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京大附図

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

1994

杉 本 幸 彦

**A Study for Structure and Function
of the Prostaglandin E Receptor**

1994

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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, PGE₂ in particular produces a broad range of biological actions in diverse tissues. These actions of PGE₂ include the contraction or relaxation of vascular and nonvascular muscles, and stimulation or suppression of secretion of the neurotransmitters and hormones (Fig. 0-2).

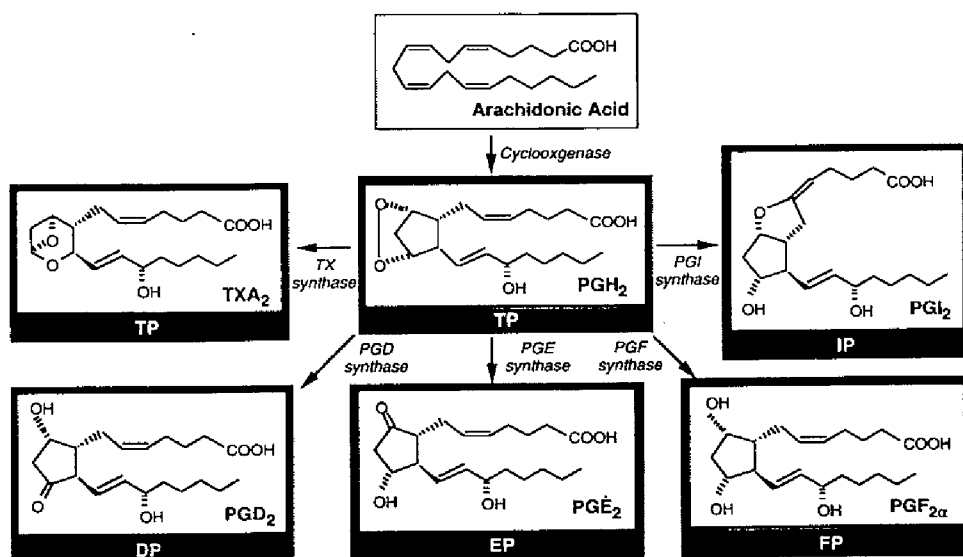


Fig. 0-1. Structures of the prostanoids. Prostanoids are synthesized from arachidonic acid by cyclooxygenase. Prostanoids exert their biological activities through specific cell-surface receptors.

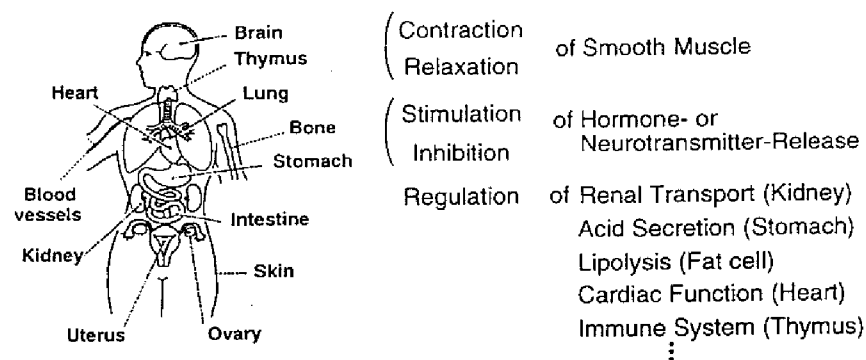


Fig. 0-2. Actions of prostaglandin (PG) E_2 . PGE_2 has a wide spectrum of physiological and pharmacological actions in diverse tissues.

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	<i>Gastrointestinal Smooth Muscle</i> ; Contraction <i>Central Neurons</i> ; Hyperalgesia
EP_2	cAMP I	<i>Trachea</i> ; Relaxation <i>Vascular Smooth Muscle</i> ; Relaxation <i>Chief Cells</i> ; Gastric Nonparietal Secretion <i>Hypothalamus</i> ; Pyrexia
EP_3	cAMP I	<i>Adipocytes</i> ; Inhibition of Lipolysis <i>Kidney</i> ; Inhibition of Sodium and Water Reabsorption <i>Parietal Cells</i> ; Inhibition of Gastric acid Secretion <i>Uterus</i> ; Contraction <i>Central and Peripheral Neurons</i> ; 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 EP₃ 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 PGE₂, but also development of more subtype-specific PGE analogues for therapeutic purposes.

The abbreviations used in the text are as follows;

AppNHp, adenylyl-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

EC₅₀, 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 hydrolase

GTP γ S, guanosine 5'-O-(3-thiotriphosphate)

IC₅₀, 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

[Ca²⁺]_i, intracellular Ca²⁺ concentration

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

HPL, the human thromboxane A₂ receptor

MK643, the mouse PGE receptor subtype EP₁

MP412, the mouse PGE receptor subtype EP₂

MP660, the mouse PGE receptor subtype EP₃ (EP₃α isoform)

MP653, the mouse PGE receptor subtype EP₃ (EP₃β isoform)

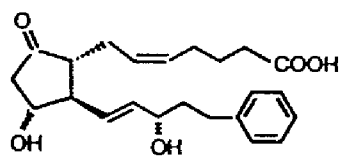
LT3, the mouse thromboxane A₂ receptor (a partial cDNA)

ML64, the mouse PGE receptor subtype EP₃ (a partial cDNA)

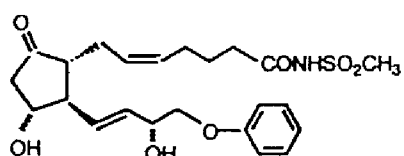
ML42, the mouse PGE receptor subtype EP₁ (a partial cDNA)

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

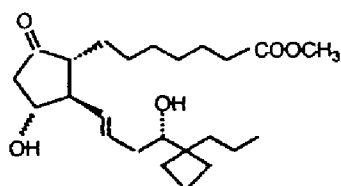
Agonists



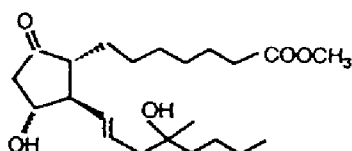
17-phenyl- ω -trinor PGE₂ (EP₁)



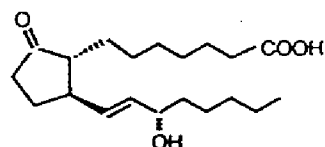
Sulprostone (EP₁, EP₃)



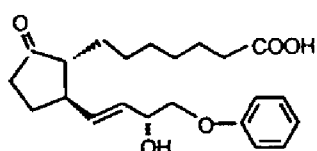
butaprost (EP₂)



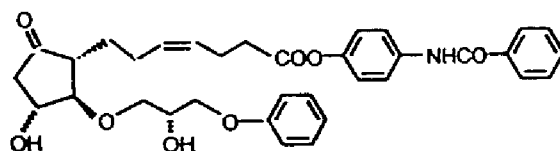
misoprostol (EP₂, EP₃)



11-deoxy-PGE₁ (EP₂, EP₃)

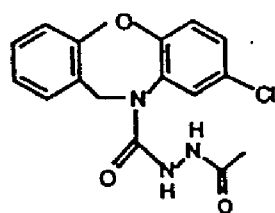


M&B28767 (EP₃)

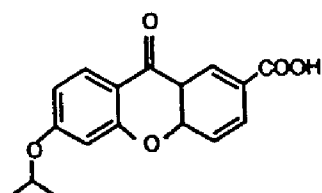


GR63799X (EP₃)

Antagonists



SC-19220 (EP₁)



AH 6809 (EP₁)

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 TXA₂ receptor and cross-hybridization screening. These receptor together with TX receptor belong to a new family of G protein-coupled receptors.

The mouse EP₃ receptor consists of 365 amino acid residues with an estimated molecular weight of 40,077. [³H]PGE₂ specifically bound to the membrane of mammalian COS cells transfected with the EP₃ cDNA. The binding of EP₃ was displaced with unlabeled PGs in the order of PGE₂ = PGE₁ > iloprost (a prostacyclin agonist) > PGD₂ > PGF₂α. The EP₃-selective agonists, M&B28767 or GR63799X, potently competed for the [³H]PGE₂ binding, but no competition was found with EP₁- or EP₂-selective ligands. PGE₂ and M&B28767 decreased forskolin-induced cAMP formation in a concentration-dependent manner in Chinese hamster ovary (CHO) cells permanently expressing EP₃. Northern blot analysis demonstrated that the EP₃ 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 EP₂ receptor consists of 513 amino acid residues with an estimated molecular weight of 56,157. [³H]PGE₂ specifically bound to the membrane of COS cells transfected with the EP₂ cDNA. The binding to the membrane was displaced with unlabeled PG in the order of PGE₂ = PGE₁ >> iloprost (a prostacyclin agonist) ≥ PGF₂α ≥ PGD₂. The binding was also inhibited by misoprostol (an EP₂ and EP₃ agonist), but not by sulprostone (an EP₁ and EP₃ agonist), and SC-19220 (an EP₁ antagonist). PGE₂ markedly increased cAMP level in COS cells transfected with the EP₂

cDNA. The EP₂ mRNA is widely expressed in various tissues, the abundant expression being observed in ileum, thymus and mastocytoma P-815 cells.

The mouse EP₁ receptor consists of 405 amino acid residues with an estimated molecular weight of 42,966. [³H]PGE₂ specifically bound to the membrane of CHO cells stably expressing EP₁. The binding to the membrane was displaced with unlabeled PGs in the order of PGE₂ > iloprost (a prostacyclin analogue) > PGE₁ > PGF₂α > U-46619 (a thromboxane A₂ analogue) > PGD₂. The binding was also inhibited by 17-phenyl trinor PGE₂ (an EP₁ agonist), and sulprostone (an EP₁ and EP₃ agonist), but not by 11-deoxy PGE₁ (an EP₂ and EP₃ agonist), and butaprost (an EP₂ agonist). PGE₂ induced a rapid increase in intracellular Ca²⁺ concentration in CHO cells expressing the receptor. The mRNA for EP₁ 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 PGF₂ α , and to PGD₂ in addition to PGE₂ or PGE₁. Recently, Hirata et al. identified the structure of the human TXA₂ 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*".

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 EP₃ receptor, MP412 for the EP₂ receptor, and MK643 for the EP₁ receptor. The EP₃ polypeptide consists of 365 amino acid residues with an estimated molecular weight of 40,077. The EP₂ polypeptide consists of 513 amino acid residues with an estimated molecular weight of 56,157. The EP₁ 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 EP₁ receptor has two potential *N*-glycosylation sites at the extracellular amino- (N-) terminal region, and the EP₂ and EP₃ 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 EP₂ receptor possesses a relatively longer third intracellular loop (59 amino acid residues) than the EP₃ 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-D₁ (Dearry et al. 1990) receptors.

