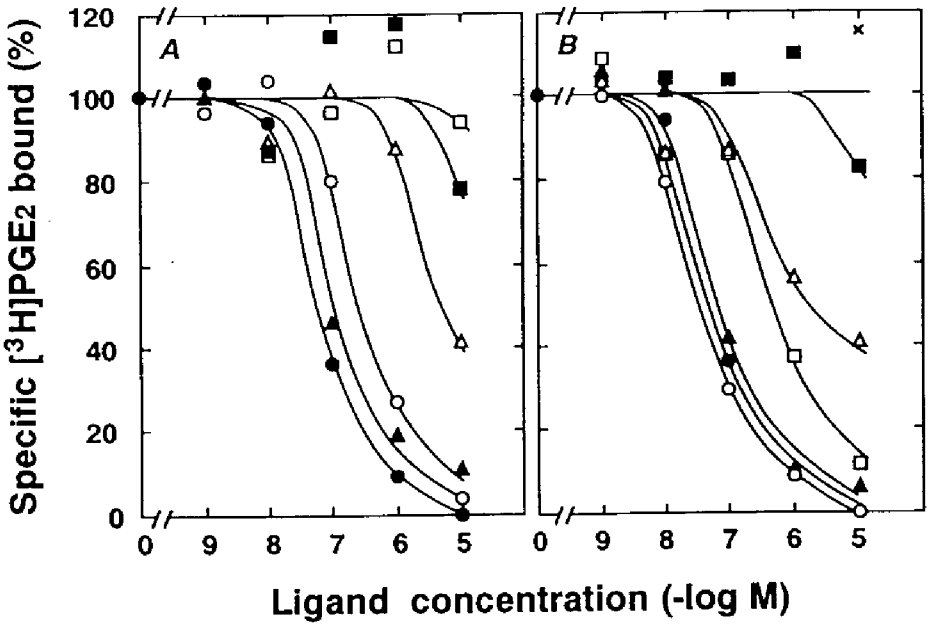

Fig. 1-5. Binding of $[^3H]PGE_2$ to EP_1 in the CHO cell membrane. A, displacement of $[^3H]PGE_2$ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations, and specific $[^3H]PGE_2$ binding was determined. ● PGE_2; ▲ iloprost; O PGE_1; Δ $PGF_{2\alpha}$; □ PGD_2; ■ U-46619. B, displacement of $[^3H]PGE_2$ binding by agonist or antagonist for PGE receptor subtypes. ● PGE_2; O 17-phenyl trinor PGE_2; ▲ sulprostone; □ M&B28767; Δ 11-deoxy PGE_1; x butaprost; ■ AH 6809.

4. Signal transduction pathways of the PGE receptors.

As shown in Fig. 1-6, the CHO cells stably expressing the EP_3 showed a dose-dependent decrease to PGE_2 in forskolin-induced cellular cAMP accumulation. M&B28767 (an EP_3-specific agonist) also inhibited forskolin-induced cAMP synthesis and was more potent than PGE_2 (IC_{50} of M&B28767= 1×10^{-12} M; IC_{50} of PGE_2= 1×10^{-10} M). Neither agonist alone did not increase cAMP accumulation. Phosphatidylinositol turnover was also examined but up to 1 μM of M&B28767 revealed no significant increase in inositol phosphates content over the basal levels (data not shown). These results demonstrated that the EP_3 receptor is coupled exclusively to inhibition of adenylate cyclase.

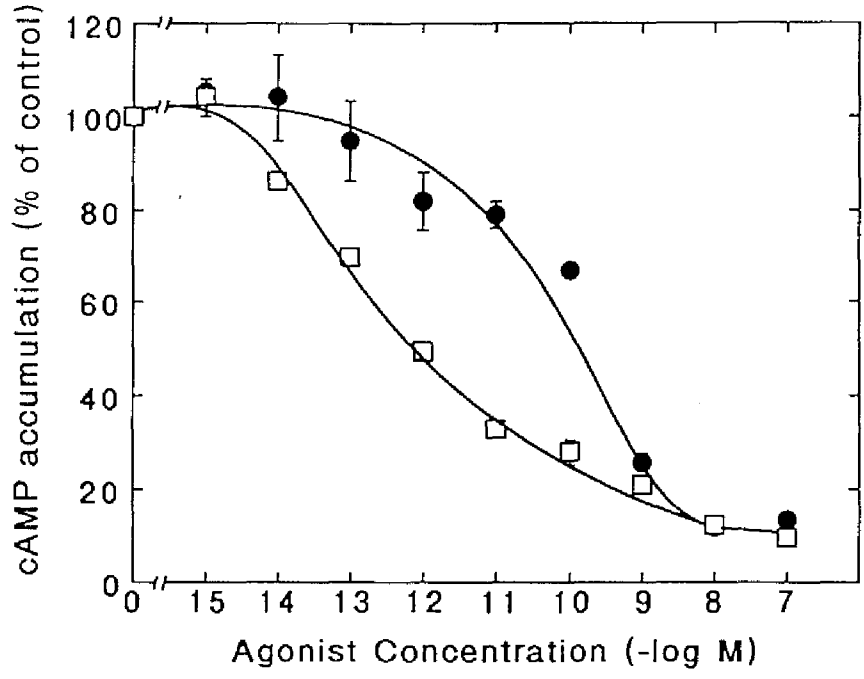

Fig. 1-6. Inhibition of forskolin-induced cAMP accumulation by PGE_2 and EP_3-selective agonist in CHO cells expressing the EP_3 receptor. CHO cells permanently expressing the EP_3 receptor were incubated with 1 μM forskolin in the presence of indicated concentrations of PGE_2 (●) or M&B28767 (□) , and cAMP accumulation was determined. The incubation buffer contained 1 mM 3-isobutyl-1-methylxanthine. Each point represents the mean±S.E. of triplicate determinations.

As shown in Fig. 1-7, PGE_2 dose-dependently increased cAMP level in EP_2 cDNA-transfected COS cells, the maximal level being 4.05 pmol/10^5 cells which is 2.8-fold higher than that accumulated by PGE_2 in untransfected cells. On the other hand, PGE_2 neither inhibited forskolin-induced cAMP formation and nor accumulated inositol phosphates (data not shown). These results demonstrate that EP_2 receptor is coupled to stimulation of adenylate cyclase.

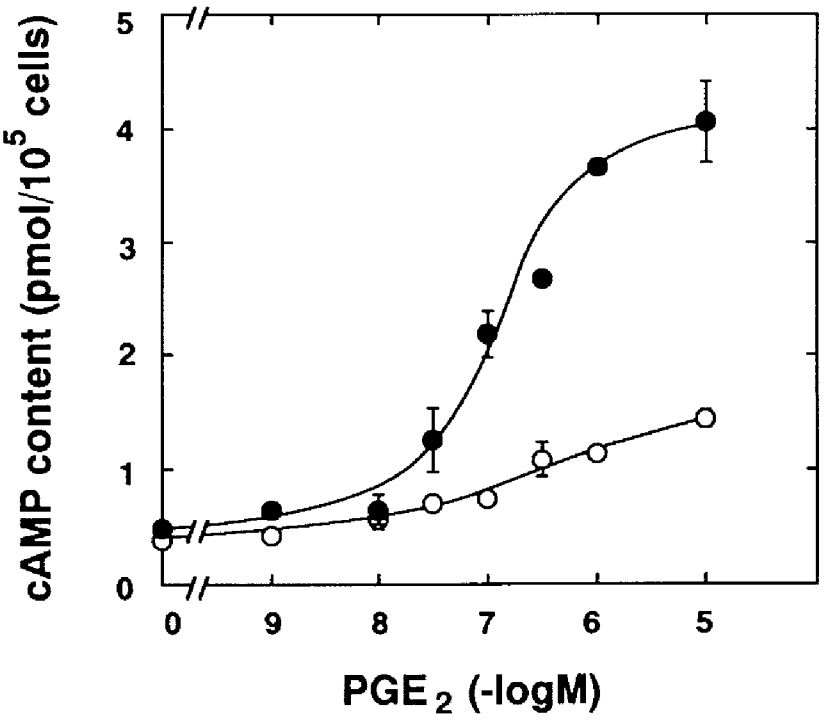

Fig. 1-7. Effect of PGE_2 on cAMP level in EP_2-expressing cells. MP412-transfected (●) or untransfected (○) COS-1 cells were incubated with the indicated concentrations of PGE_2 in the presence of 1 mM 3-isobutyl-1-methylxanthine, and cAMP accumulation was determined. The results shown are the means ± S. E. for triplicate determinations.

As shown in Fig. 1-8, PGE_2 induced a rapid increase in $[Ca^{2+}]i$ within 10 sec in the CHO cells stably expressing the EP_1. The level decreased quickly but remained above the basal for over 3 min after the stimulation. This increase in $[Ca^{2+}]i$ was completely abolished by removal of extracellular Ca^{2+} (data not shown), suggesting that the PGE_2-induced increase in $[Ca^{2+}]i$ is due to the entry of extracellular Ca^{2+}. No such response was observed in mock-transfected cells (data not shown). Under this condition, PGE_2 induced a rapid but limited IP_3 formation of 120% of the control. On the other hand, PGE_2 neither stimulated nor inhibited adenylate cyclase (data not shown). These results demonstrate that this receptor is of the EP_1 subtype coupled exclusively to Ca^{2+} mobilization.

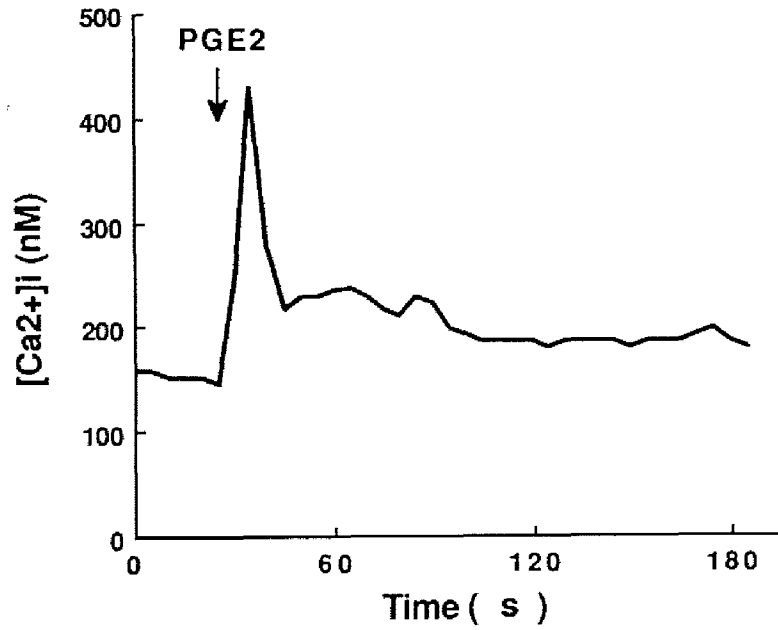

Fig. 1-8. Effect of PGE_2 on $[Ca^{2+}]i$ change in EP_1-expressing CHO cells. Fura-2-loaded cells were challenged with 0.1 μM PGE_2. A trace shown is a representative of $[Ca^{2+}]i$ responses of more than 20 individual cells. Pairs of fluorescence images at 340/480 nm were captured at 5-sec intervals. The *arrow* indicates the addition of PGE_2.

5. Tissue distribution of the PGE receptors.

Poly $(A)^+$ RNAs were prepared from various mouse tissues, and hybridized with a fragment of the EP_3 cDNA (Fig. 1-9). A positive band was seen at 2.3-kilobase in a number of tissues in which PGE_2 has pharmacological effects and/or specific binding sites (4). High expression of EP_3 mRNA was seen in the kidney, in uterus, and in stomach. The EP_3 receptor was also expressed in brain, heart, lung, thymus and spleen. On the other hand, EP_3 mRNA was not detectable in testis, and little was found in liver and ileum. The abundant amount of EP_3 mRNA is also expressed in mastocytoma P-815 cells. Another hybridizing band was also detected at an estimated mRNA size of 7.0 kilonucleotides in kidney, uterus, brain and mastocytoma P-815 cells.

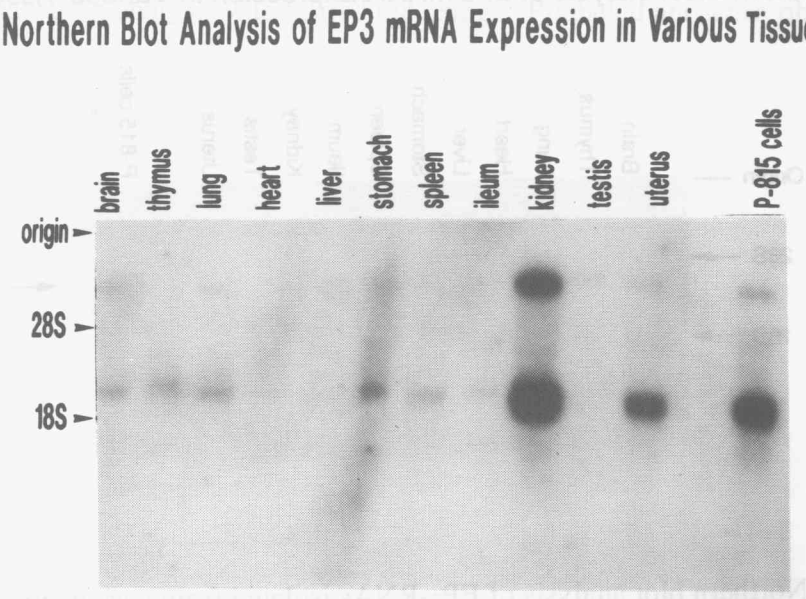

Fig. 1-9. Northern blot analysis of EP_3-RNAs isolated from various mouse tissues and P-815 cells. Poly$(A)^+$ RNAs were isolated from the following tissues and a cell line, and 10 μg of RNA was applied in each lane except that 5 μg was used for P-815 cells. Hybridization analysis was carried out using the 1,072-bp *Eco* RI-*Bam* HI fragment excised from clone MP660 as a probe.

Fig. 1-10 shows the distribution of the EP_2 receptor mRNA. A positive band is observed at 3.9 kilobase in most of tissues, suggesting widespread distribution of the EP_2 receptor. The tissues highly expressing EP_2 mRNA were ileum and thymus. A significant band was also observed in lung, spleen, heart or uterus. EP_2 mRNA was not detectable in testis and liver.

Fig. 1-11 shows the distribution of the EP_1 receptor mRNA. The 2.4-kilobase mRNA of this receptor was abundantly expressed in kidney and an additional band of 6.0-kilobase was detected. Significant expression of the mRNA of the receptor was also observed in the lung. Despite use of the RNA probe with high specific radioactivity, no significant expression was observed in other tissues.

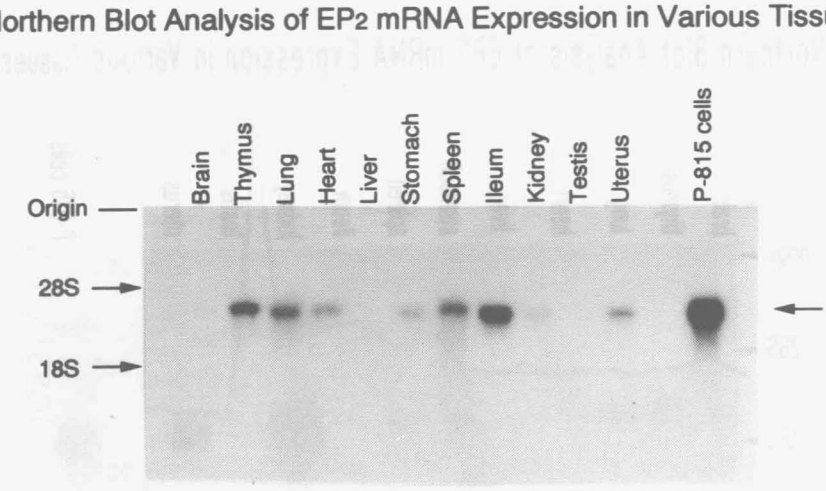

Fig. 1-10. Northern blot analysis of EP_2-RNAs isolated from various mouse tissues and P-815 cells. Poly(A)$^+$ RNAs were isolated from the tissues listed below and a cell line, and 10 µg of RNA was applied in each lane except that 5 µg was used for P-815 cells. Hybridization analysis was carried out using the 1859-bp EcoRI-XhoI fragment excised from clone MP412 as a probe.