a

CAGTGCACCTCTGCTGCTATCCCGCAGCTGAGCCGGGAGGCTCCGGCCCCGTGCGCCCTACCGTGGCCCCGCACT	-118	GCGGCGGGCGATGGAGAGCAGAGCCTGGGCTCCGGCTGTCCCC	-76
M A S M W A P E H S A E A H S N L S S T T D D C G			25
ATGGCTAGCATGTGGGCGCCGAGCACTCTGCTGAAGCGCACAGCAACCTGTCAAGTACTACCGACGACTGCGGC			75
S V S V A F P I T M M V T G F V G N A L A M L L V	I		50
TCCGTGTCCGTGGCCTTCCCATCACCATGATGGTCACTGGCTTCGTGGGCAACGCGCTGGCCATGCTGCTGTG			150
S R S Y R R R E S K R K K S F L L C I G W L A L T		II	75
TCGCGCAGCTACCGGCGCCGAGAGCAAGCGCAAGAAGTCTTTCCTGCTGTGCATTGGCTGGCTGGCGCTCACC			225
D L V G Q L L T S P V V I L V Y L S Q R R W E Q L			100
GACTTAGTGGGGCAGCTCCTGACCGCCCGGTGGTCACTCCTCGTGTACCTGTACAGCGACGCTGGGAGCAGCTC			300
D P S G R L C T F F G L T M T V F G L S S L L V A	III		125
GACCCATCGGGCGTCTGTGCACTTCTTCGGGCTAACCATGACAGTGTTCGGGCTATCCTCGCTCCTGGTGGCC			375
S A M A V E R A L A I R A P H W Y A S H M K T R A			150
AGCGCCATGGCCGTGGAGCGCCCTGGCCATCCGTGCGCCGCACTGGTATGCCAGCCACATGAAGACTCGCGCC			450
T P V L L G V W L S V L A F A L L P V L G V G R Y	IV		175
ACGCGGTACTGCTGGGCGTGGCTGTCTGTGCTCGCCTTCGCGCTGCTGCGGCTGTGGGCGTGGGCGGTAC			525
S V Q W P G T W C F I S T G P A G N E T D P A R E			200
AGCGTGCACTGGGCGGCGACGTTGGTGTTCATCAGCACCGGCGCGGCGGCAACGAGACAGACCCTGCGCGCGAG	V		600
P G S V A F A S A F A C L G L L A L V V T F A C N			225
CCGGCAGCGTGGCCTTTGCCCTCCGCTTCGCGCTTGGCTTGGCTCTGGTGGTGACCTTTGCCTGCAAC			675
L A T I K A L V S R C R A K A A V S Q S S A Q W G			250
CTGGCGCCATCAAGCCCTGGTGTCCCGCTGTGCGGCAAGCGCCGCTCTCGCAGTCCAGCGCCAGTGGGGC			750
R I T T E T A I Q L M G I M C V L S V C W S P L L	VI		275
AGAATCACCACGGAGACGGCCATCCAGCTCATGGGATCATGTGTGCTGTCCGTCTGTTGGTGGCGCTATTG			825
I M M L K M I F N Q M S V E Q C K T Q M G K E K E			300
ATAATGATGTTGAAAATGATCTTCAATCAGATGTGGTTGAGCAATGCAAGACACAGATGGGAAAGGAGAGGAG	VII		900
C N S F L I A V R L A S L N Q I L D P W V Y L L L			325
TGCAATTGCTTTCTAATTGCAAGTTCGCTGGCTTCGCTGAACAGATCTTGGATCCCTGGGTTTATCTGCTGCTA			975
R K I L L R K F C Q I R D H T N Y A S S S T S L P			350
AGAAAGATCCTTCTCGGAAGTTCTGCCAGATCAGAGACCACCAACTATGCTTCCAGCTCCACCTCCTTGCCC			1050
C P G S S A L M W S D Q L E R			365
TGCCCAGGCTCCTCAGCCCTGATGTGGAGTGACCGAGTGGAAAGATGATGAACAACCTGAAGTGGACTTTCATTG			1125
CAGTACCTGTTTCCCTGGGCTGTGAGAATTTCTTCTCCAGGGAAGGATGACTGAGTATTTGGATTGTATCTTCT			1200
TTTGGCCCTCAATTTTAAGTTTTCCTTGCCATTAACACACCGAGACAAGCTTCTTAGGATAATCTGAGAGTCTG			1275
GTTGTTAGCTGGTTCCTGTGAAGACTGAAGACTCTGCCTTGAGACGGGGGCAAGACGACACAGAGCAGCATGGA			1350
GAGACTCAGTCAGAAATATCTCCAGCCTCAGAACCTTTGTGGACATGGACACCTTCATGTATTGATAGTCTGAC			1425
TCCTTAAATAGGCTGTGAAAAGCAGCATAAGTTTAAACAGTGAAGCATCAATGTGTGAGAGCAATGTTCA			1500
TCTAATAAGCCATGAGCCAAACAGACAAAAGTCTACATGAGAGGCAAGAGAGATTCTGCAAGGGTATTTGTG			1575
CCAAGAAGGTATACAGTACCACAGAGTTGTCTCCTCAGTGAAGTGGGAAATAAGTTTCTAATTTAATTCTAATT			1650
ACTGGCTCCTCAGTAATTCAGGAATCGTCCCATCATTTCCCTGCTTTTAAAGGGAGAACTTTAGCTAAAGACACA			1725
TTCCAGGTGTCACTAACAGTTCCAAAGCTAGGTGACTAAATGTTTCAGCTAGAGCTGTTAAAGGAAAACAGCTA			1800
ATTATCATTTCCAGTCCAATGCTATTTTGAATTACTATCTACTTAAGATTTCTCATAATTTGTGCTCAGGCAGCA			1875
CAATAAAAGGGGGGGGCAAAATTACTAAGTGACAGTTATTCTGCATCTAAGTCTGTGACTTTTATGAAATAA			1950
AATGATTTGTCTGTTGAAATAAAAAAAAAAAAAA			1989

Fig. 1-2. Nucleotide and deduced amino acid sequences of EP3- (a), EP2- (b) and EP1-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.

	-121
TTCCAAGCTTTTTTGAAGCAAGATACTCTGACCTCAGTTCCGGAAAGTTGGCAGCCACCGAGCCCCGGTTCGGAGACAGCAAAAAGCTTGACAATTCGCGACTCGGTGGGAAGAGACTG	-1
ATGGCTGAGGTTGGAGGTACCATTCTAGATCGAACCGTAGCTCCAACGCTGTGTGTACTAACCACCACCATCATGTCCATCCCCGAGTCAACGCGTCTTCTCTCCACTCCGGAG	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 T I H S I P G V N A S F S S T P E	40
AGGCTGAACAGCCCGGTGACCATCCCAGCAGTGGTTTCATCTCGGGGTGGTGGGCANCTGGTGGCCATCTGATGATTGTGCAAGTCCGCGAAGGACAGAGAAGACGACCTTTTAC	240
R L N S P V T I P A V H F I F G V V G A V L V L G C K S R K E Q K E T T F Y	80
I	
ACTCTAGTATGTGGCTGGCTGTCACTGACCTTCTGGCACCTTGTGGTAAGCCCGGTGACCATCGCCACATACATGAAGGCCAGTGGCCGGAGACAGGCACTGTGTGACTATAGC	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	
ACCTTCATCCTACTTTTCTTCGGTCTGCGGGTCTCAGCATCATCTGTGCCATGAGCATCGAGCGCTACCTGCCATCAACCAACCGCTACTTCTACAGCCACTACGTGGACAAGCGGCTG	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	
GCGGGCTCACACTCTTCGCCATCTATGCATCTAAGTGCTGTTCTGCGCGCTGCCAACATGGGCTTGGGAGATCCGAGCGGCAGTACCGCGGCACCTGGTGCTTCATCGACTGGAC	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	
ACCAAAGTAAAGCGCTACGCGCCCTTCTTACATGTACGCGGCTTCAGGTCCTTCTCATCTTGCACCGTGCTCTGCAAGTGCTGGTGTGCGGCGCGCTGCTCCGCATGACACCGG	720
T N V C T A A C F S S F A G F S F L I A V N V L V C G G L A T R M H R	240
V	
CAGTTTCAGCCCGCACCTGTGGGACGAGCAGCACCATCGGGCTGCCCGCCGCGGTAGCTTCGGTGCCCTGTGGGGCCACGCTGGGGCTCCCGCAGCTCGAGCGCTCAGC	840
C F M H R R T S L G T E Q H H A A A A A V A S V A C R G H A G A S P A L R G L S	280
★	
GACTTTCGCGCCCGCAGGAGTTTCCGGCGCATCGCGGGTGGGAGATCCAGATGGTCATCTTACTCATCGCCACCTCTCTGGTGGTGCTCATCTGCTCCATTCGCGTCTGGTGGAGTG	960
D F R R R R S F R R I A G A E I O 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	
TTCAATTAACAGTTATATCAGCCAAAAGTGGTGAAGACATCAGCAGAAACCCAGATTTCAGCGCATCAGGATTGCTTCTGTGAACCCCATCTGGACCCCTGGATTACATCTTCTT	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	
CGGAAGACTGTGCTCAGTAAAGCATAGAGAAGATCAAGTGCTCTTCTGCGCGATGGGCGGTTCCGGCAGACAGCTCGGCCAGCAGCTGCTCAGAGAGTCGAGGACATCTTCGCGC	1200
R K T V L S X 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
ATGTCCGGGCACTCTCGCTCTCTCGCCCGGAGGTTAAAGGAGATCAGCAGCAGCTCCAGACCTCTCTGTACCTGCCAGACCTGACTGAAGCAGGCTCGGAGGAGCGGAATTGCTT	1320
H S G H S R S F L A R E L K E I S T S Q T L L Y L P D L T E S S L G G R N L R	440
CCAGGTTTCGATGCGCTGGGCTGACCCAAAGCAGACACCACCTCGCTGAGAATTTTGGGAATTTCCGAGACCTCAGACTCTCCAGGGCCAGGACTCTGAGAGTGTCTGTTGGTGGAT	1440
P G S H G H 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
GAGGTTAGTGGGAGCCACAGAGAGGAGCCTGCCTCTAAAGGAAACTCTCTGCAAGTCACTTCCCGAGTGAAGCTCTGAAATATCTGAAAAATGTATATAGTAGCTAAAGGGGAATCT	1560
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
TATAAAATCTGTGTAATAGACATACATGATCTCAGAAAGGCGTCTTCTCATCTGGACTCCCACTAGAGAAACAGGCGAGCTCTCGAGGCTCGAGACTGAGGTCCTT	1680
GATGCGCCAGCTTGAAGCACATTGGCTGTCTTCTGATGTGACTCGAGATTGCAAGTTGCAACTTGGCAGCTTTTCTACTGGACAGGAAGTCCGAAGCTACGCTATTGTGCATAGC	1800
AAAAGAGCTTPTCTATTGTGGCACAATACCAGGGGTCCAGCTACTGGAAGGGCTCTACCCCAAATCTGAGGACTACCTTACAGCTGACTTAAAGTGCTCACTAAAGCATGAAATGTGAATTT	1920
TTATTTGTGGAATATAATTTAAGGTTATTTATGTTCTCTCTGTGAGAAAGGTTATGTTTAATACAGGTTATAAAACACATGATAGCCCTCTCTGCCAATATAACAGCTAAATATT	2040
TCGATGTGTTATTTTATTTCCATAAACAGTTCAGGCCAAGGTGTAAGGACAGATGAAACTAATATCTATAAATAGATATAAATTTTAAATAGTTTAAAGTATCATCAAGAGAAA	2160
ATAAAGTAGTATTTAAGATGTGAATAAACAACCTAAAATATATTTTCCAAGCTATATAATAATGAAGAAAATAAAACATTACATTATTTATCCAGAAAATCTGTGATTTAGGATT	2280
ACCTAACATTGCTGGTAATATTTTCAAC	2309

	-71	CGACGAGGAGCAAGTTCACGAGCATGGGACCTGCATCTGAGCAGCACTGGCCCTCTTGCCCACTGAT	-1
ATGAGCCCCGCGGGCTTAACCTGAGCCTAGCGGATGAGGCAGCAACGTCGCACACCCAGGCTCCCCAATACATCTGTGGTGTGCGCAACAGGCGGATAATGGCACATCACCAAGCGCTC	120		
M S P C G L N L S L A D E A A T C A T P R L F N T S V V L P T G D N G T S P A L	40		
↑		↑	↑
CCTATCTTCTCCATGAGCGTGGGTGCTGTGTCCAACGCTGCTGGCGCTGGCGCTGCTGGCCAGGTTGCAGGCCGAATGCGCGCGCGCGCTGCGCTGCCACCTTCTCTGTGTGTCTGCTGCC	240		
P I F S M 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 R S A A T F L L F V A	80		
AGCCTCCTTGCATCGACCTAGCAGGCCATGTGATCCCGGGCGCCCTGGTGTCTCGCTGTATACTCGCGGGCGTGCCTCTGCTGGAGGGCGGTGTCATTTCCTGGGTGGCTGCATGGTC	360		
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	
TTCTTCGGGCTGTGCCCACTTTTGCTTGGCTGGGCAGCGCGTAGAGCGCTGTGTGGGTGTACACGAGCCACTGATCCACGCGCGCGGTATCTGTGGCCCGCGCACGCGCTGGCACTA	480		
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 G V S V A R A R L A L	160		
		IV	
GCCGTGCTGGCTGCCATGGCTTTGGCAGTGGCGCTGCTGCCACTGGTACACGTGGGTGCTACAGATTACAGTACCCCTGGCACCTGGTGTTTTATTAGCCTTGGGCTCTGTGGAGGTTGG	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		
CGCCAGGCGTGTCTTCCCGGCCCTCTTCGCGCGCCTTGGCCTGGCCGCGCTCTCTTCGGGCATTAGTGTGCAATACGCTCAGCGGCGTGGCGCTCTCTCCGTGCGCGCTGGAGACGGCGTGC	720		
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	240		
V			
TCTCGACGATTCGGAAGACCGCAGGTCGCGATGATCGCGGCGCTGGGATCTCGTGGACCCGCTTGGCTCCGCTCGTCTGCCTCATCCATCACTTACGCCACAGCCACCTCCGC	840		
S R R F R K T C C G A P D D C R R W G S R G P R L A S A S S I G T S A T A T L R	280		
AGCTCTCGGGCGGCGGCTCCGCGCGCAGGTTCCACGCACACGATGTGGAATGGTGGGCCAGCTCGTGGGCATCGTGGTGTGCTGTCATCTGTCGAGGCCCTGCTGGTGTGGTG	960		
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			
GTGTGTGCCATCGGGGCTGGAACCTCTAACTCCCTGCACGCGCCACTCTTCTGGCTGTACGCGCTCGCATCTGTGGAACAGATCTTGGACCATGGGTGTACATCTCTGCTGGCGCCAGGCC	1080		
V L A I G G W N S N S L Q R P L F L A V V R L A S W N Q I L D P W V Y I L L R Q A	360		
VII			
ATGCTCGCCAACTGCTTCCGCTCTTACCCCTGAGGCTCAGTGCACCAAGGCTGGTCCAACGGAGCTGGGCTTAACCAAGAGTGCCTGGGAAGCCAGTTCACTGCGTAGTCTCCCGGCACAGT	1200		
H L R Q L L R L L P L R V S A K G G P T E L G L T K S A W E A S S L R S S R H S	400		
GTTTCAGTCACTCTGTAATGTGCCCGAAGCTAAGTCAAA	1241		
G F S H L	405		

3. Binding characters of the three PGE receptors.

The cloned mouse EP₃ receptor was expressed in COS-1 cells and its membranes were subjected to binding assays using [³H]PGE₂. Scatchard analysis of this binding yielded a dissociation constant (K_d) 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]PGE₂ was inhibited by unlabeled PGs in the order of PGE₂ = PGE₁ > iloprost, a PGI₂ analogue > PGF₂α > PGD₂. We further characterized the specificity of this [³H]PGE₂ binding using ligands specific for PGE receptor subtypes. As shown in Fig. 1-3b, among various PGE analogues, only EP₃-specific agonists, GR63799X and M&B28767, specifically competed for the [³H]PGE₂ binding with equal potency, and they were more potent than PGE₂ itself. On the other hand, no competition was found at all with either SC-19220 (an EP₁-specific antagonist), or butaprost (an EP₂-specific agonist).

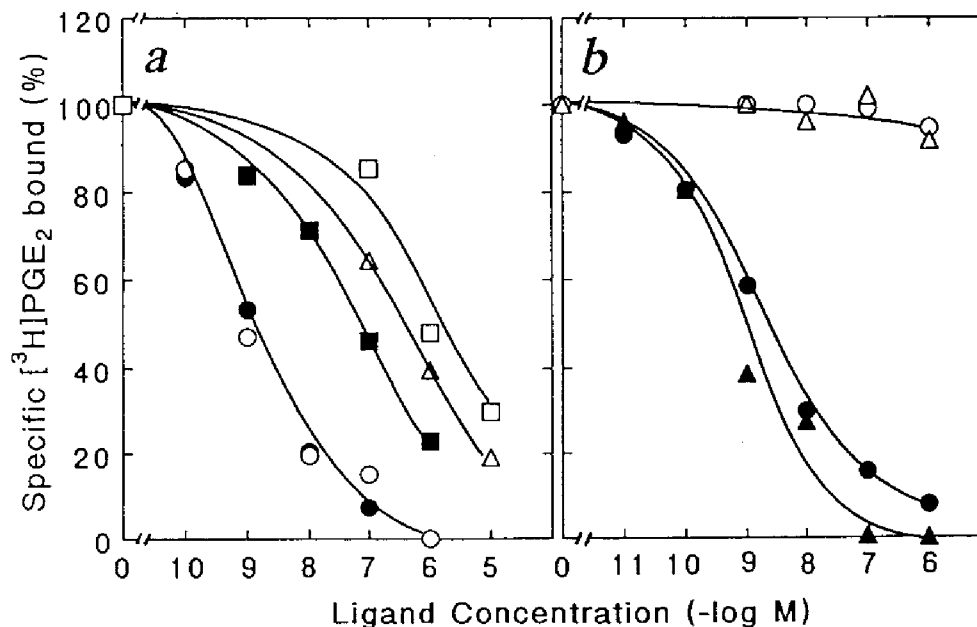


Fig. 1-3. Binding of [³H]PGE₂ to EP₃ in the MP660-transfected COS-1 cell membranes. *a*. Displacement of [³H]PGE₂ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations and [³H]PGE₂ binding was determined. PGE₂ (○), PGE₁ (●), iloprost (■), PGF₂α (△) and PGD₂ (□). *b*. Displacement of [³H]PGE₂ binding by subtype-selective PGE analogues. Ligands used are M&B28767 (▲), GR63799X (●), butaprost (△) and SC-19220 (○).

When the EP₂ receptor was expressed in COS-1 cells, [³H]PGE₂ 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]PGE₂ binding to the membrane of untransfected cells was almost negligible (data not shown). Fig. 1-4a shows the binding specificity of the EP₂ receptor. Specific [³H]PGE₂ binding was inhibited by unlabeled PG in the order of PGE₂ = PGE₁ >> iloprost, a stable PGI₂ analogue $\geq$ PGF₂ α $\geq$ PGD₂. Among these PGs, the EP₂ receptor shows higher specificity for PGE₁ and PGE₂ than EP₃ receptor. As shown in Fig. 1-4b, the binding of EP₂ receptor was inhibited by misoprostol (an EP₂ and EP₃ agonist) and more weakly by M&B28767 (an EP₃ agonist). On the other hand, sulprostone (an EP₁ and EP₃ agonist), SC-19220 (an EP₁ antagonist), and butaprost (an EP₂ agonist), did not inhibit it.

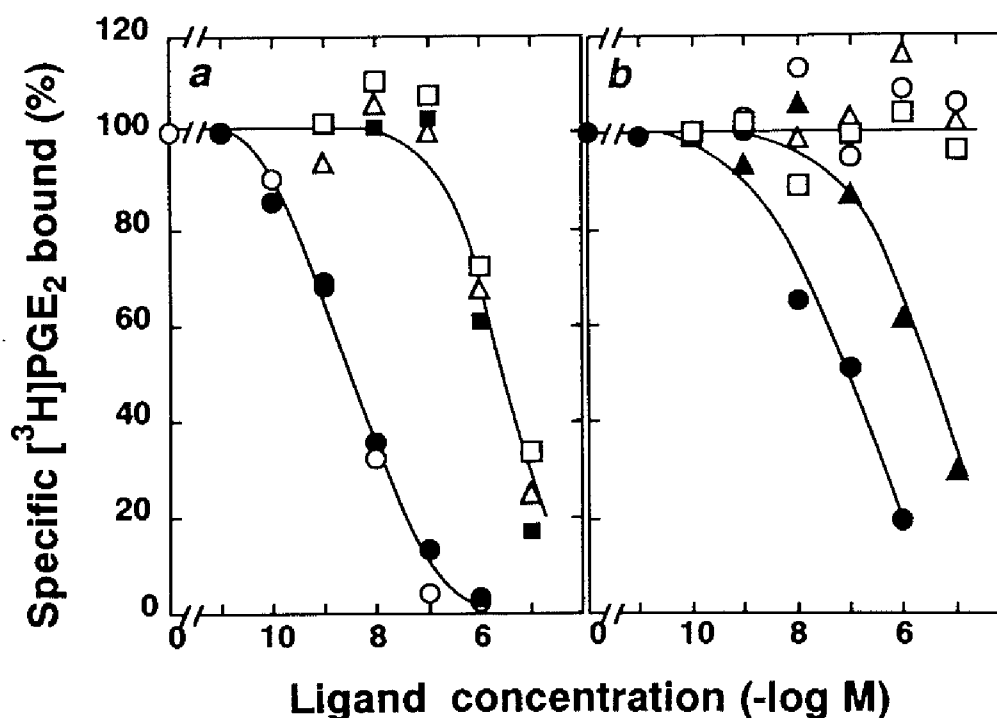


Fig. 1-4. Binding of [³H]PGE₂ to EP₂ in the MP412-transfected COS-1 cell membrane. a, displacement of [³H]PGE₂ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations, and specific [³H]PGE₂ binding was determined. ○, PGE₂; ●, PGE₁; ■, iloprost; △, PGF₂ α , and □, PGD₂. b, displacement of [³H]PGE₂ 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 EP₁ receptor, and the binding activities of this receptor was investigated by using [³H]PGE₂. [³H]PGE₂ 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 EP₁ receptor yielded a dissociation constant of 21 nM. Fig. 1-5A shows the binding specificity of EP₁ receptor. Specific [³H]PGE₂ binding was inhibited by unlabeled PGs in the order of PGE₂ > iloprost (a stable prostacyclin analogue) > PGE₁ > PGF₂α > U-46619 (a stable TXA₂ analogue) > PGD₂. In contrast to EP₂ and EP₃ receptors, this receptor shows higher affinity to PGE₂ than PGE₁. As shown in Fig. 1-5B, the [³H]PGE₂ binding of EP₁ receptor was inhibited by 17-phenyl-18,19,20-trinor PGE₂ (an EP₁ agonist), and by sulprostone (an EP₁ and EP₃ agonist), while the inhibition by M&B28767, by 11-deoxy PGE₁ (an EP₂ and EP₃ agonist), and by butaprost, was weak or negligible. On the other hand, AH6809 (an EP₁ antagonist) showed only weak inhibition of the binding.

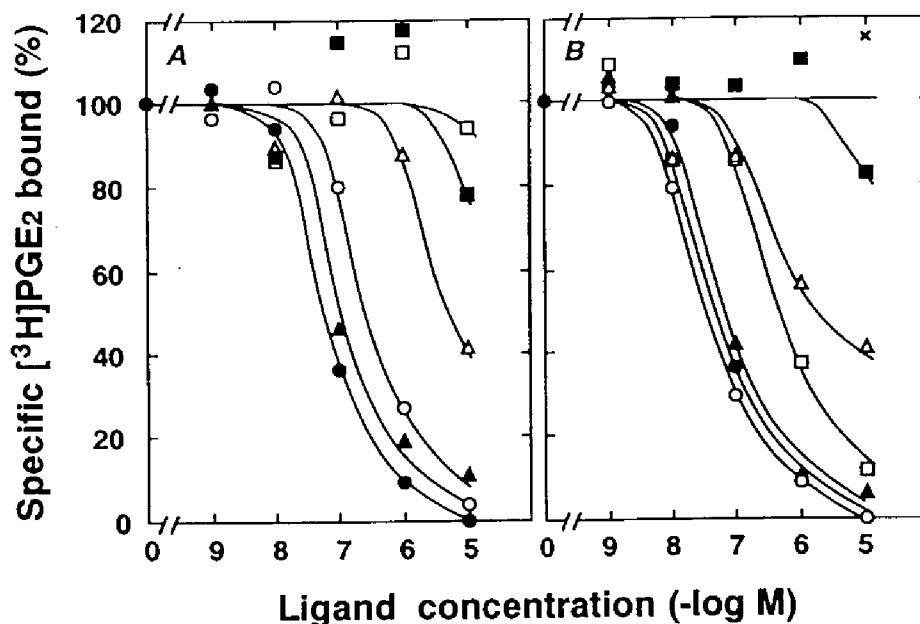


Fig. 1-5. Binding of [³H]PGE₂ to EP₁ in the CHO cell membrane. A, displacement of [³H]PGE₂ binding by various PGs. Unlabeled PGs were added to the binding assay mixture at indicated concentrations, and specific [³H]PGE₂ binding was determined. ● PGE₂; ▲ iloprost; ○ PGE₁; △ PGF₂α; □ PGD₂; ■ U-46619. B, displacement of [³H]PGE₂ binding by agonist or antagonist for PGE receptor subtypes. ● PGE₂; ○ 17-phenyl trinor PGE₂; ▲ sulprostone; □ M&B28767; △ 11-deoxy PGE₁; x butaprost; ■ AH 6809.