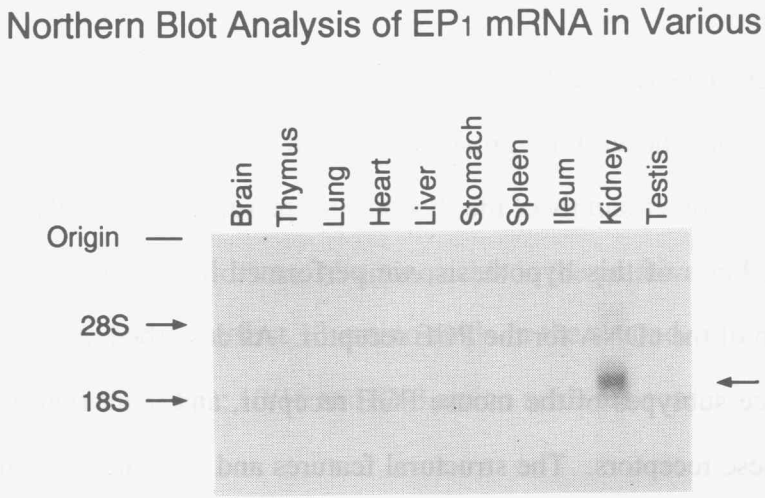

Fig. 1-11. Northern blot analysis of EP_1-RNAs isolated from various mouse tissues. Poly $(A)^+$ RNAs were isolated from the tissues, and 5 μg of RNA was applied in each lane. Hybridization analysis was carried out using ^{32}P-labeled antisense riboprobe.

DISCUSSION

1. Structural features and similarity of the PGE receptors.

We supposed that the various prostanoid receptors including the TX receptor construct a novel receptor subfamily of the rhodopsin-type superfamily with homologous structures. On the basis of this hypothesis, we performed homology cloning for the purpose of isolation of the cDNA for the PGE receptor. As described above, we cloned the cDNAs for three subtypes of the mouse PGE receptor, and identified the primary structures of the these receptors. The structural features and sequence identities of the PGE receptors are summarized in Fig. 1-12. The sequence homology analyses indicate that PGE receptors share significant sequence similarity with each other. However, it is interesting that among three PGE receptors, EP_2 shows the lowest identities with other prostanoid receptors, and that EP_3 shares higher sequence homology with TX receptor than with the other two PGE receptors. It is possible that these sequence homology among prostanoid receptors may depend on pathway of their coupling signal transduction, rather than on the kind of their own natural ligand.

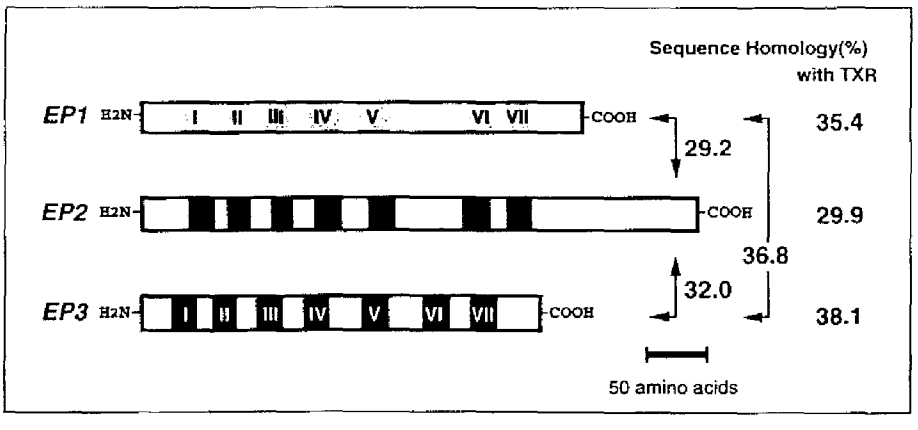

Fig. 1-12. Structures of the mouse PGE receptor subtypes and sequence identity among the prostanoid receptors.

Fig. 1-13 shows the comparison of the amino acid sequences of the mouse PGE receptor subtypes and mouse TX receptor (Namba et al. 1992). The amino acid sequence identity in the putative transmembrane segments (TMs) are as follows: 27.5% between EP_1 and EP_2, 33.2% between EP_1 and EP_3, and 36.2% between EP_2 and EP_3. EP_1 shows higher homology to TX receptor (35.4%) than other PGE receptor subtypes, especially in the TM III (75%) and VI (68%). The most highly conserved regions among these four prostanoid receptors are 16 amino acids in the putative TM VII (from Arg-341 to Leu-356 in EP_1) and the first 13 amino acids in the second extracellular loop (from Gly-179 to Phe-191 in EP_1); 10/16 and 8/13 amino acids are conserved, respectively.

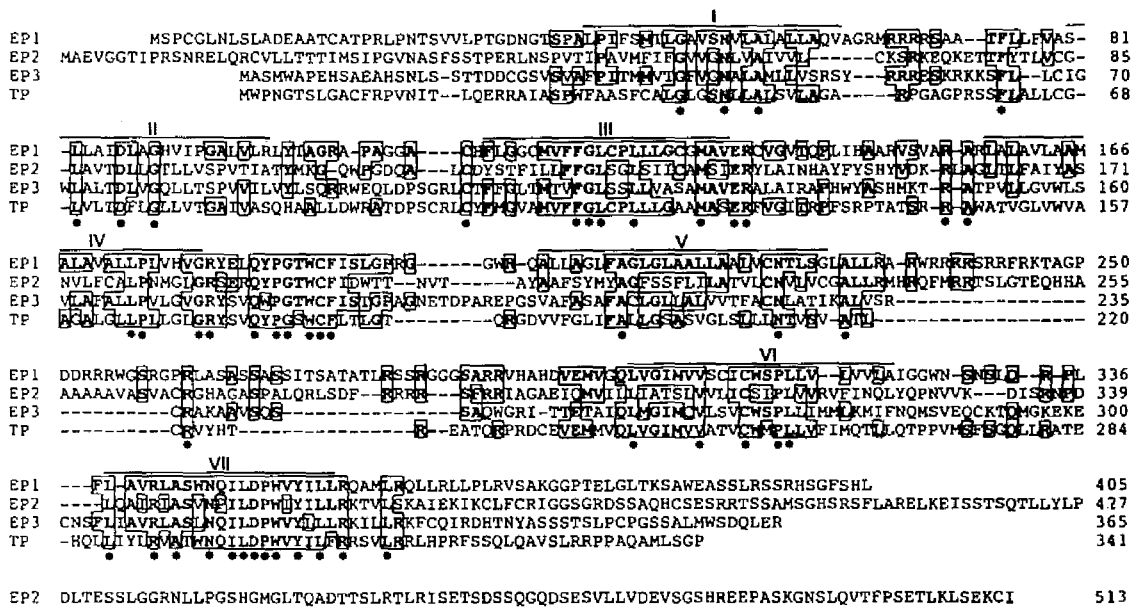

Fig. 1-13. Comparison of the amino acid sequences among the mouse EP_1, EP_2, EP_3 and TX receptor (TP). The amino acid sequences are aligned to achieve the maximal homology using a computer program. Boxes represent conserved amino acid residues between EP_1 and other prostanoid receptors. *Dashes* show deletions of the amino acid residues when compared among the four sequences.

Arg residue within the TM VII, such as Arg-341 in EP_1, is conserved among these prostanoid receptors. Considering that retinal is attached to Lys-296 of bovine rhodopsin in TM VII (Findlay and Pappin 1986), the structural features of highly conserved segment VII including Arg residue may reflect the acidic nature of the ligand for the prostanoid receptors as first suggested by the structure of the human TX receptor (Hirata et al. 1991). These prostanoid receptor share the features common to members of the G protein-coupled superfamily of receptors (Dohlman et al. 1991). First, there are two potential *N*-linked glycosylation sites in the putative extracellular amino terminus (EP_1), or in this region and the second extracellular loop region (EP_2 and EP_3). Second, conserved cysteine residues are found in the extracellular loop 1 and 2. There is a heavy bias toward basic residues throughout the intracellular loop regions in each receptor. There are also multiple serine and threonine residues, potential phosphorylation sites, throughout the C-terminal tail and the cytoplasmic loops, which may be involved in receptor desensitization (Hausdorff et al. 1990; Kennelly and Krebs 1991).

2. Ligand binding characters of the PGE receptors.

Three PGE receptors revealed high affinities to [^{3}H]PGE_2 with Kd of 3.3 (EP_3), 11.2 (EP_2), and 21 nM (EP_1) (Fig. 1-14). In contrast to EP_2 and EP_3 receptors, the only EP_1 receptor shows higher affinity to PGE_2 than PGE_1 and high affinity to iloprost. These properties are consistent with those reported for the EP_1 receptor. For example, it has been shown that PGE_2 has higher agonist potency than PGE_1 on the EP_1 receptor (Coleman et al. 1990), and that iloprost acts as an agonist not only at the prostacyclin receptor but also at the EP_1 receptor (Dong et al. 1986; Armstrong et al. 1989). It is also noted that the binding properties of the EP_1 agrees well with those described for the [^{3}H]PGE_2 binding in canine kidney papilla membrane (Smith et al. 1987); this binding has a similar Kd value for PGE_2, and the Ki of PGE_1 was about one order of magnitude higher than that of PGE_2. These results strongly suggest that the cloned EP_1 receptor is identical to the PGE receptor found in kidney papilla. On the other hand, EP_2 and EP_3 showed a similar rank order of prostaglandin ligands.

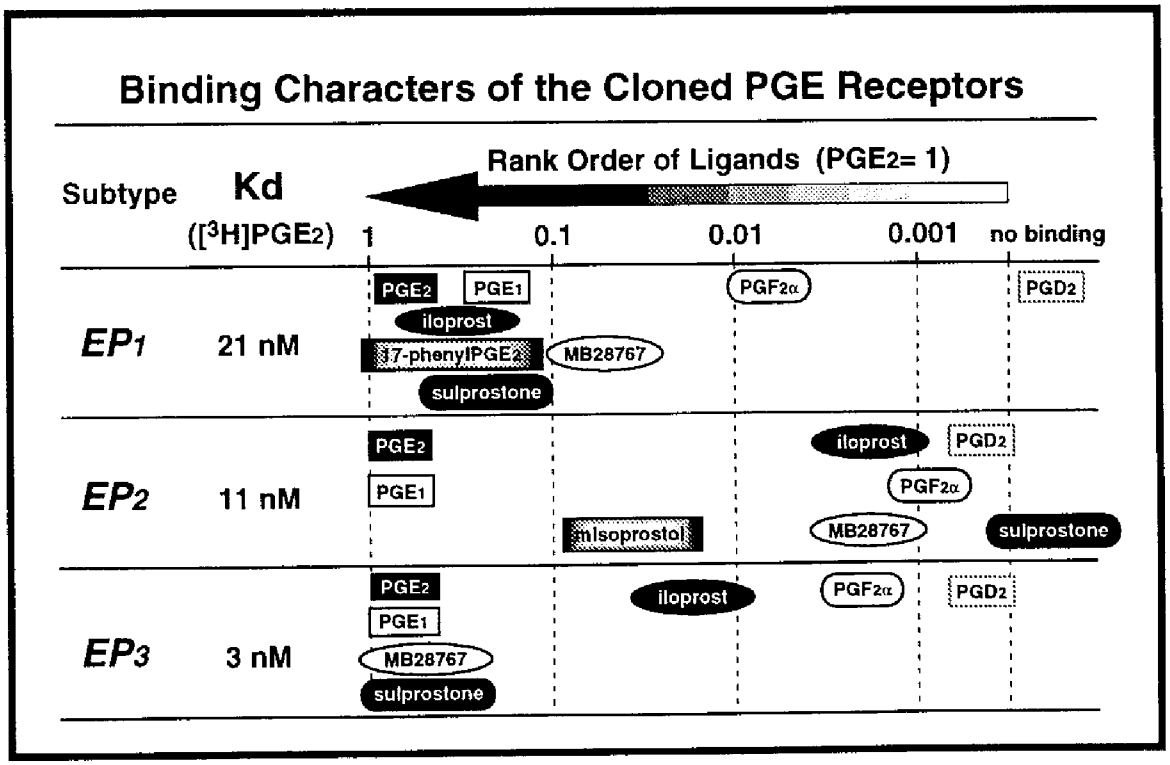

Fig. 1-14. Binding characters of the cloned PGE receptors. The Kd values for [^{3}H]PGE_2 of the receptors are shown. Rank order of ligands (PGE_2 = 1) for each subtype is also shown.

Among the cloned PGE receptors, EP_3 receptor showed the typical characteristic about subtype-selective ligand binding which agrees well to those previously reported. As to the binding of the EP_3, only EP_3-specific agonists, GR63799X and M&B28767, specifically competed with equal potency, and they were more potent than PGE_2 itself. However, EP_2 and EP_1 revealed the binding characters different from those reported. At first, the binding of EP_2 is inhibited by misoprostol (an EP_2 and EP_3 agonist) and more weakly by M&B28767 (an EP_3 agonist). On the other hand, sulprostone (an EP_1 and EP_3 agonist), SC-19220 (an EP_1 antagonist), and butaprost (an EP_2 agonist), did not inhibit it. The ability of misoprostol to inhibit the binding of EP_2 and no ability of sulprostone suggest that MP412 indeed encodes the EP_2 subtype of PGE receptor, and this was also supported by weak cross-reaction of M&B28767 to EP_2 (Lawrence et al. 1992). The lack of binding activity of butaprost in mouse EP_2 might indicate that the action of butaprost is species specific. Secondly, the binding of EP_1 was inhibited by 17-phenyl-18,19,20-trinor PGE_2 (an EP_1 agonist), and by sulprostone (an EP_1 and EP_3 agonist), while the inhibition by M&B28767 (an EP_3 agonist), by 11-deoxy PGE_1 (an EP_2 and EP_3 agonist), and by butaprost (an EP_2 agonist), was weak or negligible. The 11-hydroxy residue is important for EP_1 agonist activity but not essential for activity of either EP_2 or EP_3 (Carpio et al. 1987). On the other hand, AH6809 (an EP_1 antagonist) showed only weak inhibition of the binding. This result suggests that there may be other forms of EP_1 receptor sensitive to AH6809, or that the action of AH6809 is species-specific and does not work on the mouse receptor.

3. Physiological roles of the PGE receptors.

The most remarkable difference among these three PGE receptor subtypes is that in intracellular signal transduction pathways (Fig. 1-15). As previously suggested by a number of pharmacological studies, EP1 is coupled to Ca^{2+} mobilization, and EP_2 and EP_3 is coupled to stimulation and inhibition of adenylate cyclase, respectively. As to EP_3 receptor, it was suggested that this receptor can be coupled to phospholipase C (Coleman et al. 1990). However, this signaling pathway could not detected in our CHO expression system using MP660.