4. Signal transduction pathways of the PGE receptors.

As shown in Fig. 1-6, the CHO cells stably expressing the EP₃ showed a dose-dependent decrease to PGE₂ in forskolin-induced cellular cAMP accumulation. M&B28767 (an EP₃-specific agonist) also inhibited forskolin-induced cAMP synthesis and was more potent than PGE₂ (IC₅₀ of M&B28767 = 1×10^{-12} M; IC₅₀ of PGE₂ = 1×10^{-10} M). Neither agonist alone did not increase cAMP accumulation. Phosphatidylinositol turnover was also examined but up to 1 μ M of M&B28767 revealed no significant increase in inositol phosphates content over the basal levels (data not shown). These results demonstrated that the EP₃ receptor is coupled exclusively to inhibition of adenylate cyclase.

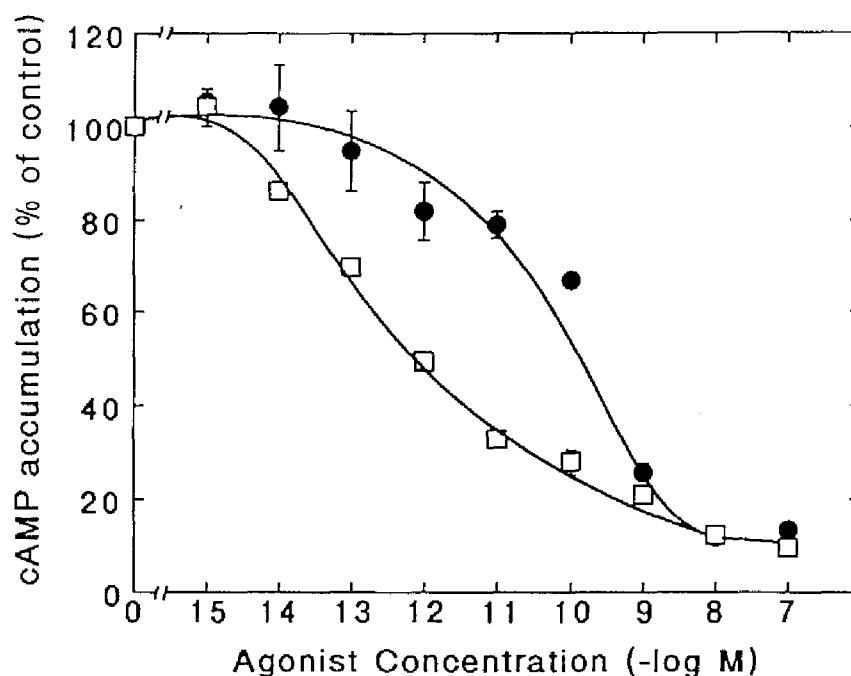


Fig. 1-6. Inhibition of forskolin-induced cAMP accumulation by PGE₂ and EP₃-selective agonist in CHO cells expressing the EP₃ receptor. CHO cells permanently expressing the EP₃ receptor were incubated with 1 μ M forskolin in the presence of indicated concentrations of PGE₂ (●) or M&B28767 (□), and cAMP accumulation was determined. The incubation buffer contained 1 mM 3-isobutyl-1-methylxanthine. Each point represents the mean $\pm$ S.E. of triplicate determinations.

As shown in Fig. 1-7, PGE₂ dose-dependently increased cAMP level in EP₂ cDNA-transfected COS cells, the maximal level being 4.05 pmol/10⁵ cells which is 2.8-fold higher than that accumulated by PGE₂ in untransfected cells. On the other hand, PGE₂ neither inhibited forskolin-induced cAMP formation and nor accumulated inositol phosphates (data not shown). These results demonstrate that EP₂ receptor is coupled to stimulation of adenylate cyclase.

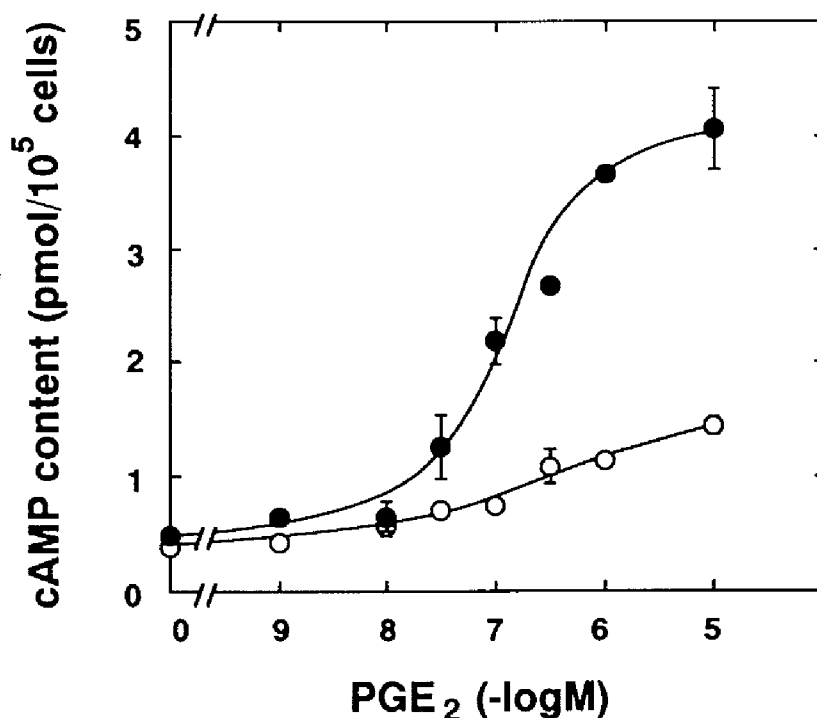


Fig. 1-7. Effect of PGE₂ on cAMP level in EP₂-expressing cells. MP412-transfected (●) or untransfected (○) COS-1 cells were incubated with the indicated concentrations of PGE₂ in the presence of 1 mM 3-isobutyl-1-methylxanthine, and cAMP accumulation was determined. The results shown are the means $\pm$ S. E. for triplicate determinations.

As shown in Fig. 1-8, PGE₂ induced a rapid increase in [Ca²⁺]_i within 10 sec in the CHO cells stably expressing the EP₁. The level decreased quickly but remained above the basal for over 3 min after the stimulation. This increase in [Ca²⁺]_i was completely abolished by removal of extracellular Ca²⁺ (data not shown), suggesting that the PGE₂-induced increase in [Ca²⁺]_i is due to the entry of extracellular Ca²⁺. No such response was observed in mock-transfected cells (data not shown). Under this condition, PGE₂ induced a rapid but limited IP₃ formation of 120% of the control. On the other hand, PGE₂ neither stimulated nor inhibited adenylate cyclase (data not shown). These results demonstrate that this receptor is of the EP₁ subtype coupled exclusively to Ca²⁺ mobilization.

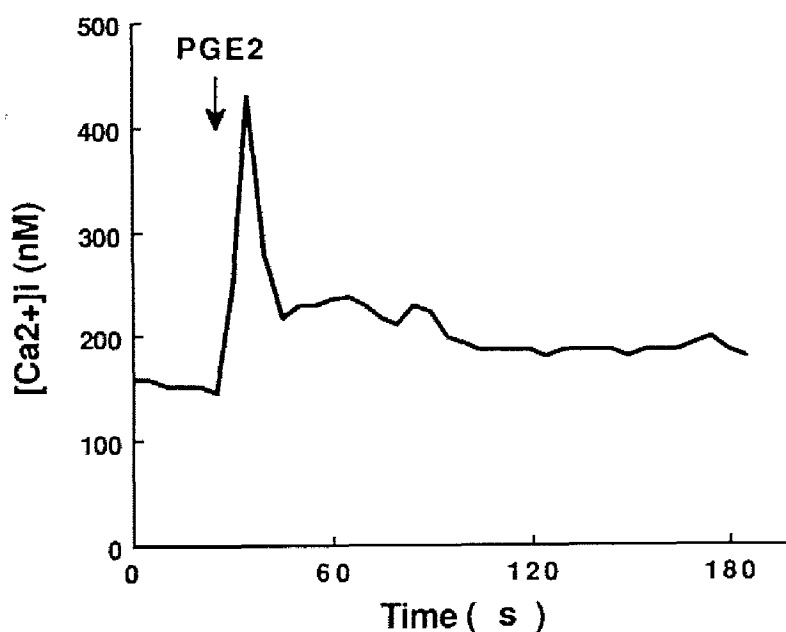


Fig. 1-8. Effect of PGE₂ on [Ca²⁺]_i change in EP₁-expressing CHO cells. Fura-2-loaded cells were challenged with 0.1 μM PGE₂. A trace shown is a representative of [Ca²⁺]_i responses of more than 20 individual cells. Pairs of fluorescence images at 340/480 nm were captured at 5-sec intervals. The *arrow* indicates the addition of PGE₂.

5. Tissue distribution of the PGE receptors.

Poly (A)⁺ RNAs were prepared from various mouse tissues, and hybridized with a fragment of the EP3 cDNA (Fig. 1-9). A positive band was seen at 2.3-kilobase in a number of tissues in which PGE₂ has pharmacological effects and/or specific binding sites (4). High expression of EP3 mRNA was seen in the kidney, in uterus, and in stomach. The EP3 receptor was also expressed in brain, heart, lung, thymus and spleen. On the other hand, EP3 mRNA was not detectable in testis, and little was found in liver and ileum. The abundant amount of EP3 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.

Northern Blot Analysis of EP3 mRNA Expression in Various Tissues

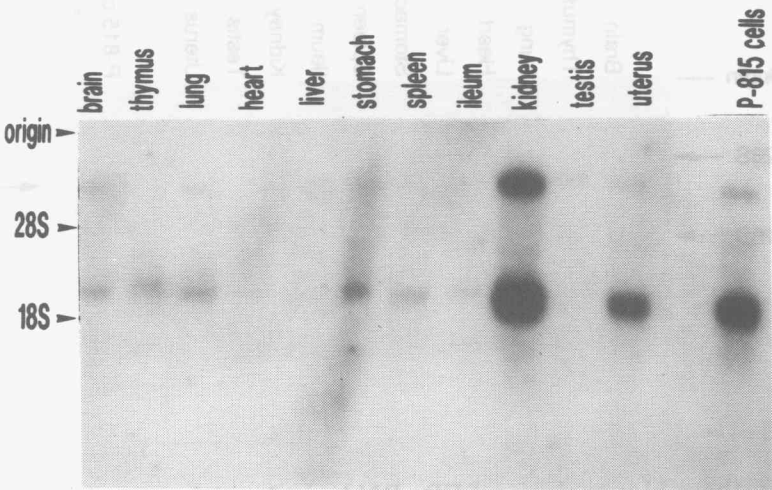


Fig. 1-9. Northern blot analysis of EP3-RNAs isolated from various mouse tissues and P-815 cells. Poly(A)⁺ RNAs were isolated from the following tissues and a cell line, and 10 µg of RNA was applied in each lane except that 5 µg was used for P-815 cells. Hybridization analysis was carried out using the 1,072-bp *Eco* RI-*Bam* HI fragment excised from clone MP660 as a probe.

Fig. 1-10 shows the distribution of the EP₂ receptor mRNA. A positive band is observed at 3.9 kilobase in most of tissues, suggesting widespread distribution of the EP₂ receptor. The tissues highly expressing EP₂ mRNA were ileum and thymus. A significant band was also observed in lung, spleen, heart or uterus. EP₂ mRNA was not detectable in testis and liver.