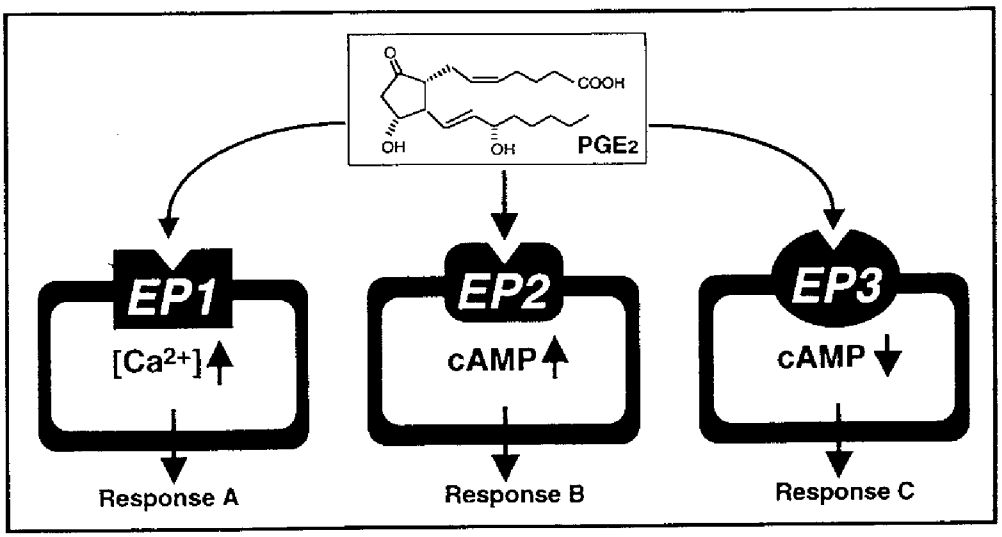

Fig. 1-15. Signal transduction pathways of the PGE receptor subtypes.

Northern hybridization analyses revealed distinct tissue distribution of the three PGE receptor mRNAs (Fig. 1-16).

The tissue most highly expressing EP_3 mRNA was kidney in which PGE_2 exerts inhibitory effect on sodium and water reabsorption by inhibiting adenylate cyclase via G_i (Sonnenburg and Smith 1988; Watanabe et al. 1991). A significant band was also observed in stomach, suggesting that the receptor we cloned is indeed involved in inhibition of histamine-induced gastric acid secretion in this tissue (Chen et al. 1988). This analysis also showed that uterus expressed mRNA of this receptor much higher than most tissues. It is known that PGE_2 exerts contractile response in uterine smooth muscle. The EP_3 receptor we found may mediate this contractile action. Uterine contraction has been observed as a side effect of several EP_3 agonists used as gastric anti-secretory prostanoid drugs (Collins 1986). The EP_3 receptor was also expressed in heart, lung, thymus and spleen. Although PGE_2 causes inhibition of sympathetic neurotransmitter release in some of these tissues (Hedqvist 1972), major functions of this receptor remain to be investigated. Our results also showed that EP_3 mRNA is significantly expressed in brain. The exact function of this receptor in this tissue is again not known at present. As described above, the abundant amount of EP_3 mRNA is expressed in and a functional clone was obtained from mastocytoma P-815 cells, we have not yet identified a role of this receptor in these cells.

The tissues highly expressing EP_2 mRNA were ileum and thymus in which PGE_2 induces relaxation of ileum circular muscle (Lawrence et al. 1992) and inhibits proliferation of T cells by increasing intracellular cAMP levels (Phipps et al. 1991). Significant expression was also observed in lung, spleen, heart or uterus. It is known that PGE_2 has the relaxant activity in trachea (Gardiner 1986) and myometrium (Senior et al. 1991).

A hybridizing band for EP_1 receptor was abundantly detected in kidney. It is known that PGE_2 induces Ca^{2+} mobilization and inhibits the action of arginin-vasopressin in the rabbit collecting duct (Hébert et al. 1991). This action may be mediated

by EP_1 receptor, and it plays a critical role in the renal function. Significant expression of the mRNA of the receptor was also observed in the lung, in which PGE_2 induces contraction of tracheal smooth muscle (Coleman et al. 1990).

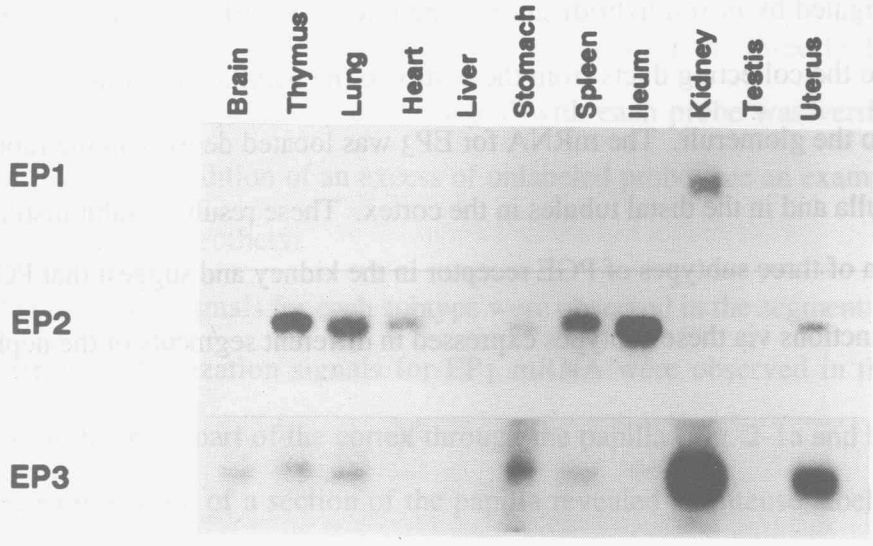

Fig. 1-16. Northern blot analyses of mRNAs of the PGE receptor subtypes.

2. Distinct cellular localization of the mRNAs for three subtypes of the PGE receptor in the mouse kidney

SUMMARY

Distribution of the mRNAs for three subtypes of PGE receptor in the mouse kidney was investigated by *in situ* hybridization. The mRNA for EP_1 subtype was specifically localized to the collecting ducts from the cortex to the papilla. The mRNA for EP_2 was localized to the glomeruli. The mRNA for EP_3 was located densely in the tubules in the outer medulla and in the distal tubules in the cortex. These results exhibit distinct cellular localization of three subtypes of PGE receptor in the kidney and suggest that PGE_2 exerts multiple functions via these subtypes expressed in different segments of the nephron.

RESULTS

[^{35}S]-Labeled riboprobes prepared from the EP_1, EP_2 and EP_3 cDNAs were used and the regional distribution and cellular localization of mRNA for each subtype were investigated in kidney sections by in situ hybridization. In situ hybridization of kidney sections revealed distinct macroautoradiographic patterns for the three PGE receptor mRNAs (Fig. 2-1). The specificity of the signal with each probe was verified by its disappearance with the addition of an excess of unlabeled probe (see an example in Fig. 1d for EP_1, not shown for others).

The hybridization signals for each subtype were observed in the segments along the nephron. Strong hybridization signals for EP_1 mRNA were observed in the tubular structures from the inner part of the cortex through the papilla (Fig. 2-1a and Fig. 2-2a). Microscopic examination of a section of the papilla revealed an intense labeling of the tubular cells in the collecting ducts (Fig. 2-3a). A large number of punctate hybridization signals for EP_2 mRNA were observed in the cortex (Fig. 2-1b), and this hybridization signals were specifically located in the glomeruli (Fig. 2-3b). No significant labeling of EP_2 mRNA was observed in the other part of the kidney. In the glomeruli, silver grains were located over the mesangial cells, but their presence in podocytes and endothelial cells cannot be excluded. Strong hybridization signals for EP_3 mRNA were observed in tubular structures with the highest density in the outer medulla and also in the cortex, but no signal was detected in papilla (Fig. 2-1c, Fig. 2-2b and 2-2c). Microscopic examination in the outer medulla showed the intense labeling in the tubular epithelia (Fig. 2-3c). In the cortex, no significant hybridization signals are detected in the glomeruli and proximal tubules. On the other hand, prominent expression of EP_3 mRNA was observed in the neighboring distal tubules (Fig. 2-3d). The positive signals were also found over the macula densa (Fig. 2-3d, arrow).

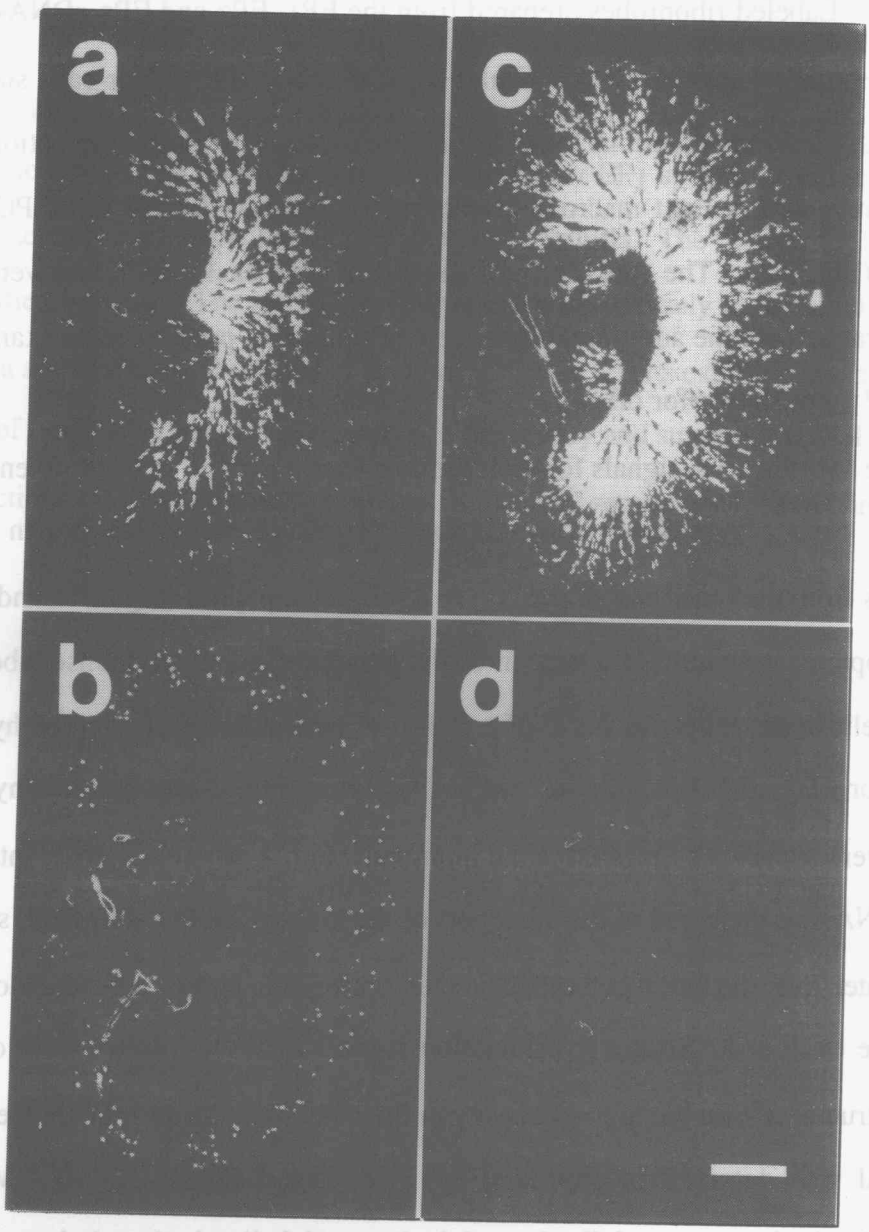

Fig. 2-1. Dark-field photomicrographs, showing hybridization signals for three PGE receptor mRNAs in the kidney . Sections were hybridized with antisense riboprobe for EP_1 (a), EP_2 (b), and EP_3 mRNA (c). The hybridization signals for EP_1 shown in *a* were abolished in the control experiments using the same probe in the presence of an excess amount of the unlabeled probe (d). Bar, 1 mm.

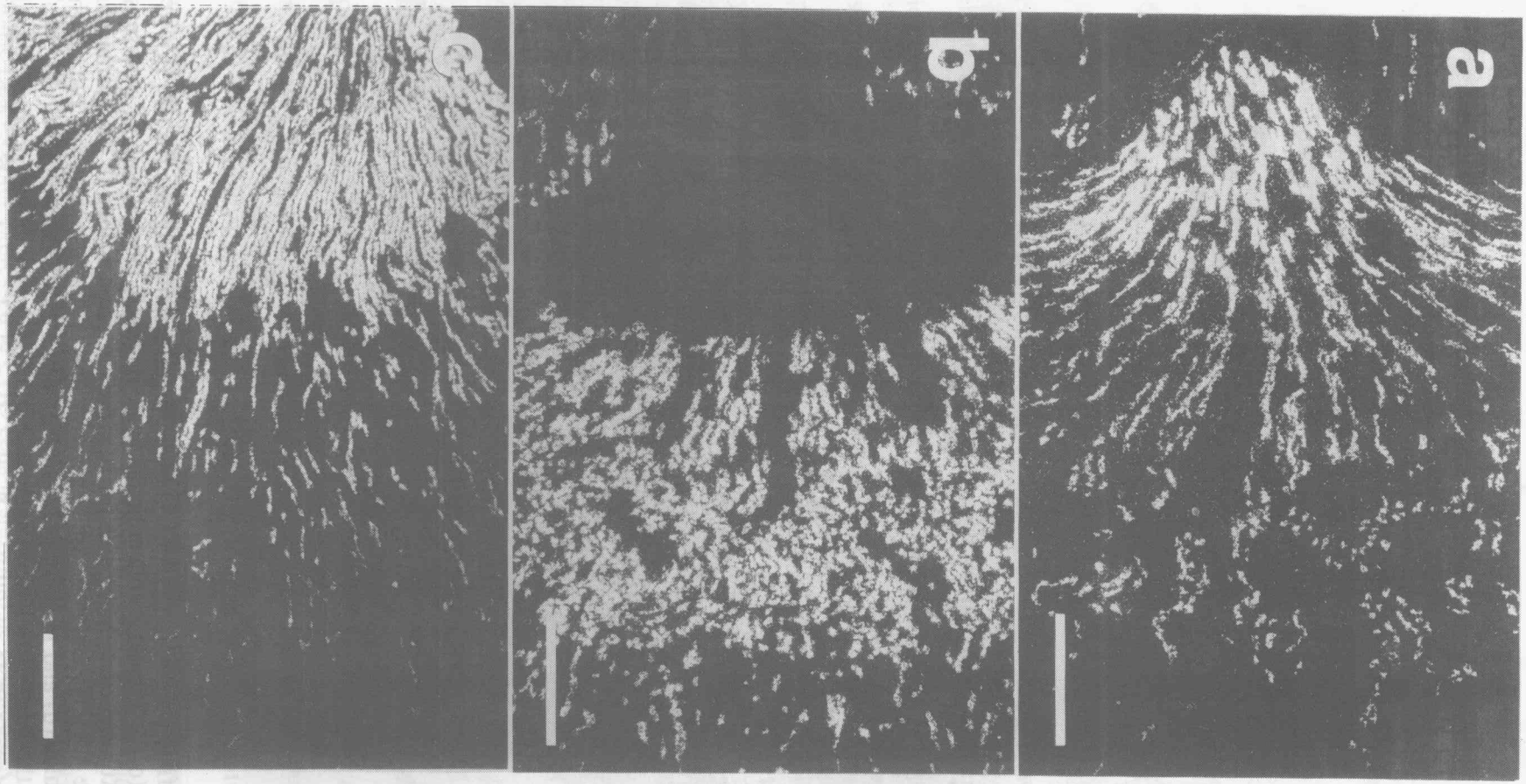

Fig. 2-2. Enlarged dark-field photomicrographs of the emulsion-dipped sections showing the hybridization signals for EP_1 (a) and EP_3 mRNA (b, c). *a* and *b* were taken from the corresponding regions of continuous sections showing signals for EP_1 and EP_3, respectively. EP_1 mRNAs were located in the collecting ducts from the renal cortex to the papilla. EP_3 mRNAs were located in tubules in the cortex and the outer medulla, but not in the papilla. Bars, 500 μm.