Fig. 1-11 shows the distribution of the EP₁ 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.

Northern Blot Analysis of EP₂ mRNA Expression in Various Tissues

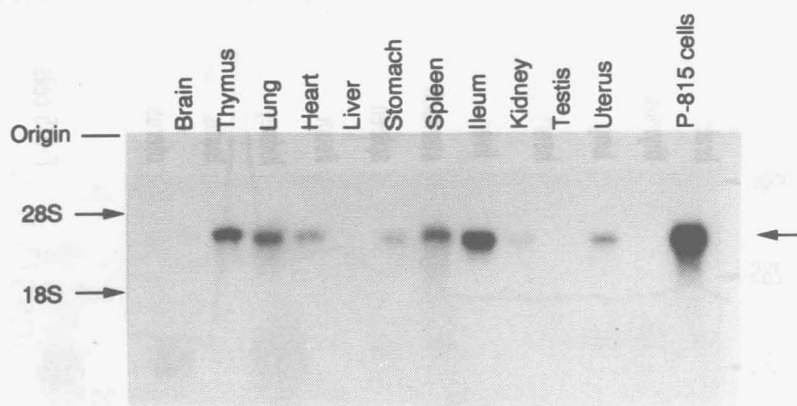


Fig. 1-10. Northern blot analysis of EP₂-RNAs isolated from various mouse tissues and P-815 cells. Poly(A)⁺ RNAs were isolated from the tissues listed below and a cell line, and 10 µg of RNA was applied in each lane except that 5 µg was used for P-815 cells. Hybridization analysis was carried out using the 1859-bp EcoRI-XhoI fragment excised from clone MP412 as a probe.

Northern Blot Analysis of EP₁ mRNA in Various Tissues

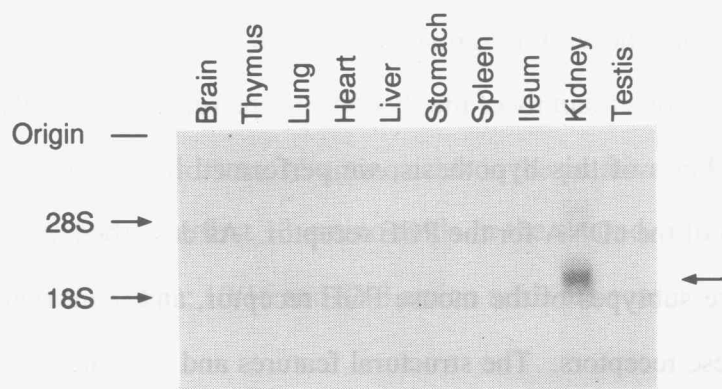


Fig. 1-11. Northern blot analysis of EP₁-RNAs isolated from various mouse tissues. Poly (A)⁺ RNAs were isolated from the tissues, and 5 µg of RNA was applied in each lane. Hybridization analysis was carried out using ³²P-labeled antisense riboprobe.

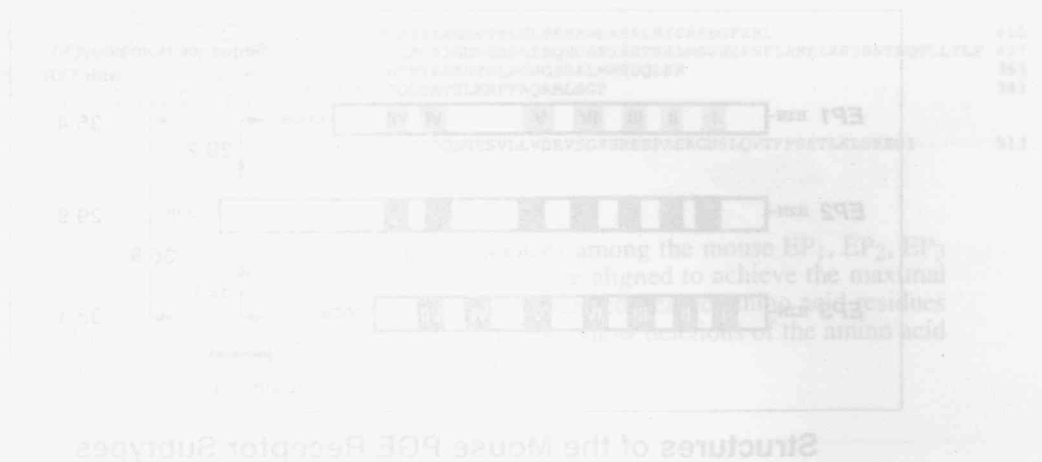
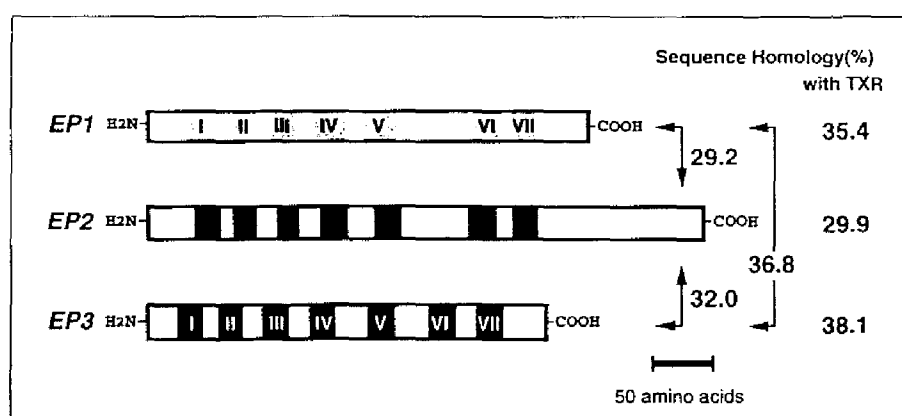


Fig. 1-12. Structures of the mouse PGE receptor subtypes. The diagram shows the relative positions of the EP1, EP2, and EP3 genes on the chromosome.

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, EP2 shows the lowest identities with other prostanoid receptors, and that EP3 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.



Structures of the Mouse PGE Receptor Subtypes

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 EP₁ and EP₂, 33.2% between EP₁ and EP₃, and 36.2% between EP₂ and EP₃. EP₁ 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 EP₁) and the first 13 amino acids in the second extracellular loop (from Gly-179 to Phe-191 in EP₁); 10/16 and 8/13 amino acids are conserved, respectively.

EP1	MSPCGLNLSDAEAAATCATPRLPNTSVVLPETGDNQIS	EP2	MAEVGGTIPRSNRELQRCVLLTTTMSIPGVNASFSSTPERLNSPVTI	EP3	MASHWAPESAEASNLNLS-STDDCCGSV	TP	MWPNGTSLGACFRPVNIT--LQERRAIAS	81
EP1	-----	EP2	-----	EP3	-----	TP	-----	85
EP1	-----	EP2	-----	EP3	-----	TP	-----	70
EP1	-----	EP2	-----	EP3	-----	TP	-----	68
EP1	-----	EP2	-----	EP3	-----	TP	-----	166
EP1	-----	EP2	-----	EP3	-----	TP	-----	171
EP1	-----	EP2	-----	EP3	-----	TP	-----	160
EP1	-----	EP2	-----	EP3	-----	TP	-----	157
EP1	-----	EP2	-----	EP3	-----	TP	-----	250
EP1	-----	EP2	-----	EP3	-----	TP	-----	255
EP1	-----	EP2	-----	EP3	-----	TP	-----	235
EP1	-----	EP2	-----	EP3	-----	TP	-----	220
EP1	-----	EP2	-----	EP3	-----	TP	-----	336
EP1	-----	EP2	-----	EP3	-----	TP	-----	339
EP1	-----	EP2	-----	EP3	-----	TP	-----	300
EP1	-----	EP2	-----	EP3	-----	TP	-----	284
EP1	-----	EP2	-----	EP3	-----	TP	-----	405
EP1	-----	EP2	-----	EP3	-----	TP	-----	477
EP1	-----	EP2	-----	EP3	-----	TP	-----	365
EP1	-----	EP2	-----	EP3	-----	TP	-----	341
EP2	DLTESSLGGRNLLPGSHGMGLTQADTTSLRTLRISETSDSSQ							513

Fig. 1-13. Comparison of the amino acid sequences among the mouse EP₁, EP₂, EP₃ 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 EP₁ 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 EP₁, 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 (EP₁), or in this region and the second extracellular loop region (EP₂ and EP₃). 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]PGE₂ with K_d of 3.3 (EP₃), 11.2 (EP₂), and 21 nM (EP₁) (Fig. 1-14). In contrast to EP₂ and EP₃ receptors, the only EP₁ receptor shows higher affinity to PGE₂ than PGE₁ and high affinity to iloprost. These properties are consistent with those reported for the EP₁ receptor. For example, it has been shown that PGE₂ has higher agonist potency than PGE₁ on the EP₁ receptor (Coleman et al. 1990), and that iloprost acts as an agonist not only at the prostacyclin receptor but also at the EP₁ receptor (Dong et al. 1986; Armstrong et al. 1989). It is also noted that the binding properties of the EP₁ agrees well with those described for the [3 H]PGE₂ binding in canine kidney papilla membrane (Smith et al. 1987); this binding has a similar K_d value for PGE₂, and the K_i of PGE₁ was about one order of magnitude higher than that of PGE₂. These results strongly suggest that the cloned EP₁ receptor is identical to the PGE receptor found in kidney papilla. On the other hand, EP₂ and EP₃ showed a similar rank order of prostaglandin ligands.

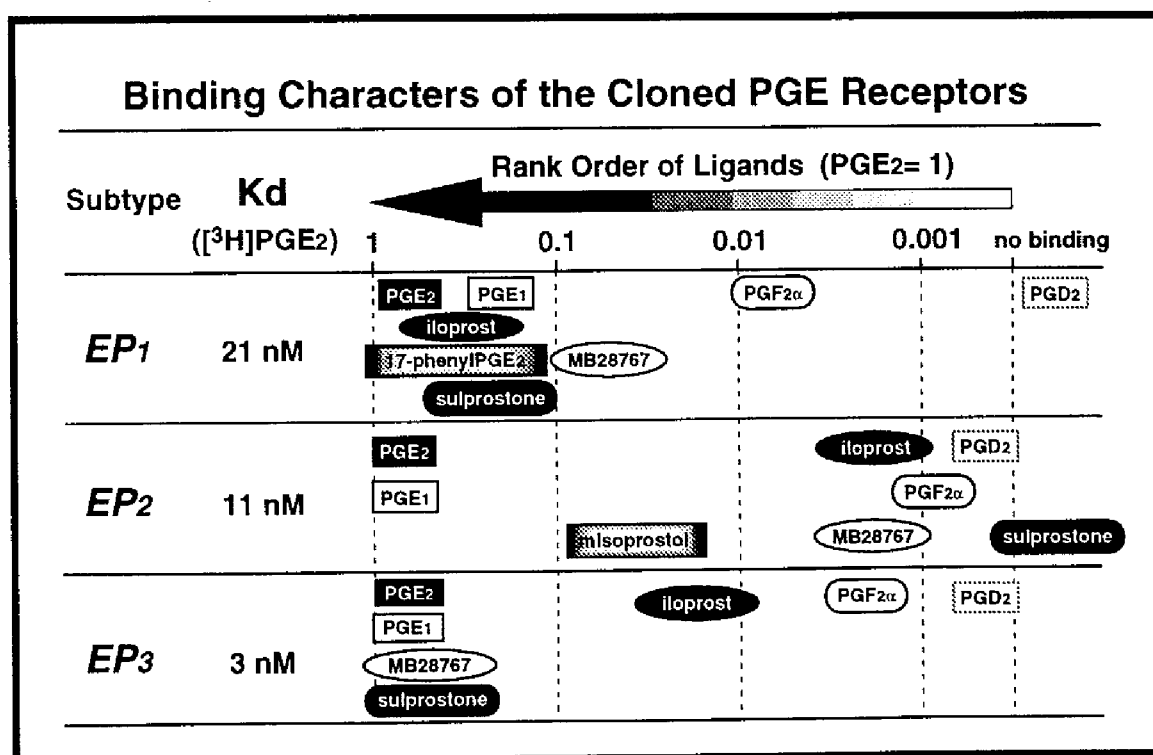


Fig. 1-14. Binding characters of the cloned PGE receptors. The K_d values for [3 H]PGE₂ of the receptors are shown. Rank order of ligands (PGE₂ = 1) for each subtype is also shown.