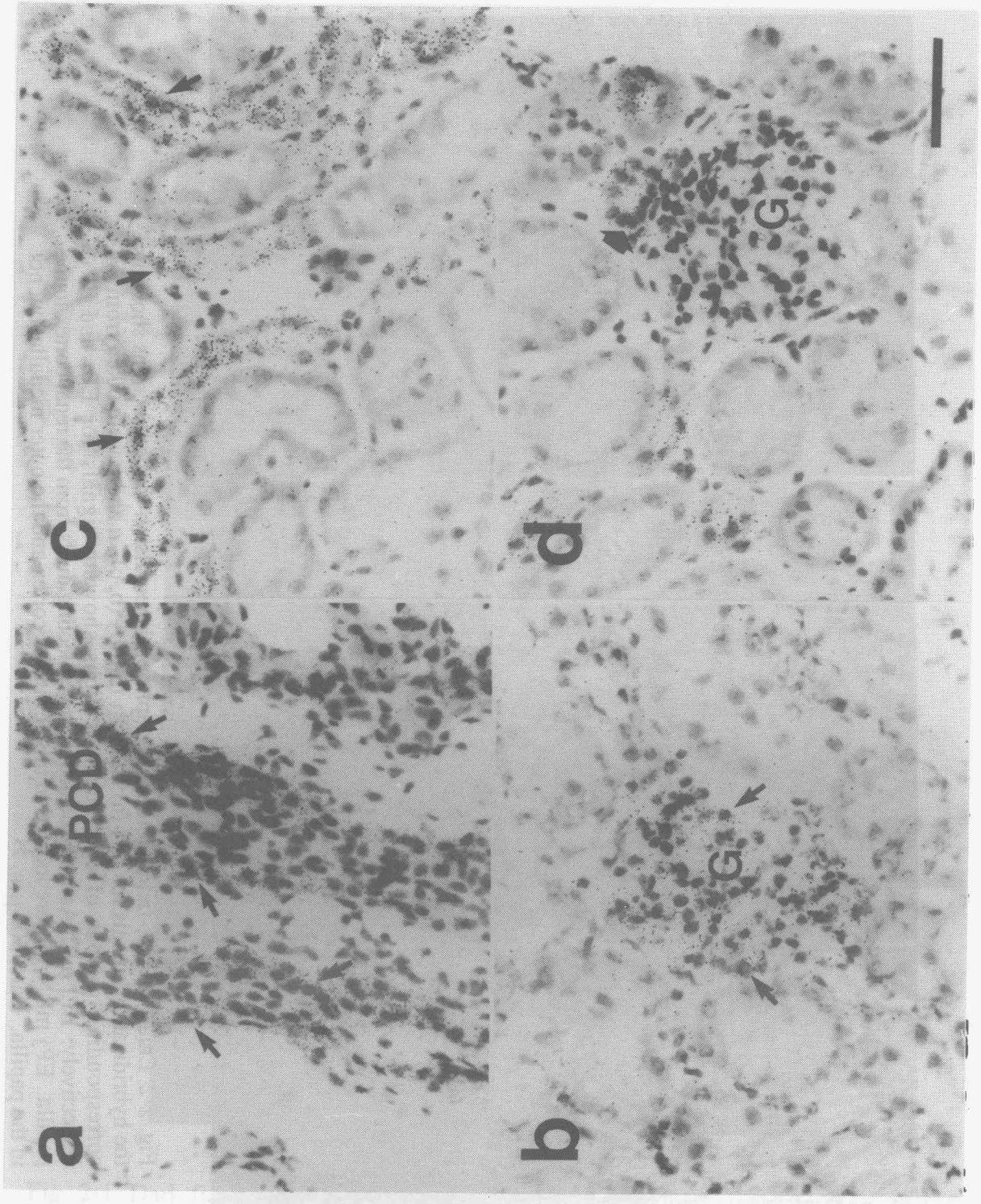

Fig. 2-3. Bright-field photomicrographs, showing hybridization signals for EP_1 (a), EP_2 (b), EP_3 mRNA (c, d). Papillary collecting ducts are intensely labeled with EP_1 antisense riboprobe (a). Autoradiographic grains for EP_2 mRNA are confined to the glomerulus (b), while those for EP_3 mRNA are observed not in the glomerulus, but in the neighboring tubules (d) and in the tubules in the cortex-medulla boundary (c). *Small arrows* indicate the labeled cells with each probe. *Bold arrow* shows positive signals over the macula densa. PCD, papillary collecting duct; G, glomerulus. *Bar*, 100 μm.

DISCUSSION

PGE_2 is a major arachidonate metabolite synthesized by cyclooxygenase in the mammalian kidney (Badr and Jacobson 1991). Its biosynthesis has been reported in various regions of the kidney, including glomeruli, cortical arterioles, thick ascending limbs of Henle's loop, collecting tubules and medullary interstitial cells (Bonvalet et al. 1987). PGE_2 exerts various physiological functions in the kidney (Badr and Jacobson 1991). They include regulation of water and electrolytes reabsorption (Bonvalet et al. 1987; Garcia-Perez and Smith 1984), regulation of renal blood flow and glomerular filtration (Gerber et al. 1982), and regulation of renin release (Freeman et al. 1984). However, contribution of these subtypes to each renal action of PGE_2 has not yet been established except its action on water reabsorption in the collecting duct, where PGE_2 appears to act on the EP_3 receptor and antagonizes vasopressin by inhibiting adenylate cyclase via G protein, Gi (Grantham and Orloff 1968; Sonnenburg and Smith 1988). Northern blot analyses revealed the significant expression of these subtypes in the kidney (see Section 1). In order to establish their contribution to each renal action of PGE_2, the cellular distribution of the mRNAs for the three subtypes was examined in mouse kidney by *in situ* hybridization.

The cellular distribution of the subtypes of PGE receptor in the kidney is summarized in Fig. 2-4. EP_1 receptor transcripts are abundantly found in the papillary collecting ducts. The collecting ducts are involved in the concentration of the urine (Stein and Reineck 1974), which is stimulated by vasopressin-induced cAMP system (Handler 1988). The action of vasopressin in these segments is regulated in two ways; one by intracellular Ca^{2+} concentration and the other by inhibiting adenylate cyclase (Breyer et al. 1990; Dillingham et al. 1987). It has been reported that PGE_2 attenuates the vasopressin action by both mechanisms (Breyer et al. 1990; Hébert et al. 1990; Nadler et al. 1986). Mouse EP_1 receptor expressed in CHO cells is shown to be coupled to Ca^{2+} mobilization.

It is, therefore, likely that EP_1 receptor in the collecting duct modifies the vasopressin-induced osmotic water permeability by the former mechanism. The transcripts of the EP_2 receptor are specifically found in glomerulus with intense labeling over the mesangial cells. In the cultured mesangial cells, PGE_2 elicited cAMP accumulation and attenuated contractility induced by vasoconstrictors such as TXA_2, angiotensin II, platelet-activating factor and endothelin (Dunlop and Larkins 1990; Méne and Dunn 1988).

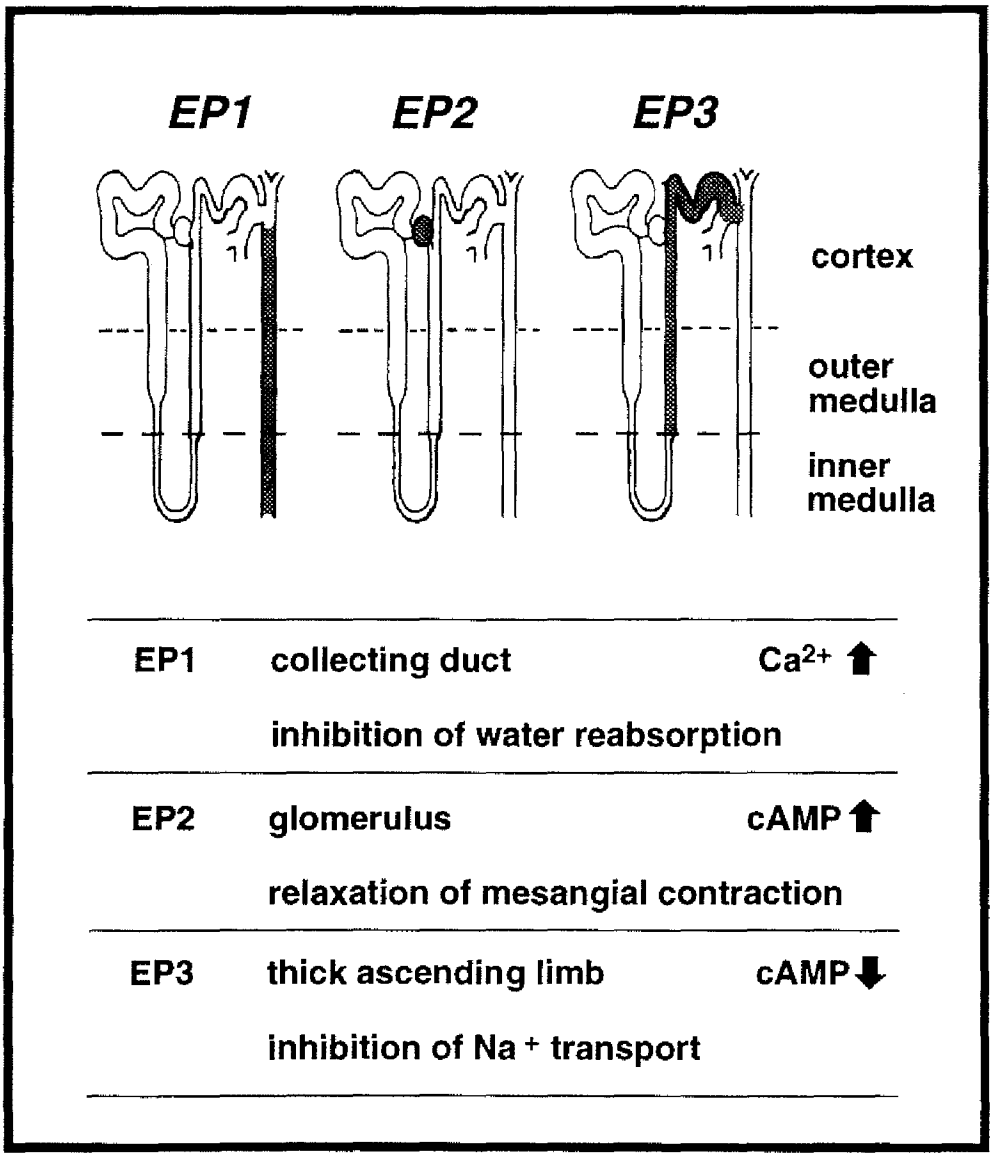

Fig. 2-4. Summary of distribution of three PGE receptor subtypes in the kidney.

This inhibitory action of PGE_2 was blocked by the inhibitor of adenylate cyclase, 2',5'-dideoxyadenosine (Méne and Dunn 1988), and mimicked by 8-bromo cAMP (Dunlop and Larkins 1990). These results suggested that EP_2 receptor coupled to cAMP generation mediates the PGE_2-induced inhibition of the mesangial contractility to regulate glomerular filtration (Méne and Dunn 1988).

EP_3 receptor transcripts are detected in the tubules in the outer medulla and in the distal tubules in the cortex. Previous PCR distribution analysis on the nephron segments microdissected from mouse and rat kidney showed significant amplification of the EP_3 mRNA in medullary and cortical thick ascending limbs of Henle's loop, and also in distal tubules and in the cortical and outer medullary collecting ducts (Takeuchi et al. 1993; Taniguchi et al. 1992). Our result together with these reports indicate the presence of EP_3 receptor in the thick ascending limb as well as in the collecting duct. PGE_2 is synthesized in a large amount in the thin limbs of Henle's loop and the cortical and medullary collecting ducts (Bonvalet et al. 1987). PGE_2 attenuates vasopressin-induced NaCl reabsorption in the mouse thick limb (Hebert and Andreoli 1984), and also inhibits vasopressin-induced water reabsorption in the collecting ducts (Grantham and Orloff 1968; Nadler et al. 1992). These actions of PGE_2 are mediated by inhibiting adenylate cyclase in a pertussis toxin-sensitive manner (Nakao et al. 1989; Sonnenburg and Smith 1988). Mouse EP_3 receptor expressed in CHO cells is coupled to inhibition of adenylate cyclase. These findings suggest that PGE_2 synthesized locally acts on EP_3 receptor and exerts inhibitory action on vasopressin-enhanced NaCl and water reabsorption by inhibiting cAMP accumulation. In addition to these effects, it has been suggested that PGE_2 inhibits net Na^+ transport in isolated perfused medullary thick ascending limb or cortical collecting duct (Iino and Imai 1978; Stokes and Kokko 1977), and this action is proposed to be mediated by inhibition of the basolateral Na^+-K^+-ATPase (Cohen-Luria et al. 1993; Warden and Stokes 1993). Whether such an action is mediated also by the EP_3 receptor remains to be tested. PGE_2 is also known to increase renin release and two renal sites are proposed for this action; one is the intrarenal baroreceptor and the other the

macula densa (Freeman et al. 1984). It is interesting in this respect that the EP_3 receptor mRNA is expressed densely in the latter structure.

While the present study revealed cellular localization of the PGE receptor subtypes relevant to its actions in nephron, there remain several unsolved issues. One regards on PGE receptor in the renal blood vessels. Although a large literature described potent vasodilatory action of PGE_2 in the kidney (reviewed in Gerber et al. 1982), we did not detect specific signals in vascular beds in the present sections. However, it does not imply that there is no PGE receptor expression in the renal blood vessels. Such expression should be examined systematically on vessels at various anatomical sites.

In summary, the present in situ hybridization study has revealed that the three subtypes of PGE receptor show distinct cellular localization in the kidney, and suggests that PGE_2 exerts multiple functions via these subtypes expressed in different segments of the nephron. The mode of PGE_2 actions in the renal system is based on its physiological role as a local mediator, which is distinguished from hormones such as AVP (Fig. 2-5).

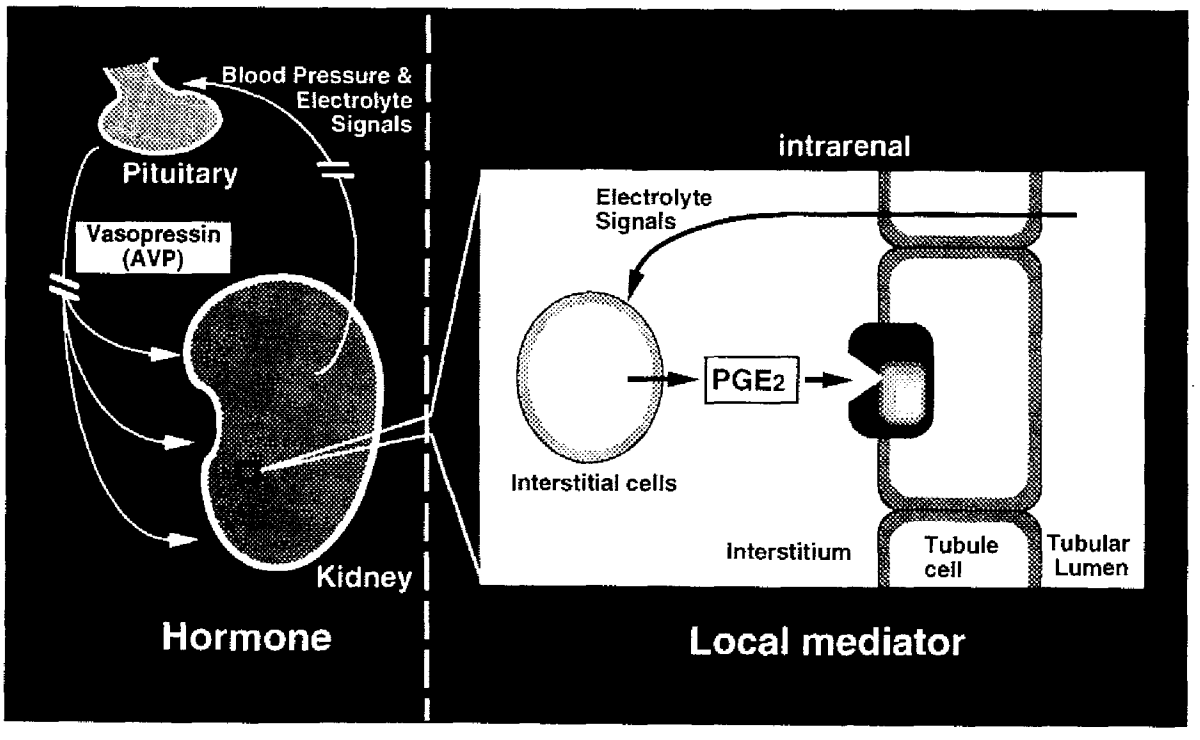

Fig. 2-5. Physiological role of PGE_2 as a local mediator in the kidney. Pituitary hormones such as AVP (arginine vasopressin) act on the whole target organs through circulation system. On the other hand, the local mediators (autacoids) such as PGE_2 act on only local target segments neighboring its secreting cells.

3. Identification of two isoforms of the PGE receptor subtype EP_3

SUMMARY

Functional cDNA clones for two isoforms of mouse EP_3 receptor derived from alternative RNA splicing were obtained. The two isoforms are different only in sequence of the putative cytoplasmic C-terminal tail and their hydrophobicity; one isoform named $EP_{3\alpha}$ has a hydrophilic tail and the other named $EP_{3\beta}$ a hydrophobic tail. When expressed, the two receptors displayed identical ligand binding properties but different responses to GTPγS. Without a change in B_{max} values, GTPγS increased Kd for PGE_2 of $EP_{3\beta}$ and decreased that of $EP_{3\alpha}$. These effects were abolished by the treatment of the membranes with pertussis toxin, and restored by the addition of G_{i2}. While both isoforms exerted inhibition of forskolin-induced cAMP accumulation, three orders lower concentrations of agonists were required for $EP_{3\alpha}$ than $EP_{3\beta}$ for 50% inhibition of cAMP formation. Similar difference in agonist potency was observed also in agonist-induced stimulation of GTPase activity in the membranes. Thus, the two receptors with the different C-terminal tails show different coupling to the Gi protein, leading to the opposite responses to GTP in the ligand binding affinity and to different affinities of the agonist-occupied receptor to the G protein.

RESULTS

1. cDNA cloning of two molecular forms of the mouse EP_3 receptor.

In the homology screening for the PGE receptor cDNA, we first obtained a partial EP_3 cDNA clone from mouse lung cDNA library, and using this fragment as a probe, we isolated several clones from mouse mastocytoma P-815 library. Restriction analysis of the isolated clones displayed at least two types of cDNAs; five clones belong to one type represented by MP660, and three clones belong to another type represented by MP653. We first characterized MP660, found that it is a functional mouse EP_3 receptor cDNA. We then characterized another group of the clones represented by MP653. Sequencing analyses revealed that MP653 had a 1,083-base pair (-bp) open reading frame, and was identical to MP660 in the nucleotide sequence except deletion of an 89-bp sequence in the coding region of the putative C-terminal tail of the receptor in MP660-encoded receptor (Figure 3-1a). Figure 3-1b shows the cDNA and deduced amino acid sequences of MP653 as compared with those of MP660. Deletion of this 89-bp sequence creates another reading frame downstream from this junction, which extends coding region until a new stop codon placed on 77-bp downstream from the stop codon of MP660. As a consequence, a 30-amino-acid C-terminal fragment of the MP660-encoded receptor (peptide-α) was replaced with a new 26-amino-acid fragment (peptide-β) in the C-terminal end of MP653-encoded receptor. Thus, the cytoplasmic C-terminal domain of the two EP_3 isoforms consists of the common 10 amino acids in the amino-terminal region followed by these different peptides.

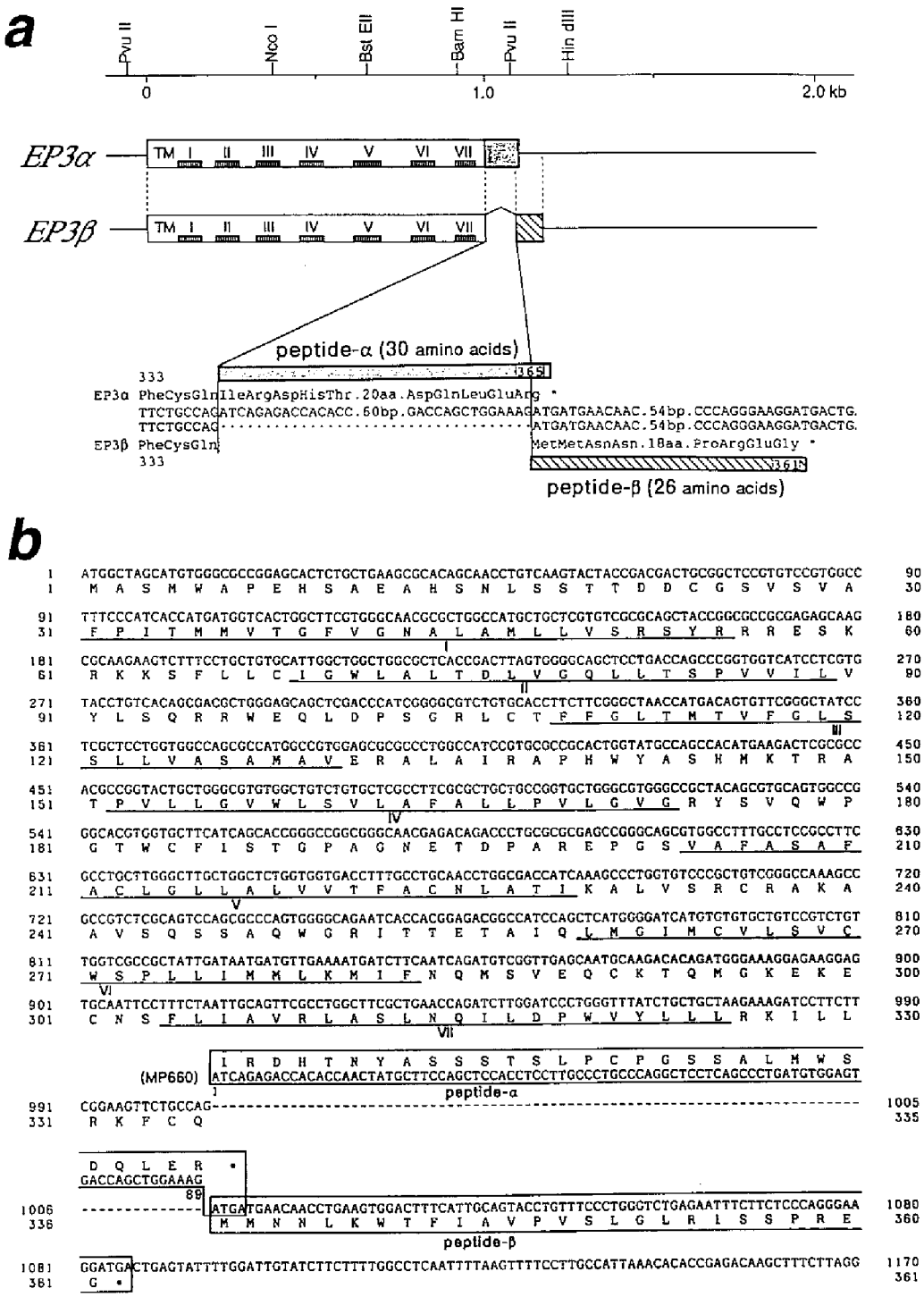

Fig. 3-1. Comparison of cDNA structures of two EP_3 receptor isoforms.
a. Schematic representation of mouse EP_3 receptor cDNA clones, MP660 and MP653. Boxes represent coding sequences; open box is a corresponding coding sequence between the two cDNA, grey one is the sequence coding peptide-α, hatched one is the sequence coding peptide-β. The putative transmembrane domains are indicated by striped boxes.
b. Nucleotide and deduced amino acid sequences of MP653. The deduced amino acid sequence is shown under the nucleotide sequence using single letter code. Positions of the putative transmembrane segments I-VII are indicated by underlines below the amino acid sequence. The region deleted in MP653 is represented by hyphens. The deleted 89-bp sequence is indicated above them, and their deduced translation in MP660 is shown over the nucleotide sequence. The peptide-α and peptide-β are boxed with the termination codon indicated as asterisks.

Hydrophobicity analyses according to the method of Kyte and Doolittle (1982) revealed that the peptide-β forms a hydrophobic domain, while the peptide-α is of hydrophilic nature (Fig. 3-2). The peptide-α contains nine residues of serine and threonine, potential phosphorylation sites (reviewed by Dohlman et al. 1991), while the peptide-β has only four.

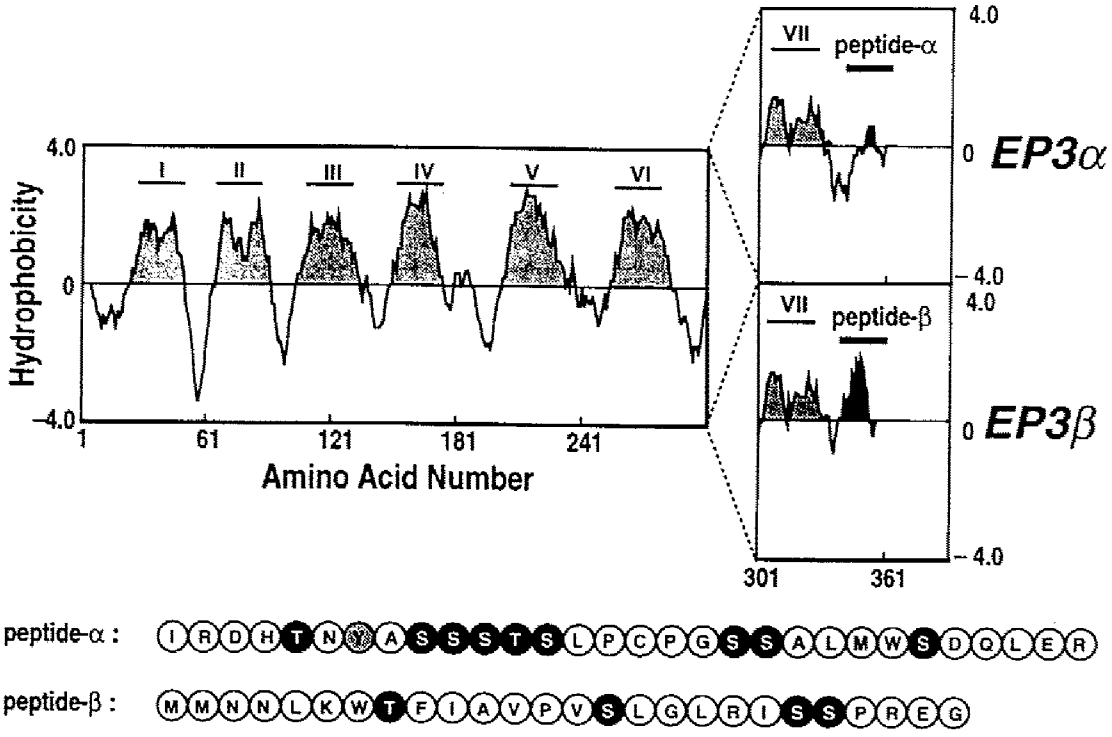

Fig. 3-2. Comparison of hydrophobicity of the EP_3 sequence coded by MP660 (upper) and MP653 (lower) analyzed by the method of Kyte and Doolittle (1982). The positions of the transmembrane domains and of peptide-α and peptide-β are indicated by plain and bold lines. Amino acids are numbered underneath. Sequence of each C-terminal peptide are shown below.

The two cDNAs were expressed in COS-1 cells and their ligand binding properties were compared. Specific [^{3}H]PGE$_2$ binding was observed in the membranes of COS-1 cells transfected with MP660 and those with MP653. Figure 3-3 shows the effect of various unlabeled PGs and PGE receptor subtype-specific ligands on these bindings. The binding of [^{3}H]PGE$_2$ to MP660- or MP653-transfected cell membranes was inhibited in a similar concentration-dependent manner by unlabeled ligands. These binding properties indicate that the two receptors have the identical specificity and are classified to the EP$_3$ subtype of PGE receptor (Armstrong et al. 1990). Hence, we have designated MP660-encoding receptor as EP$_{3\alpha}$ (containing the peptide-α), and MP653-encoding one as EP$_{3\beta}$ (containing the peptide-β).

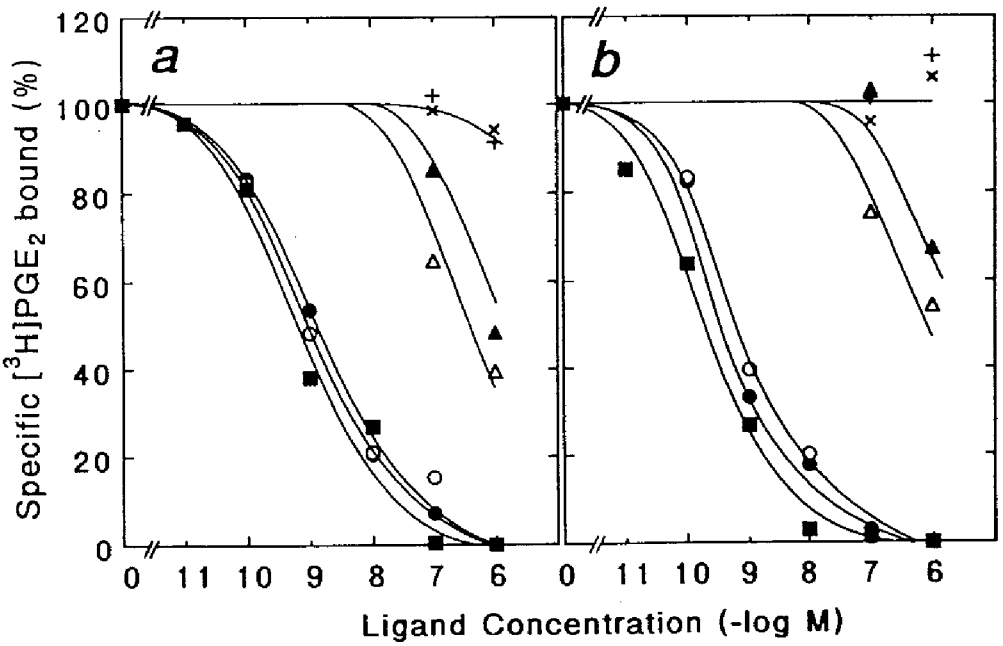

Fig. 3-3. Displacement of [^{3}H]PGE$_2$ binding to the two EP$_3$ isofoms in transfected COS-1 cells by various prostaglandins and PGE sustype-selective ligands. Unlabeled ligands were added to the binding assay mixture at indicated concentrations, and [^{3}H]PGE$_2$ binding to MP660-transfected COS-1 cell membranes (a) and MP653-transfected cell membranes (b) was determined. Prostaglandins used are PGE$_2$ (●), PGE$_1$ (○), PGF$_{2\alpha}$ (△), and PGD$_2$ (▲). Subtype-selective ligands used are SC-19220 (×, for EP$_1$), butaprost (+, for EP$_2$), M&B28767 (■, for EP$_3$).

2. GTPγS conversely affects the binding affinity for [^{3}H]PGE$_2$ of the two receptors via the same G protein.

The binding characterization of the two expressed receptors revealed that the C-terminal sequences have no effect on the binding affinity and specificity of the receptors. The C-terminal peptide of receptors has been shown to participate in the receptor-G protein coupling (O'Dowd et al. 1988), suggesting that the two receptors couple to a G protein differently. The receptor-G protein coupling can be examined in several ways. One is to examine modulation of the binding affinity of the receptor by guanine nucleotides (reviewed by Gilman 1987). We therefore examined the effect of a guanine nucleotide on [^{3}H]PGE$_2$ binding to the two EP$_3$ isoforms. Figure 3-4 shows the results of Scatchard plot analyses of the specific [^{3}H]PGE$_2$ binding to each receptor in the presence or absence of GTPγS, a nonhydrolyzable GTP analogue. In the absence of GTPγS, both receptors displayed indistinguishable binding affinity with a dissociation constant (Kd) of 3.0 nM for EP3α, and 2.8 nM for EP3β. The addition of GTPγS at 100 μM decreased Kd of the EP3α (1.1 nM), while it increased Kd of the EP3β (5.9 nM), without any change in B_{max}.