Among the cloned PGE receptors, EP₃ receptor showed the typical characteristic about subtype-selective ligand binding which agrees well to those previously reported. As to the binding of the EP₃, only EP₃-specific agonists, GR63799X and M&B28767, specifically competed with equal potency, and they were more potent than PGE₂ itself. However, EP₂ and EP₁ revealed the binding characters different from those reported. At first, the binding of EP₂ is inhibited by misoprostol (an EP₂ and EP₃ agonist) and more weakly by M&B28767 (an EP₃ agonist). On the other hand, sulprostone (an EP₁ and EP₃ agonist), SC-19220 (an EP₁ antagonist), and butaprost (an EP₂ agonist), did not inhibit it. The ability of misoprostol to inhibit the binding of EP₂ and no ability of sulprostone suggest that MP412 indeed encodes the EP₂ subtype of PGE receptor, and this was also supported by weak cross-reaction of M&B28767 to EP₂ (Lawrence et al. 1992). The lack of binding activity of butaprost in mouse EP₂ might indicate that the action of butaprost is species specific. Secondly, the binding of EP₁ was inhibited by 17-phenyl-18,19,20-trinor PGE₂ (an EP₁ agonist), and by sulprostone (an EP₁ and EP₃ agonist), while the inhibition by M&B28767 (an EP₃ agonist), by 11-deoxy PGE₁ (an EP₂ and EP₃ agonist), and by butaprost (an EP₂ agonist), was weak or negligible. The 11-hydroxy residue is important for EP₁ agonist activity but not essential for activity of either EP₂ or EP₃ (Carpio et al. 1987). On the other hand, AH6809 (an EP₁ antagonist) showed only weak inhibition of the binding. This result suggests that there may be other forms of EP₁ 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 EP2 and EP3 is coupled to stimulation and inhibition of adenylate cyclase, respectively. As to EP3 receptor, it was suggested that this receptor can be coupled to phospholipase C (Coleman et al. 1990). However, this signaling pathway could not be detected in our CHO expression system using MP660.

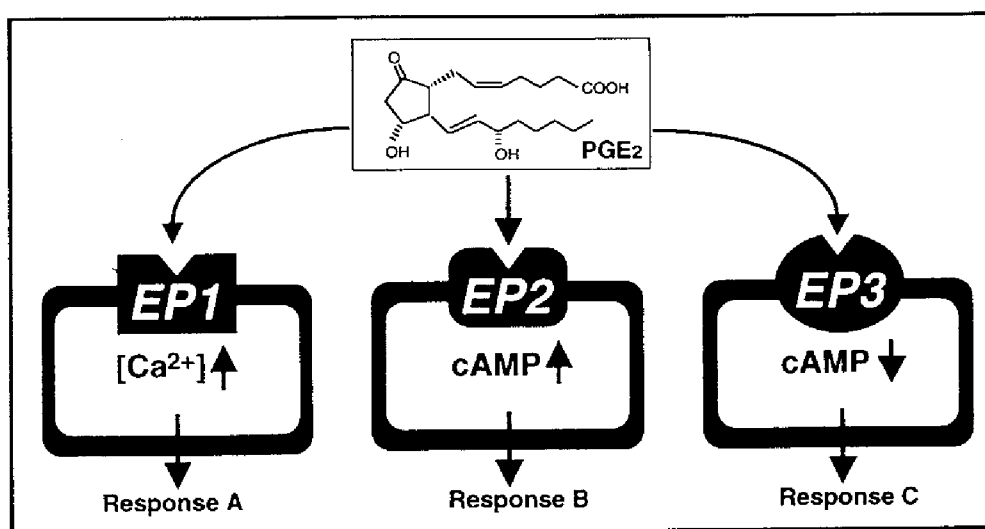


Fig. 1-15. Signal transduction pathways of the PGE receptor subtypes.

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

The tissue most highly expressing EP₃ mRNA was kidney in which PGE₂ 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 PGE₂ exerts contractile response in uterine smooth muscle. The EP₃ receptor we found may mediate this contractile action. Uterine contraction has been observed as a side effect of several EP₃ agonists used as gastric anti-secretory prostanoid drugs (Collins 1986). The EP₃ receptor was also expressed in heart, lung, thymus and spleen. Although PGE₂ 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 EP₃ 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 EP₃ 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 EP₂ mRNA were ileum and thymus in which PGE₂ 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 PGE₂ has the relaxant activity in trachea (Gardiner 1986) and myometrium (Senior et al. 1991).

A hybridizing band for EP₁ receptor was abundantly detected in kidney. It is known that PGE₂ induces Ca²⁺ mobilization and inhibits the action of arginin-vasopressin in the rabbit collecting duct (Hébert et al. 1991). This action may be mediated

by EP1 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 PGE2 induces contraction of tracheal smooth muscle (Coleman et al. 1990).

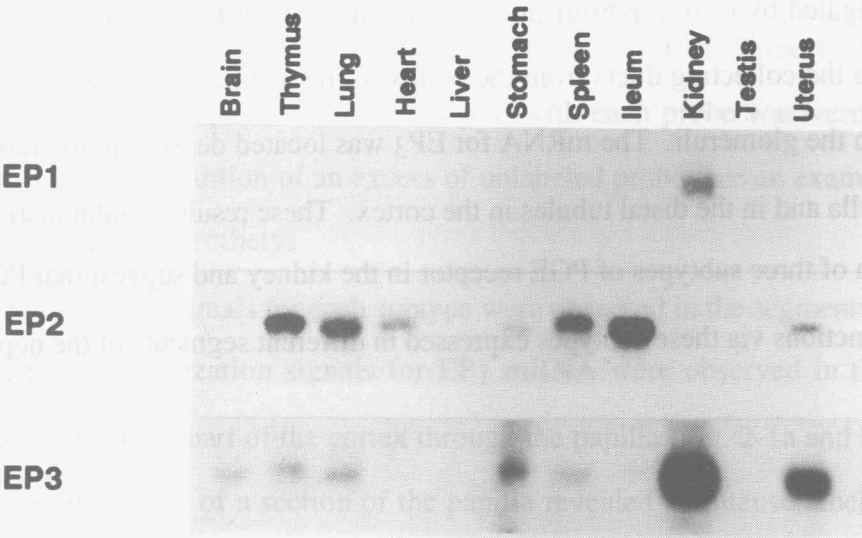


Fig. 1-16. Northern blot analyses of mRNAs of the PGE receptor subtypes.

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 EP₁ subtype was specifically localized to the collecting ducts from the cortex to the papilla. The mRNA for EP₂ was localized to the glomeruli. The mRNA for EP₃ 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 PGE₂ exerts multiple functions via these subtypes expressed in different segments of the nephron.

RESULTS

[³⁵S]-Labeled riboprobes prepared from the EP₁, EP₂ and EP₃ 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 EP₁, not shown for others).

The hybridization signals for each subtype were observed in the segments along the nephron. Strong hybridization signals for EP₁ 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 EP₂ 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 EP₂ 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 EP₃ 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 EP₃ 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).

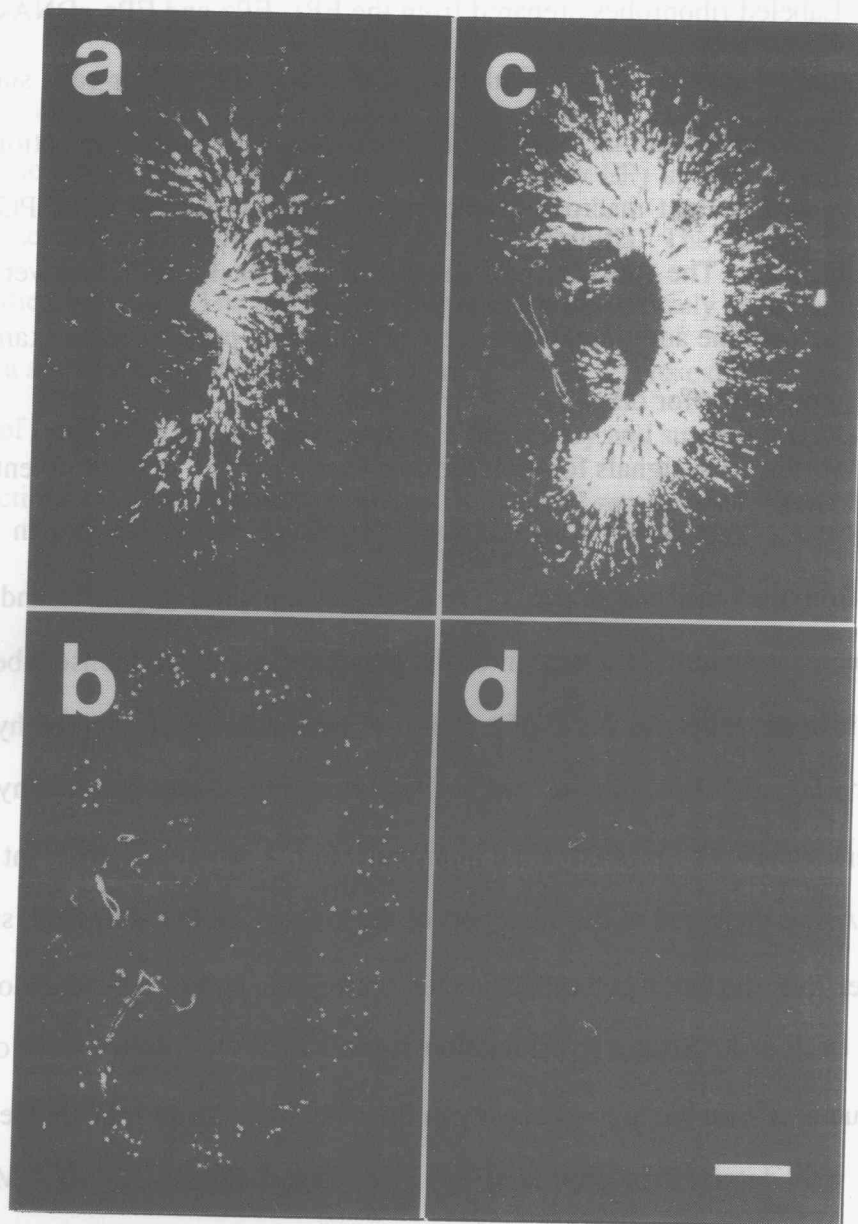


Fig. 2-1. Dark-field photomicrographs, showing hybridization signals for three PGE receptor mRNAs in the kidney . Sections were hybridized with antisense riboprobe for EP₁ (a), EP₂ (b), and EP₃ mRNA (c). The hybridization signals for EP₁ shown in a were abolished in the control experiments using the same probe in the presence of an excess amount of the unlabeled probe (d). Bar, 1 mm.