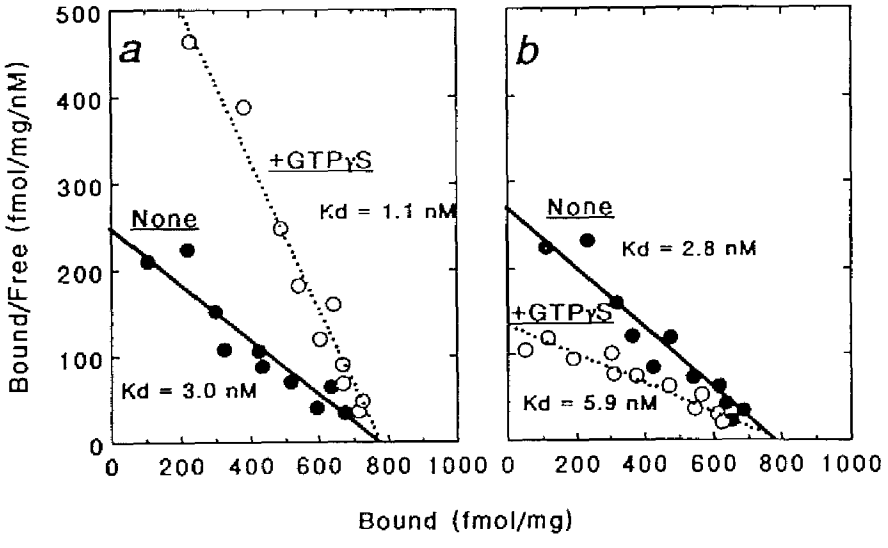

Fig. 3-4. Scatchard plot analyses of the PGE$_2$ binding to each isoform in COS cell membranes in the presence or absence of GTPγS. The specific binding of [^{3}H]PGE$_2$ (0.5-30 nM) to MP660 (EP3α; a) or MP653 (EP3β; b) in transfected COS-1 cell membranes (50 μg) was determined in the presence (○) or absence (●) of 100 μM GTPγS. The Scatchard plot was transformed from the value of specific [^{3}H]PGE$_2$ binding.

The opposite responses of $EP_{3\alpha}$ and $EP_{3\beta}$ to GTPγS might be due to difference in types of G protein coupling to each receptor. This was examined in CHO cells stably expressing the two receptors. We first examined effect of pertussis toxin (PT) treatment (Table 1). At the fixed concentration of the ligand (2.0 nM), the addition of GTPγS enhanced $[^3H]PGE_2$ binding in $EP_{3\alpha}$-expressing cell membranes. PT treatment also enhanced this binding and additional stimulation by GTPγS was not observed. On the other hand, the GTPγS addition reduced $[^3H]PGE_2$ binding in $EP_{3\beta}$-expressing cells. PT treatment of this membrane also reduced this binding into the same level as that reduced by GTPγS and abolished the inhibitory effect of GTPγS. As a result, in either isoform, PT-treatment mimicked the effects of GTPγS and abolished them in these membranes. This suggests that both $EP_{3\alpha}$ and $EP_{3\beta}$ receptors couple to a PT-sensitive G protein, probably G_i. In order to examine whether G_i can participate in these receptor systems, we reconstituted G_{i2} purified from bovine spleen into each of the PT-treated cell membranes. The addition of G_{i2} restored the effect of GTPγS on PGE_2 binding in each membranes (Table 1), indicating that the opposite responses of $EP_{3\alpha}$ and $EP_{3\beta}$ to GTPγS are ascribed not to difference in types of coupling G protein, but to that in C-terminal tails of the two receptors.

Table 1. Effects of pertussis toxin treatment and subsequent G_{i2} reconstitution on $[^3H]PGE_2$ binding to CHO cell membranes expressing the two EP_3 isoforms.

	Specific PGE_2 binding (pmol/mg protein)			
	$EP_{3\alpha}$		$EP_{3\beta}$	
Treatment	-GTPγS	+GTPγS	-GTPγS	+GTPγS
None	0.723± 0.084	1.63 ± 0.043	1.59 ± 0.084	1.05 ± 0.058
Pertussis toxin	1.73 ±0.095	1.70 ± 0.062	1.06 ± 0.081	0.970± 0.060
Pertussis toxin+G_{i2}	0.699± 0.10	1.65 ± 0.11	1.54 ± 0.057	0.956± 0.062

CHO cells stably expressing EP_3 receptor isoforms were cultured in the presence or absence of 20 ng/ml pertussis toxin for 9h. CHO cell membranes (50 μg) were incubated with or without G_{i2} purified from bovine spleen (10 pmol/mg of the membrane protein) at 0°C for 1 h. PGE_2 binding assays were carried out with 2.0 nM $[^3H]PGE_2$ in the presence or absence of 100 μM GTPγS. Values shown are means ± S. E. for triplicate experiments.

3. Two EP_3 isoforms stimulate GTPase activity and inhibit adenylate cyclase with different efficiency.

Because both $EP_{3\alpha}$ and $EP_{3\beta}$ couple to Gi as described and $EP_{3\alpha}$ mediates inhibition of adenylate cyclase (described in Section 1), we compared potency of PGE_2 and its analogue in inhibition of adenylate cyclase in the two receptor systems. As shown in Figure 3-5, PGE_2 and M&B28767 dose-dependently inhibited forskolin-induced cAMP formation in $EP_{3\alpha}$-transfected CHO cells, the half maximal concentration for the inhibition being 1×10^{-11} M and 1×10^{-13} M, respectively. PGE_2 and M&B28767 also showed the dose-dependent inhibition in $EP_{3\beta}$-transfected CHO cells, but their half maximal concentrations for the inhibition (1×10^{-8} M for PGE_2 and 1×10^{-10} M for M&B28767) were three-orders of magnitude higher than those in $EP_{3\alpha}$-transfected CHO cells. $EP_{3\alpha}$- and $EP_{3\beta}$-transfected CHO cells showed comparable levels of the receptor expression (approximately 1.7 pmol of receptors / mg of membrane protein for both receptors), indicating that the difference in the half maximal concentration for the

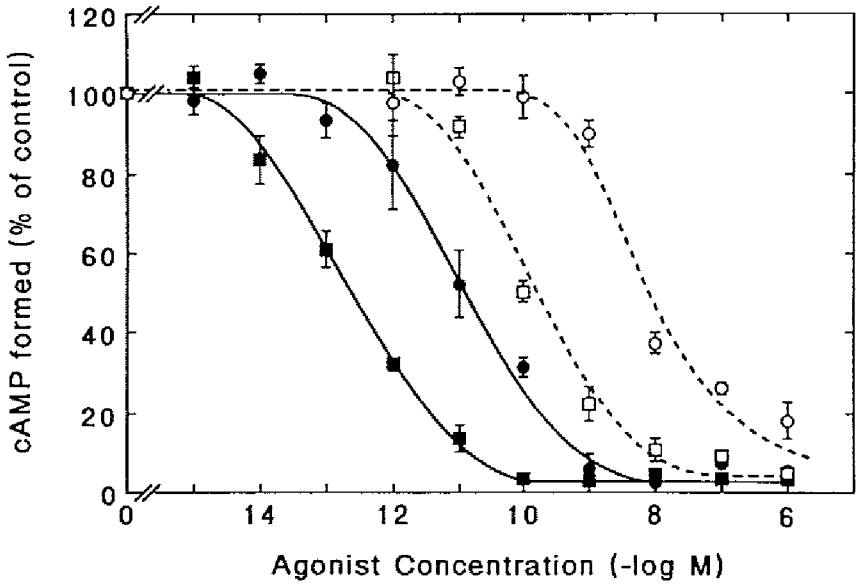

Fig. 3-5. Comparison of adenylate cyclase inhibition by the two EP_3 receptor isoforms permanently expressed in CHO cells. The $EP_{3\alpha}$-CHO cells (closed symbols) or the $EP_{3\beta}$-CHO cells (open symbols) were incubated at 37°C for 10 min with 1 μM forskolin in the presence of the indicated concentrations of PGE_2 (●, ○) or M&B28767 (■, □), and cAMP formation was determined. Each point represents the mean ± S.E. of triplicate determinations. cAMP formed in $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cells treated with forskolin was 16.6 ± 0.42 and 17.2 ± 0.32 pmol/10^5 cells, respectively.

inhibition is not due to the difference in expression level of each receptor. No significant increase in contents of cAMP and inositol phosphates was observed in either CHO cells by the addition of up to 1 μM of M&B28767 (data not shown). These results suggest that both EP_3 receptors are engaged in an identical intracellular function, i. e., inhibition of adenylate cyclase, and that the efficiency of signal transduction in $EP_{3\alpha}$ system is greatly higher than that in $EP_{3\beta}$.

Because the two receptors showed identical affinities to the ligands, the above results suggested that the different efficiency of inhibitory action of the two EP_3 systems is caused by the difference in efficiency of the ligand-receptor complexes to associate and activate G_i. We, therefore, analyzed the agonist-mediated activation of G protein by examining the effect of PGE analogues on GTPase activity in each CHO cell membranes. As shown in Fig. 3-6, PGE_2 and M&B28767 dose-dependently stimulated the GTPase activity in $EP_{3\alpha}$-transfected CHO cell membranes, the half maximal concentrations for stimulation being 1×10^{-10} M and 1×10^{-12} M, respectively. The two agonists also

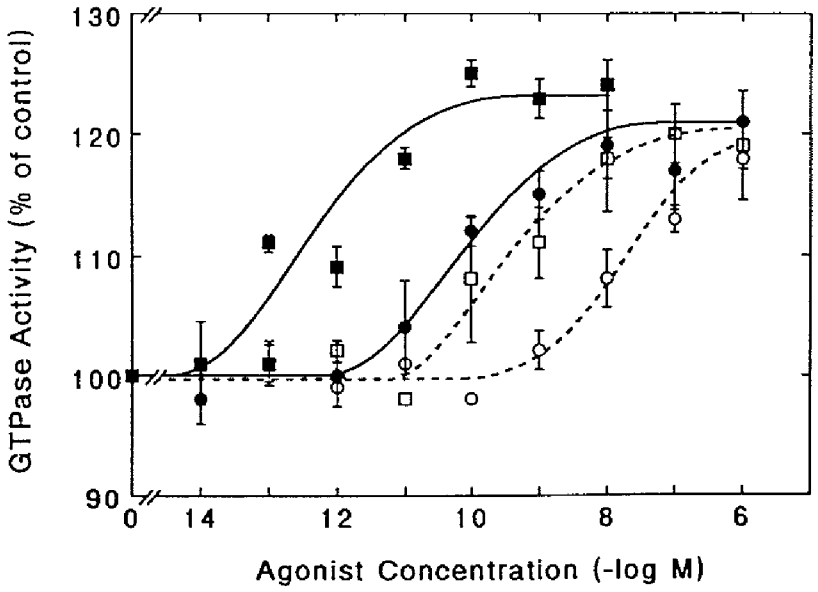

Fig. 3-6. Comparison of agonist-induced stimulation of the GTPase activity in CHO cell membranes expressing the two EP_3 receptor isoforms. GTPase activity of $EP_{3\alpha}$-CHO (closed symbols) or $EP_{3\beta}$-CHO (open symbols) cell membranes was measured in the presence of the indicated concentrations of PGE_2 (●, ○) or M&B28767 (■, □). Each point represents the mean ± S.E. of triplicate determinations. The basal GTPase activity of $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cell membranes was 16.6 ± 0.09 and 14.5 ± 0.18 pmol/min/mg of protein, respectively.

stimulated the GTPase activity in EP3β-transfected CHO cell membranes, but the half maximal concentrations (5×10^{-8} M for PGE_2 and 1×10^{-9} M for M&B28767) were about three-orders of magnitude higher than those in EP3α-transfected CHO cell membranes. The maximal increase in GTP hydrolysis by PGE_2 and M&B28767 in EP3α-transfected CHO cell membranes was about 20% above the control and was the same as that in EP3β-transfected CHO cell membranes. This result demonstrated that the different efficiency in inhibition of adenylate cyclase is due to that in the G protein activation by each receptor isoform.

4. Expression of $EP_{3\alpha}$ and $EP_{3\beta}$ in various tissues.

The relative abundance of mRNAs for the two isoforms in various tissues was investigated by PCR using the primers at both sides of the 89-bp sequence in the presence of 5'-end radiolabeled primer at one side as described by Wang et al. (1989) (Fig. 3-7). The upper and lower bands of the products corresponded to $EP3\alpha$ and $EP3\beta$, respectively. In any tissue expressing EP_3, $EP3\alpha$ was dominantly expressed over $EP3\beta$. In uterus and ileum, the $EP3\alpha$ isoform was expressed at 3~4-fold higher levels than the $EP3\beta$. In brain, very little amount of expression of $EP3\beta$ could be detected under this PCR condition in spite of significant amount of expression of $EP3\alpha$. In P-815 cells and stomach, on the other hand, the mRNA ratio for $EP3\alpha$ / $EP3\beta$ was about 1.5, which is in accordance with the results of our P-815 cDNA library screening. While the same amount of $EP3\alpha$ expression was observed in uterus and P-815 (Fig. 3-7), apparently the much higher level of expression of $EP3\beta$ was detected in P-815 cells.

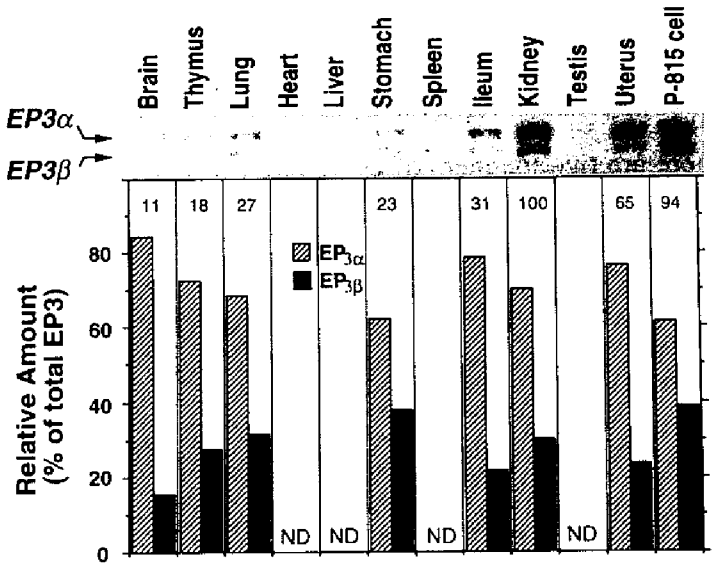

Fig. 3-7. Relative abundance of $EP3\alpha$ and $EP3\beta$ in various tissue. Aliquots (3 μl) of each PCR product were resolved in 1.5% agarose gel. Arrows point to the 732-bp (upper; $EP3\alpha$) and 643-bp (lower; $EP3\beta$) DNA fragments, acertained by sequencing. The relative expression level was calculated by the measurement of ^{32}P in each fragment. Numbers within the graph indicate the relative expression levels among tissues (kidney = 100). PCR condition and the number of amplification cycle were optimized by preliminary experiments. This figure shows an autoradiogram of one of three independent experiments.