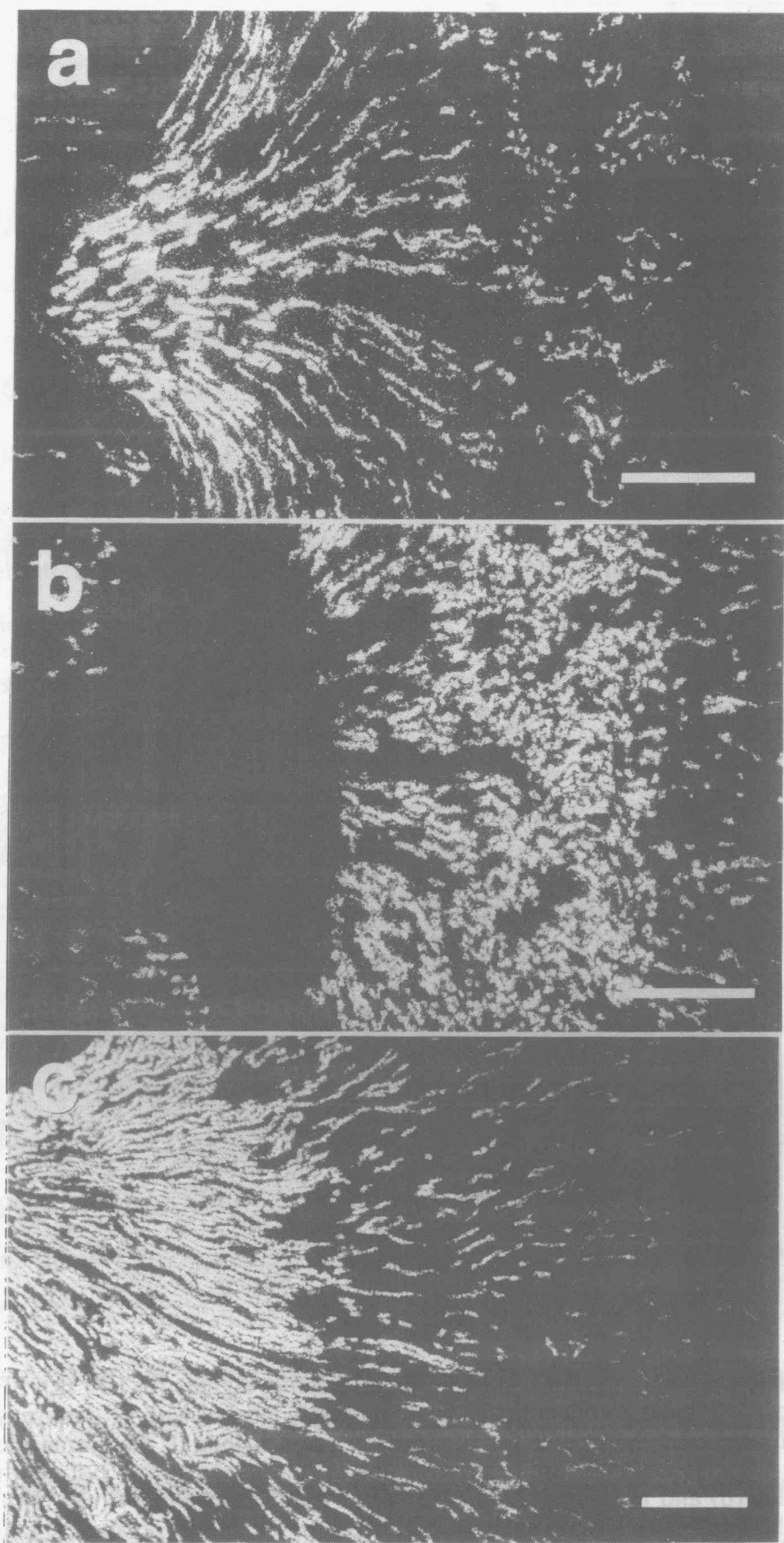


Fig. 2-2. Enlarged dark-field photomicrographs of the emulsion-dipped sections showing the hybridization signals for EP₁ (a) and EP₃ mRNA (b, c). *a* and *b* were taken from the corresponding regions of continuous sections showing signals for EP₁ and EP₃, respectively. EP₁ mRNAs were located in the collecting ducts from the renal cortex to the papilla. EP₃ mRNAs were located in tubules in the cortex and the outer medulla, but not in the papilla. Bars, 500 μ m.

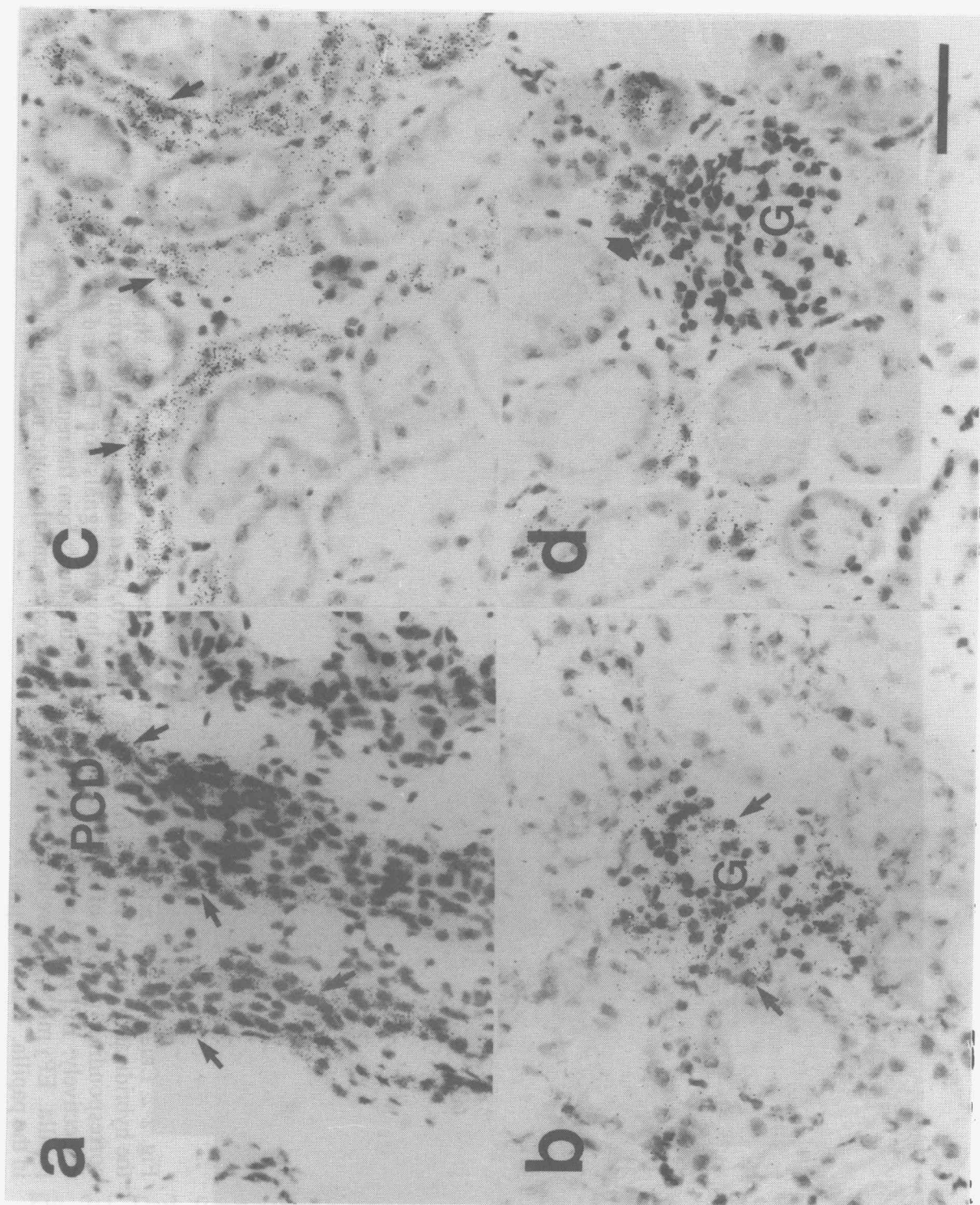


Fig. 2-3. Bright-field photomicrographs, showing hybridization signals for EP₁ (a), EP₂ (b), EP₃ mRNA (c, d). Papillary collecting ducts are intensely labeled with EP₁ antisense riboprobe (a). Autoradiographic grains for EP₂ mRNA are confined to the glomerulus (b), while those for EP₃ mRNA are observed 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 μ m.

DISCUSSION

PGE₂ 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). PGE₂ 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 PGE₂ has not yet been established except its action on water reabsorption in the collecting duct, where PGE₂ appears to act on the EP₃ 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 PGE₂, 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. EP₁ 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 Ca²⁺ concentration and the other by inhibiting adenylate cyclase (Breyer et al. 1990; Dillingham et al. 1987). It has been reported that PGE₂ attenuates the vasopressin action by both mechanisms (Breyer et al. 1990; Hébert et al. 1990; Nadler et al. 1986). Mouse EP₁ receptor expressed in CHO cells is shown to be coupled to Ca²⁺ mobilization.

It is, therefore, likely that EP₁ receptor in the collecting duct modifies the vasopressin-induced osmotic water permeability by the former mechanism. The transcripts of the EP₂ receptor are specifically found in glomerulus with intense labeling over the mesangial cells. In the cultured mesangial cells, PGE₂ elicited cAMP accumulation and attenuated contractility induced by vasoconstrictors such as TXA₂, angiotensin II, platelet-activating factor and endothelin (Dunlop and Larkins 1990; Méne and Dunn 1988).

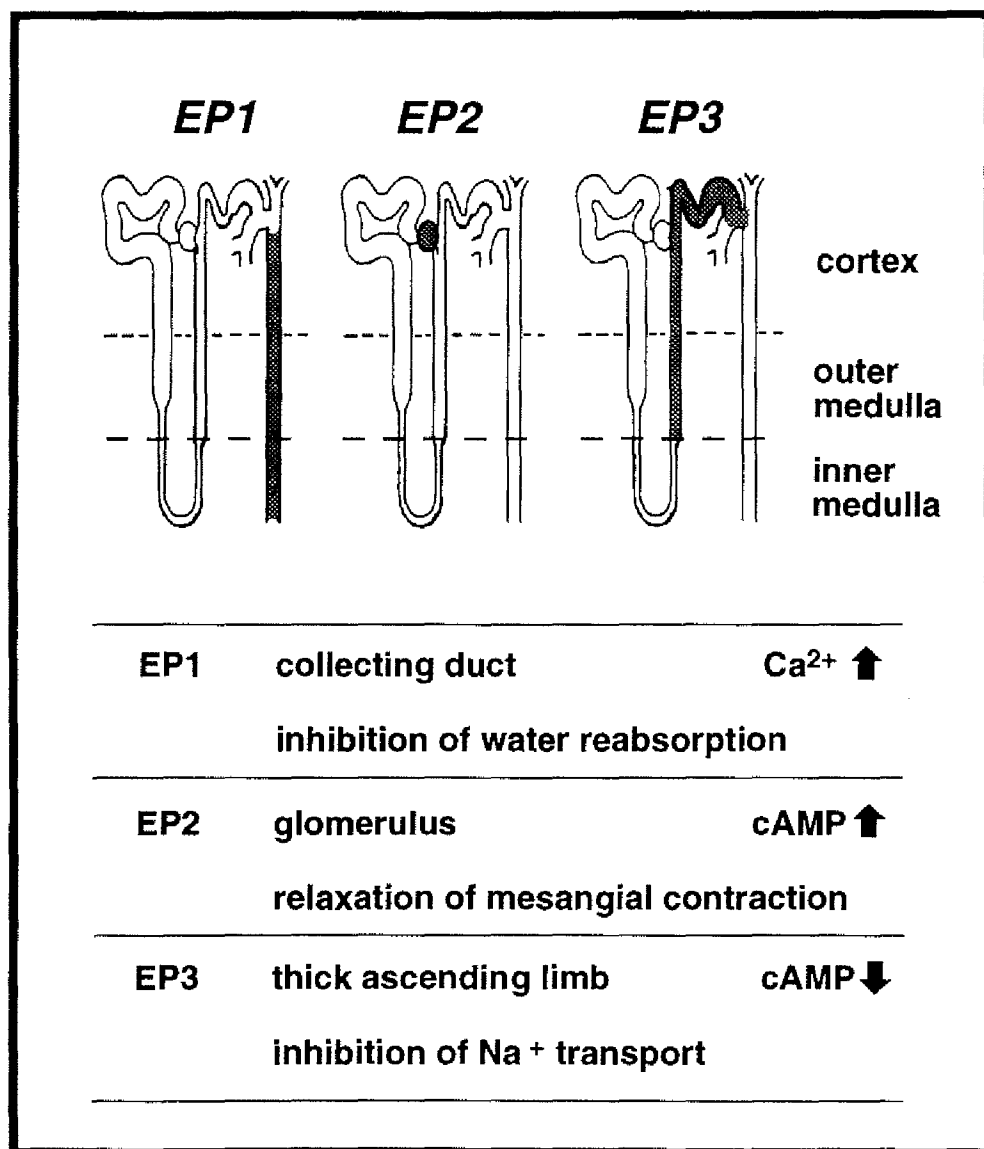


Fig. 2-4. Summary of distribution of three PGE receptor subtypes in the kidney.