DISCUSSION

The mouse EP_3 receptor from mouse mastocytoma P-815 library is a G protein-coupled rhodopsin-type receptor and engaged in inhibition of adenylate cyclase (see Section 1). Although various EP_3-mediated actions are believed to be mediated by inhibition of adenylate cyclase (Lu et al. 1988; Sonnenburg and Smith 1988), the concentrations of PGE_2 to inhibit cAMP formation varied among tissues, the IC_{50} values of PGE_2 being 10^{-8}, 10^{-10}, 10^{-12} M in rat hepatocytes (Melien et al. 1988), rat kidney cells (Torikai and Kurokawa 1983) and rat myometrium (Krall et al. 1984), respectively. In rhodopsin-type G protein-coupled receptors, guanine nucleotides have been demonstrated to modulate ligand binding affinity to receptors (reviewed by Gilman 1987; Lefkowitz and Caron 1988). Dependent on tissues, PGE receptors show different responses to guanine nucleotides in its ligand binding affinity. GTP decreases the binding affinity in bovine adrenal medulla (Negishi et al. 1987), but increases it in hamster adipocyte (Grandt et al. 1982) and canine renal medulla (Watanabe et al. 1986), suggesting that there are two manners of association of PGE receptors with G proteins.

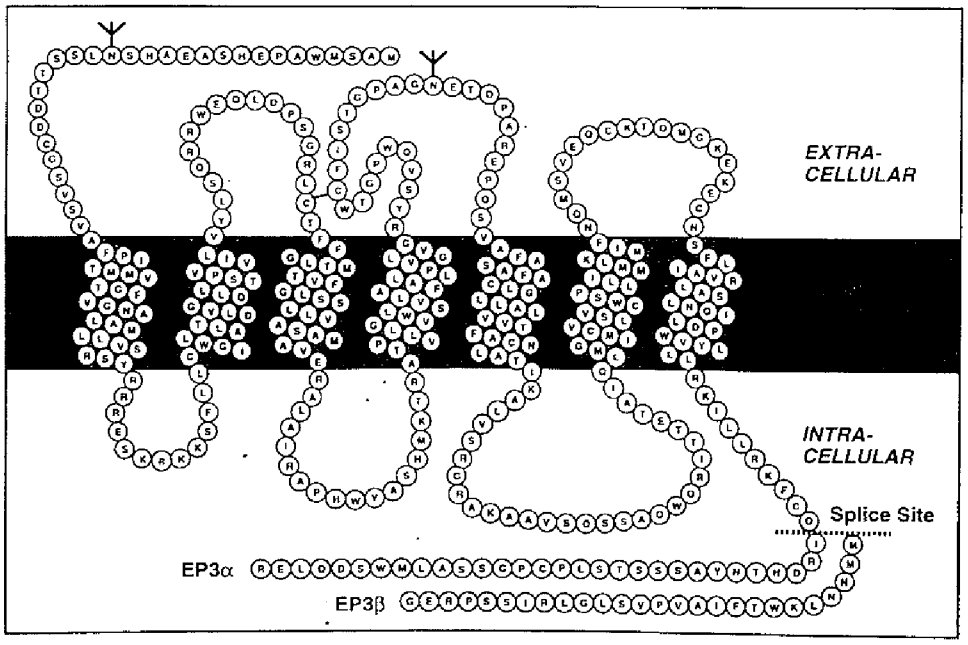

Fig. 3-8. Putative structures of the two EP_3 isoforms.

These differences in efficiency to agonists as well as responses to GTP of the EP_3 receptor implicate heterogeneity of this type of receptor.

Here two isoforms of EP_3 receptor represented by MP660 ($EP_{3\alpha}$) and MP653 ($EP_{3\beta}$) was identified (Fig. 3-8). The two isoforms show identical ligand binding specificity, couple to the same G protein, probably Gi, and inhibit cAMP accumulation. The second isoform cDNA, MP653, has the same coding sequence except deletion of 89-bp sequence in the cytoplasmic C-terminal domain. The nucleotide sequences delineating the boundaries of the 89-bp sequence in MP660 were consistent with consensus exon sequences for RNA splice junctions (Mount et al. 1982; Padgett et al. 1986). In fact, at the putative downstream splice junction of the 89-bp sequence, we have identified a 2.1 kilobase intron sequence using genomic PCR (data not shown). These results suggest that these two isoforms are generated from a single gene by alternative RNA splicing (Breitbart et al. 1987) (Fig. 3-9). Among G protein-coupled rhodopsin-type receptors, two dopaminergic D_2 receptors were the first example reported to be produced by alternative splicing which results in addition or deletion of 29 amino acids in the third cytoplasmic loop (Giros et al. 1989; Monsma et al. 1989; Dal Toso et al. 1989). However, the presence or absence of the 29 amino acids did not affect significantly the ability of the receptor to inhibit cAMP production. On the other hand, the two C-terminal

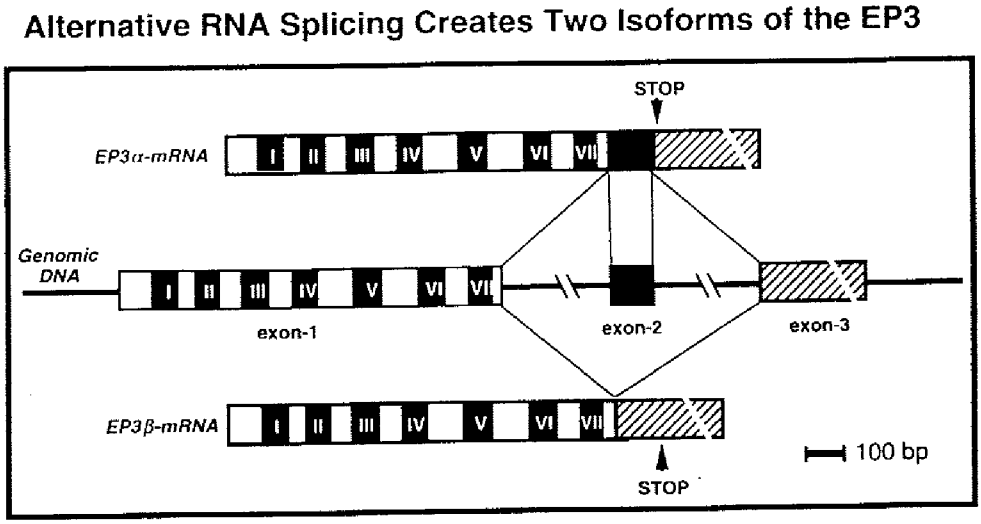

Fig. 3-9. Model system for alternative RNA spicing creating the two isoforms of the mouse EP_3 receptor.

end peptides of the EP_3 isoforms which are much the same in length but different in sequence showed significant effects on the mode of receptor-G protein interaction. Two kinds of effects were observed by analyses of the binding and signaling properties of the two isoforms. The first effect is on the binding affinity of the receptor in the G protein-free form. As shown in Fig. 3-4, the two isoforms showed identical binding affinity in the G protein-bound form. The addition of GTPγS increased Kd for PGE_2 of $EP_{3\beta}$, and decreased that of $EP_{3\alpha}$. PT treatment, by losing the ability of G_i protein to bind to a receptor (reviewed by Gilman 1987), mimicked the effect of GTPγS. These results demonstrated that ligand binding affinities in the two isoforms are either positively or negatively modulated by the cytoplasmic C-terminal tails in the Gi-free form. They also suggest that the Gi protein, by binding to these receptors, suppresses such actions of the cytoplasmic tails. The second effect is on the efficiency of the receptor to activate G protein. Although the two EP_3 isoforms couple to the same G protein, the EC_{50} values of PGE_2 and M&B28767 for G protein activation were different by three orders between the two EP_3 systems and this difference was reflected in the different IC_{50} values of the agonists to inhibit adenylate cyclase. Although the different EC_{50} may be partly attributed to the difference in the Kd values in the Gi-free receptors, it is not enough to explain the three orders of difference in G protein activation by the two isoforms. Thus, the two cytoplasmic C-terminal tails differentially affect the affinity of the ligand-occupied receptor for Gi protein; peptide-α makes the EP_3 receptor to associate Gi protein much more easily than peptide-β.

How do the two carboxyl tails cause such different effects on the receptor-G protein coupling? Hydrophobicity analyses demonstrated that the peptide-α and β show different hydrophobicity (Fig. 3-2). The peptide-α is hydrophilic, while the hydrophobicity of peptide-β was even higher than that in the TM VII. Peptide-β may be incorporated into the membranes and interact with other TMs, while peptide-α may be present in the cytoplasm. If so, this different hydrophobicity may influence the structure and function of the otherwise identical two isoforms of the EP_3 receptor. Alternatively, the difference

in the two receptors may be explained by the presence or absence of palmitoylation. In the β–adrenergic receptor, it has been reported that the cysteine residue within C-terminal tail region is palmitoylated with the fatty acid inserted in the membrane (reviewed by O'Dowd et al. 1989a), and this conformation of C-terminal domain is essential for the receptor-G protein coupling. The two isoforms contain a cysteine residue (Cys^{334}) in common at the ninth position out of the TM VII. An additional cysteine residue (Cys^{351}) is found in peptide-α at the twenty-sixth position while no cysteine is found in peptide-β. However, either position of cysteine is not consistent with the consensus position of the amino acid for palmitoylation (O'Dowd et al. 1989b). The difference in C-terminal tails may confer additional difference in receptor behaviors. The C-terminal peptide-α contains eight serine and threonine residues, while the peptide-β involves only four residues. In rhodopsin or β-adrenergic receptor, these residues within this region are proposed to be phosphorylated by their specific kinases that regulate the signaling activity of the ligand-occupied receptors (Wilden and Kuhn 1982; Benovic et al. 1989). These regulations have been suggested about PGE receptors (Robertson and Little 1983; Richelsen and Pedersen 1985). Therefore, the two C-terminal peptides might be differentially regulated by phosphorylation and involved in receptor desensitization and down-regulation.

The binding displacement experiment showed that PGE_2 and M&B28767 showed almost equal binding affinity. As for both agonists, the Kd in $EP_{3\alpha}$ system is about 1×10^{-9} M. On the other hand, the IC_{50} of agonists for inhibition of adenylate cyclase were lower (1×10^{-10} M for PGE_2; 1×10^{-12} M for M&B28767) than the Kd, indicating that agonist binding to a part of $EP_{3\alpha}$ receptor is enough to inhibit adenylate cyclase. Furthermore, in inhibition of adenylate cyclase activity and stimulation of GTPase activity, M&B28767 showed two orders lower IC_{50} and EC_{50} than PGE_2 in both EP_3 receptor systems. This suggests that the M&B28767-receptor complex couples to the G protein and, hence, to adenylate cyclase more efficiently than the PGE_2-receptor complex.

The result in the amplification of RNA (Fig. 3-7) demonstrated that in every tissue expressing EP_3, $EP_{3\alpha}$ is dominantly expressed but the two EP_3 mRNA coexist with

different relative abundance. The pattern of effect of GTPγS observed in $EP_{3\alpha}$ closely resembles that reported previously for the PGE binding sites in hamster adipocyte (Grandt et al. 1982) and canine renal outer medulla (Watanabe et al. 1986) which are believed to be EP_3 receptor. Thus, $EP_{3\alpha}$ appears to be responsible for the GTP sensitivity in these tissues. Considering $EP_{3\alpha}$ and $EP_{3\beta}$ giving different IC_{50} values of PGE_2 for adenylate cyclase inhibition, the proportion of the two isoforms would affect the ability of individual tissues to respond to PGE_2. The different proportions of the two EP_3 isoforms might, therefore, underlie the diversity of EC_{50} values of PGE_2.

Thus, the present study on two EP_3 receptor isoforms contribute to understanding not only the heterogeneity of the actions of PGE_2 but also functions of the cytoplasmic C-terminal tail of the G protein-coupled receptors. The C-terminal tail plays a role in signaling both from G protein to receptor and from receptor to G protein by modulating the ligand binding affinity of the receptor and the affinity of the ligand-receptor complex for a G protein.

CONCLUSION

In the present study, the author reach a conclusion as follows;

(1) Structural and functional natures of three subtypes of the mouse PGE receptor, EP_1, EP_2 and EP_3, were characterized.

(2) The mRNAs for the three PGE receptors show different tissue distribution and distinct cellular localization in the kidney.

(3) In the EP_3 receptor, there exist functionally different molecular forms with different C-terminal structures.

The actions of PGE_2 are mediated by multiple receptor subtypes and signal transductions according to its sites of actions (Fig. 4-1). The existence of multiple EP_3 isoforms different in their functions is supported by more recent studies, also in humans, and contributes to a variety of PGE_2 actions (Irie et al. 1993; Namba et al. 1993; Adam et al. 1994).

Obtained information in the present study on PGE receptor is surely to facilitate not only understanding of the physiological functions of PGE_2, but also development of more subtype-specific PGE analogues for therapeutic purposes.

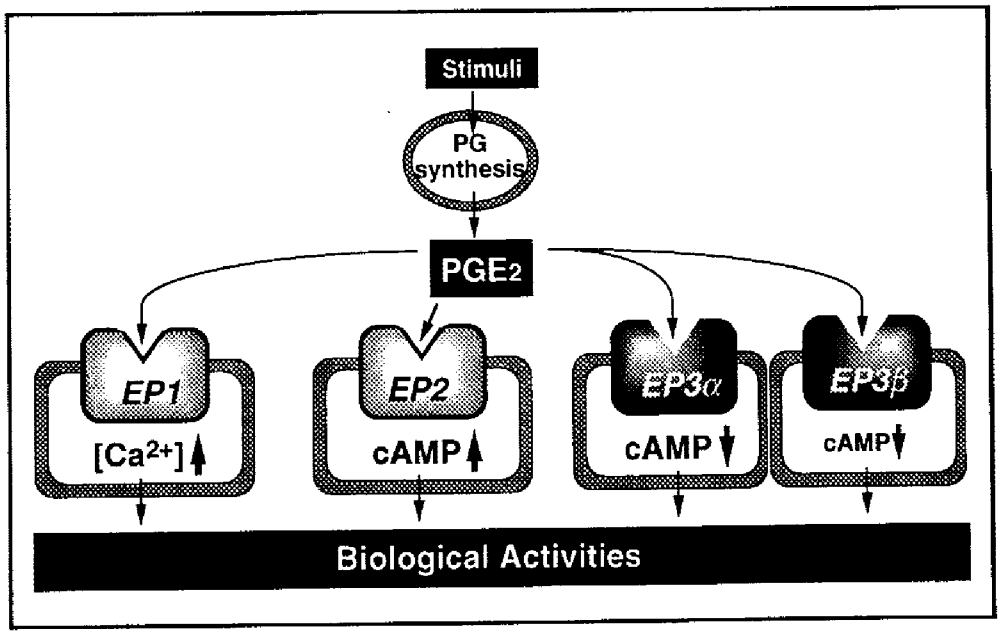

Fig. 4-1. Prostaglandin E receptors (subtypes and isoforms).

EXPERIMENTAL PROCEDURES

1. Materials

Ligands for PGE receptor subtypes were generous gifts from Dr. B. M. Bain of Glaxo Group Research Ltd. (GR63799X and AH6809), Dr. P. W. Collins of Searle (misoprostol and SC-19220), Dr. P. J. Gardiner of Bayer UK Ltd. (butaprost), Dr. M. P. L. Caton of Phone-Poulenc Ltd. (M&B28767) and Dr. K. H. Thierauch of Schering (sulprostone). [α-^{32}P]dCTP (3,000 Ci/mmol), [α-^{32}P]UTP (3,000 Ci/mmol), [α-^{35}S]CTP (1,000 Ci/mmol) and [5,6,8,11,12,14,15-^{3}H]PGE_2 (185 Ci/mmol) were obtained from Du Pont-New England Nuclear. PGE_1, PGE_2, PGD_2 $PGF_{2\alpha}$ and U46619 were purchased from Funakoshi Pharmaceuticals (Tokyo, Japan). Iloprost and the cyclic AMP [^{125}I] assay system were obtained from Amersham Corp. Forskolin and 3-isobutyl-1-methylxanthine were from Sigma. 17-phenyl trinor PGE_2 and 11-deoxy PGE_1 from Cayman Chemical (Ann Arbor, MI, USA); fura-2/AM from Dojindo Laboratory (Kumamoto, Japan). G_{i2} was purified from bovine spleen as described by Morishita et al. (1989).

Materials used in molecular biology are purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, USA), Stratagene (Heidelberg, Germany), USB (Ohio, USB), Invitrogen (Leek, Netherlands) or Promega (Madison, USA). These materials include the nucleic-acid-modifying enzymes, restriction endonucleases, sequence analysis system, cloning or expression vectors. Fundamental procedures in molecular biology are performed as described by Sambrook et al. (1989) and the protocols for kits and systems are according to manufacturer's instructions.

Synthetic oligonucleotides used in this study were all synthesized by a DNA synthesizer (Model 391; Applied Biosystems, Foster City, USA).

2. cDNA cloning of the mouse prostanoid EP_3 receptor.

2-1) Amplification of a cDNA fragment of the mouse TX receptor.

First of all, a pair of primers were designed based on sequence of the human TXA_2 receptor cDNA (HPL). Using these primers, a cDNA fragment (LT3; a fragment of the mouse TXA_2 receptor cDNA) was amplified from mouse lung cDNA.

First strand cDNA was synthesized from mouse lung total RNA by using random hexanucleotides as primers. PCR primers were designed based on the HPL sequences corresponding to the putative TM III and VI of the human TX receptor. Mouse lung cDNA served as template in 30 cycles of PCR with 1 min of denaturation at 95°C, 0.5 min of annealing at 60°C, and 1.5 min of extension at 72°C on Zymoreactor (Atto Corp., Tokyo, Japan). A single 418-bp cDNA fragment was amplified, and subcloned into pBluescript SK(+). A clone isolated (LT3) showed a sequence 78% homologous to the corresponding region of HPL.

2-2). cDNA cloning of the mouse EP_3 receptor.

Next, a cDNA clone, ML64, was isolated by cross-hybridization with LT3 from mouse lung cDNA library. This clone was a partial cDNA fragment encoding a novel receptor protein from the putative TM IV to the C-terminal tail. Northern blot analysis showed that mRNA for this clone is abundant in mouse mastocytoma P-815 cells, this cell line was chosen as a final cDNA source. Using ML64 as a probe, we isolated several positive cDNA clones identical to ML64 in the overlapping sequences. One representative clone (MP660) was picked up and used in the following expression analyses, and found to be a functional cDNA clone for the mouse PGE receptor subtype EP_3.

Mouse lung cDNA prepared by an oligo(dT) priming method was size-selected (> 1.8 kilobase) and inserted into the EcoRI site of λZAPII DNA with EcoRI adapters (Gubler and Hoffman 1983). The 1.9×10^5 clones derived from the cDNA library were screened by hybridization with LT3. Hybridization was carried out at 58°C in 6× SSC (900 mM NaCl and 90 mM sodium citrate) containing 5× Denhardt's solution (0.1%

Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin) and 0.5% SDS, and filters were washed at 60°C in 2× SSC containing 1% SDS. Among several clones hybridizing positively to LT3, we picked up one showing a signal apparently weaker than others, and subjected it to further screening. Nucleotide sequencing of the isolated clone (ML64) revealed that it was a partial clone. Using this clone as a hybridization probe, we then screened cDNA library of mouse mastocytoma P-815 cells for a full-length clone. From 7.2×10^5 clones of P-815 λZAPII library, nine clones were isolated and subjected to sequence analysis. Nucleotide sequencing was carried out on double stranded templates using dideoxy chain termination method. One clone (MP660) was a full-length clone with a 1,095-bp open reading frame.

2-3) cDNA cloning of the mouse EP_1 receptor.

In the cloning step of the cross-hybridization from the mouse lung cDNA library, we also picked up another cDNA clone, ML42. This clone was a partial clone, showing a sequence homologous but not identical to those of mouse EP_3, and TX receptors. Northern hybridization revealed that the mRNA for this clone is abundant in kidney, this tissue was chosen as a final cDNA source. Using ML42 as a probe, we isolated several positive cDNA clones identical to ML42 in the overlapping sequences. We picked up one representative clone (MK643), used in the following expression analyses, and found it functional cDNA clone for the mouse PGE receptor subtype EP_1.

2-4) cDNA cloning of the mouse prostanoid EP_2 receptor.

Previously, it was demonstrated that high level of PGE_2 binding is seen in mouse mastocytoma P-815 cells, neoplastic mast cells, and PGE_2 strongly stimulates adenylate cyclase (Yatsunami et al. 1981; Hashimoto et al. 1990), suggesting that the mastocytoma cells express EP_2 receptor. For isolation of a cDNA for the EP_2 receptor, we chose this cell line as a cDNA source, and performed homology screening using the partial cDNA fragment of the EP_3 (TM I-V), under the condition described above. A number of positive clones were isolated and subdivided into several groups by sequence and

restriction analyses. One group out of them showed sequence homologous but not identical to those of mouse EP_1, EP_3, and TX receptors. One representative clone (MP412) was picked up and used in the following expression analyses, and found to be a functional cDNA clone for the mouse PGE receptor subtype EP_2.

3. cDNA Expression in COS-1 Cells and Binding Assay.

The EcoRI inserts of the cloned cDNAs were individually subcloned into pcDNAI, a eukaryotic vector, and each resultant plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method (Sussman and Milman, 1984). After culture for 72 h, the cells were harvested and homogenized in a solution containing 25 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride using Dounce homogenizer (40 strokes). The homogenate was centrifuged at 800 × g for 10 min, and the pellet was suspended in the same buffer, homogenized, and centrifuged. The two supernatants were combined and centrifuged at 100,000 × g for 1 h. The crude membranes thus isolated were suspended in 20 mM MES, pH 6.0, containing 10 mM $MgCl_2$, 1 mM EDTA (buffer A). The 50 μg of membrane protein was used for $[^3H]PGE_2$ binding assay. As to the binding assay for EP1, a CHO cell clone stably expressing the receptor was used (described below).

For ligand binding to the two molecular forms of EP_3, each cell membranes were incubated with various concentrations (Scatchard plot analyses) or 2.5 nM (displacement experiments) of $[^3H]PGE_2$ in 100 μl of buffer A at 30°C for 1 h. The reaction was terminated by the addition of 2 ml of ice-cold buffer A, and the mixture was rapidly filtered through a Whatman GF/C filter. The filter was then washed four times with 2 ml of ice-cold buffer A, and the radioactivity associated with the filter was measured in 5 ml of Clearsol (Nacalai Tesque, Kyoto, Japan). Nonspecific binding was determined using a 1,000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

4. Establishment of the CHO cell clone showing stable expression of the cloned receptor.

cDNA transfection into CHO cells and establishment of the resultant cell line were performed essentially as described by Nakajima et al. (1992). The EcoRI fragments of the cloned cDNAs were individually inserted into EcoRI site of pdKCR-dhfr, a eukaryotic expression vector containing a mouse dihydrofolate reductase (DHFR) gene as a selection marker (Oikawa et al. 1989). The resultant plasmid was transfected to CHO cells deficient in DHFR activity (CHO-dhfr$^-$) (Urlaub and Chasin 1980) by the calcium phosphate method (Graham and van der Eb 1973) or by the lipofection method (Felgner et al. 1987). Cell populations expressing the cDNA together with DHFR were selected in α-modification of Eagle's medium lacking ribonucleosides and deoxyribonucleosides and containing 10% dialyzed fetal bovine serum (Cell Culture Laboratories). From these cell populations, clonal cell lines were isolated by single-cell cloning. Expression of the cDNA was assessed by RNA blotting. As a control, CHO cells were mock-transfected (transfected only with vector), and isolated. These cells gave no signal on RNA blotting. Crude membranes of CHO cells were prepared in the same way as those of COS cells.

5. Northern blot hybridization

Total RNAs from various mouse tissues were isolated by acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987) and poly (A)$^+$ RNAs were purified using Oligotex dT_{30}. Poly (A)$^+$ RNAs (5-10 μg) from each tissue were separated by electrophoresis on a 1.2% agarose gel, transferred onto nylon membranes (Hybond-N, Amersham), and hybridized with a ^{32}P-labeled fragment of the appropriate cDNA clone. For Northern hybridization for EP_1 mRNA, an antisense RNA probe was prepared with T7 RNA polymerase (Toyobo, Osaka, Japan), in the presence of ^{32}P-UTP. Hybridization was carried out at 68°C in 6× SSC for 12-16 h, and filters were washed twice at 68°C in 2× SSC.

6. cAMP assay

The established CHO or DNA-transfected COS-1 cells (5×10^5 cells / well) cultured in 24-well plates were washed with 0.5 ml of Hepes-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM Hepes, pH 7.4) and preincubated for 10 min in 450 µl of the solution and 1 mM 3-isobutyl-1-methylxanthine at 37°C. Then 50 µl of the test agent and forskolin (final 1 µM) in Hepes-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine was added to each well. After incubation for 10 min at 37°C, the reaction was terminated by the addition of 500 µl of the ice-cold 10 % ($^{W}/_{V}$) trichloroacetic acid. Cyclic AMP formed was measured by radioimmunoassay using Amersham's cAMP assay kit.

7. Ca^{2+} Measurement in Fura-2-loaded Single CHO Cells.

The intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) of single cells was measured by digital imaging microscopy described by Ito et al.(1991). The cells cultured on a coverglass attached to a four-well petri dish (Heraus Flexiperm-Disc, Germany) were incubated at 37 °C for 30 min with 5 µM fura-2/AM in culture medium containing 10% fetal bovine serum. After washing of the cells twice with 20 mM HEPES (pH 7.4), 115 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 13.8 mM glucose, the petri dish was placed on the thermostated stage, maintained at 37 °C in an Olympus IMT-2 inverted microscope. Paired recordings of fluorescence by excitation at 340 and 380 nm were then made at intervals of 5 sec. PGE_2 dissolved in HEPES-buffered saline solution was applied to the cells on the coverglass by the bath application method. Fluorescence images were obtained through a Hamamatsu SIT camera C2400-08H and stored in a digital image processor Argus-100. $[Ca^{2+}]i$ was calculated from the ratio of the fluorescence intensities at 340 and 380 nm on a pixel basis.

8. GTPase activity assay

Measurement of GTPase activity in $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cell membranes was described as follows;. The final reaction mixture contained 10 μg of each CHO cell membranes, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM 3-isobutyl-1-methylxanthine, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM ATP, 0.5 mM AppNHp, indicated concentrations of PGE_2 or M&B28767, and 0.25 mM [γ-^{32}P]GTP (0.3 μCi) in 100 μl of 20 mM Tris-HCl, pH 7.5. Reactions were initiated with the addition of each CHO cell membranes, conducted for 10 min at 30°C, and terminated by the addition of 0.5 ml of ice-cold 5 % Norit A and 0.1 % bovine serum albumin in 20 mM potassium phosphate, pH 7.5. Tubes were centrifuged for 5 min at 2,000 × g at 4°C, and the ^{32}Pi in 300 μl of each supernatant was counted in scintillation fluid.

9. Reconstitution of the EP_3 receptor with G_{i2} in pertussis toxin-treated membranes

Reconstitution of the EP_3 receptor with G_{i2} in PT-treated membranes was performed according to the method of Asano et al. (1985). CHO cells stably expressing EP_3 receptor isoforms were cultured in the presence or absence of 20 ng/ml PT for 9h. CHO cell membranes (50 μg) were incubated with or without G_{i2} purified from bovine spleen (10 pmol/mg of the membrane protein) at 0°C for 1 h. PGE_2 binding assays were carried out with 2.0 nM [^{3}H]PGE_2 in the presence or absence of 100 μM GTPγS.

10. Amplification of RNA

Measurement of the relative abundance of the two receptors expressed in each tissue was performed according to the method of Wang et al. (1989). Total RNA was isolated from mouse brain, thymus, lung, heart, liver, stomach, spleen, ileum, kidney, testis and uterus by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), and the RNAs were transcribed into cDNA by random hexanucleotide priming method using Moloney murine leukemia virus reverse transcriptase (Bethesda

Research Laboratories). Each cDNA derived from 2.5 μg RNA was used as template in a PCR with primers corresponding to nucleotide positions 651-680 (PCR I) and 1264-1293 (PCR II). The 5'-end ^{32}P-labeled PCR II (0.3 pmol; 1.0×10^6 cpm./pmol) was incubated in each PCR reaction (final 25 μl). Twenty-three cycles of PCR were performed using the following temperature profile: 94°C, 40 s; 60°C, 40 s; 72°C, 1.5 min. The number of amplification cycle was optimized for quantification of RNA given by preliminary experiments. DNA-resolved gel was dried and subjected to autoradiography, and the radioactivity of the gel corresponding to the bands was counted. Autoradiogram of one of three independent experiment was shown in the text.

11. Synthesis of cRNA probes

An antisense cRNA probe for mouse EP_3 was prepared as follows; a 1071-bp EcoRI-BamHI fragment of MP660 was prepared in pBluescript (Stratagene). After linearization of the template DNA, antisense riboprobes were prepared by transcription with T7 RNA polymerase in the presence of [^{35}S]CTP to a specific activity of 1.0×10^9 cpm/μg. Cold antisense riboprobes were synthesized by the same procedure with unlabeled nucleotides. After removing unincorporated nucleotides, riboprobes were degraded to ~150 base by alkaline hydrolysis. cRNA probes for EP_2 and EP_1 were similarly prepared from the respective cDNA (1857-bp EcoRI/XhoI fragment of MP412, and 1312-bp EcoRI fragment of MK643, respectively) for analyses of the PGE receptor subtype mRNAs. The nucleotide sequences of the three subtypes of PGE receptor showed low homology; at most 50% identity between EP_3 and EP_1. The three cRNA showed different patterns of hybridization, indicating that they did not cross-hybridize to the mRNA for the other subtypes of PGE receptor.

12. Preparation of kidney sections

Adult male ddY mice were anesthetized with ether and sacrificed by decapitation. Kidneys were removed immediately and frozen in isopentane at -50°C. Sections of 8 μm thickness were cut on a cryostat and thaw-mounted onto poly-L-lysine-coated slides. They were briefly air-dried and kept at -80°C until prehybridization.

13. In situ hybridization

The frozen sections were warmed to room temperature and fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min, rinsed in PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/ 0.9% NaCl for 10 min at room temperature. After dehydration in an ascending ethanol series, the sections were air-dried and stored at -80°C until use. Hybridization was carried out in a buffer containing 50% formamide, 2 × SSC, 10 mM Tris-Cl pH 7.5, 1 × Denhardt's solution, 10% dextran sulfate, 0.2% SDS, 100 mM DTT, 500 μg/ml sheared single-stranded salmon sperm DNA and 250 μg/ml yeast tRNA. The riboprobes preheated at 80°C for 3 min in 1 M DTT were added to the hybridization buffer at 7×10^4 cpm/μl. The hybridization solution was applied to the sections, which were then covered with a coverslip and sealed by rubber cement. After incubation at 57°C for 5 h, the slides were immersed in 2 × SSC to remove the coverslips, and then washed for 1 h by warming in 2 × SSC, 10 mM β-mercaptoethanol from room temperature to 60°C and cooling back to the room temperature. The sections were then treated with 20 μg/ml ribonuclease A in 0.5 M NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA, followed by an additional wash in 0.1 × SSC at 60°C for 1 h. After dehydration in ascending ethanol, the slides were air-dried and exposed to Amersham β-max film for 2 weeks at room temperature or dipped in NTB2 emulsion diluted 1:1 with distilled water. After exposure for 3-6 weeks at 4°C, the dipped slides were developed in Kodak D-19 developer, fixed, and counter-stained with hematoxylin-eosin.

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Notes for the thesis registration to 'Kyoto University Research Information Repository'

After publication of this thesis, the International Union of Pharmacology defined the classification of prostanoid receptors[1]. The type-E prostanoid receptors (EPs) have been subdivided into four subtypes, EP1, EP2, EP3 and EP4. The 'EP2' receptor referred in this thesis has been pharmacologically identified as 'EP4' receptor as reported by Nishigaki et al.[2]

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