This inhibitory action of PGE₂ 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 EP₂ receptor coupled to cAMP generation mediates the PGE₂-induced inhibition of the mesangial contractility to regulate glomerular filtration (Méne and Dunn 1988).

EP₃ 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 EP₃ 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 EP₃ receptor in the thick ascending limb as well as in the collecting duct. PGE₂ 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). PGE₂ 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 PGE₂ are mediated by inhibiting adenylate cyclase in a pertussis toxin-sensitive manner (Nakao et al. 1989; Sonnenburg and Smith 1988). Mouse EP₃ receptor expressed in CHO cells is coupled to inhibition of adenylate cyclase. These findings suggest that PGE₂ synthesized locally acts on EP₃ 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 PGE₂ 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 EP₃ receptor remains to be tested. PGE₂ 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 EP₃ 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 PGE₂ 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 PGE₂ exerts multiple functions via these subtypes expressed in different segments of the nephron. The mode of PGE₂ 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).

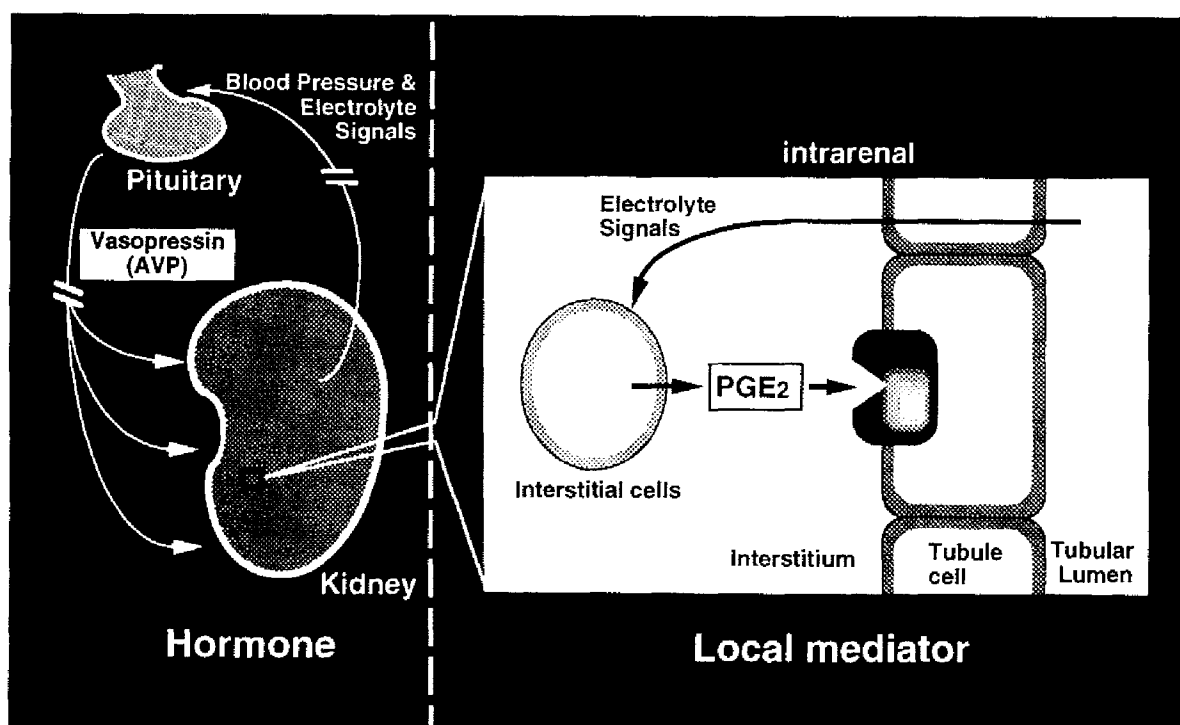


Fig. 2-5. Physiological role of PGE₂ as a local mediator in the kidney. Pituitary hormones such as AVP (arginine vasopressin) act on the whole target organs through circulation system. On the other hand, the local mediators (autacoids) such as PGE₂ act on only local target segments neighboring its secreting cells.

3. Identification of two isoforms of the PGE receptor subtype EP₃

SUMMARY

Functional cDNA clones for two isoforms of mouse EP₃ 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 EP₃ α has a hydrophilic tail and the other named EP₃ β a hydrophobic tail. When expressed, the two receptors displayed identical ligand binding properties but different responses to GTP γ S. Without a change in B_{max} values, GTP γ S increased K_d for PGE₂ of EP₃ β and decreased that of EP₃ α . 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 EP₃ α than EP₃ β 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 G_i 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 EP₃ receptor.

In the homology screening for the PGE receptor cDNA, we first obtained a partial EP₃ 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 EP₃ 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 EP₃ isoforms consists of the common 10 amino acids in the amino-terminal region followed by these different peptides.

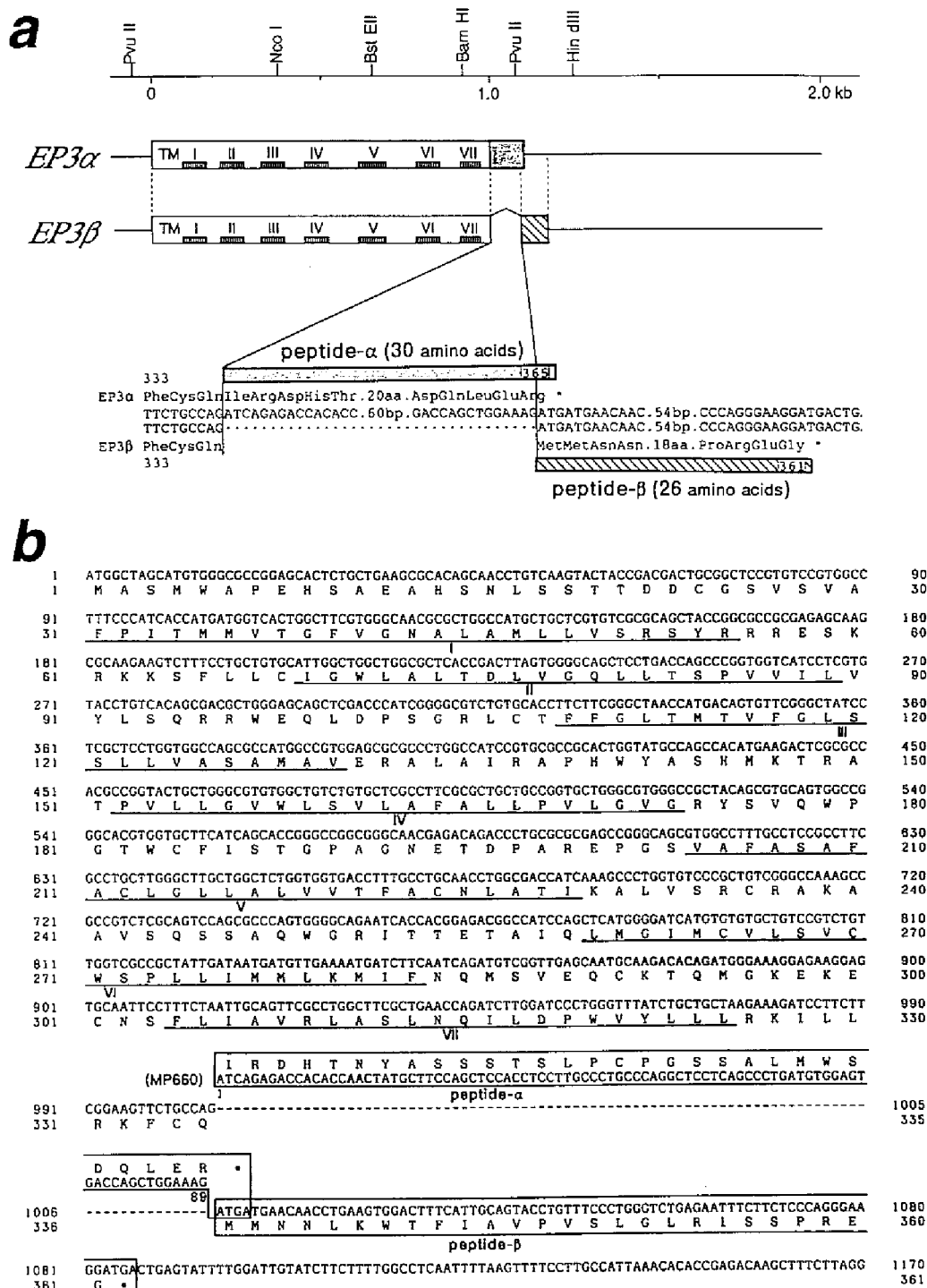


Fig. 3-1. Comparison of cDNA structures of two EP₃ receptor isoforms.
 a. Schematic representation of mouse EP₃ receptor cDNA clones, MP660 and MP653. Boxes represent coding sequences; open box is a corresponding coding sequence between the two cDNA, grey one is the sequence coding peptide-α, hatched 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.

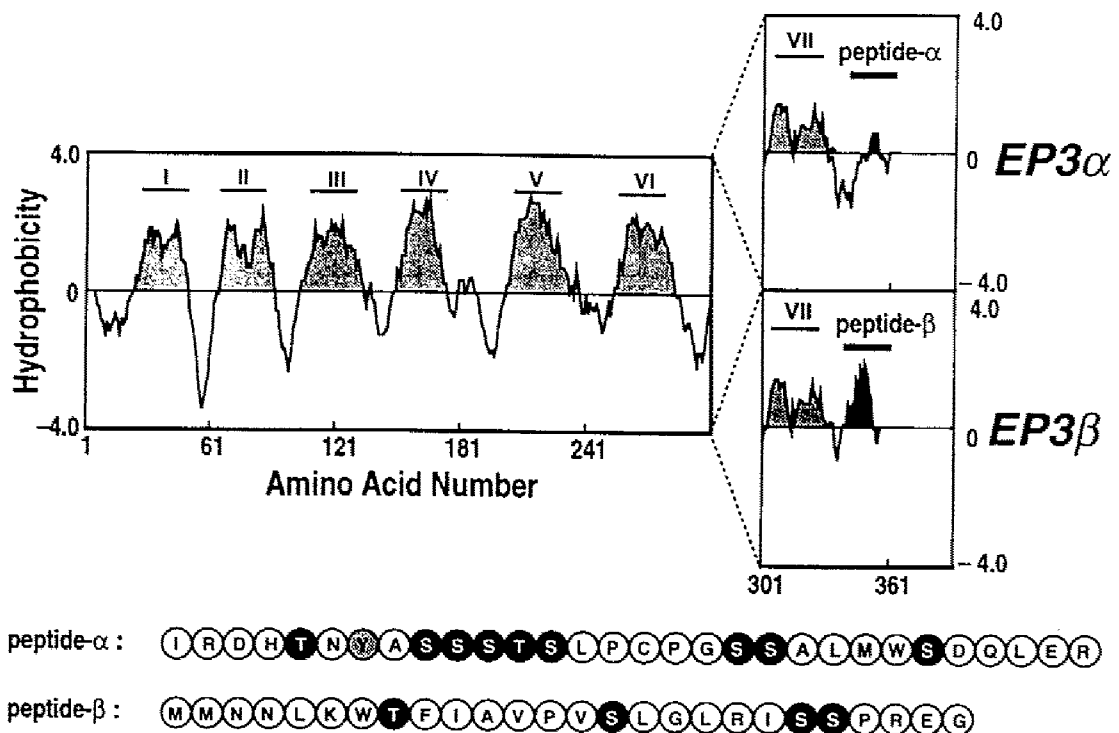


Fig. 3-2. Comparison of hydrophobicity of the EP₃ sequence coded by MP660 (upper) and MP653 (lower) analyzed by the method of Kyte and Doolittle (1982). The positions of the transmembrane domains and of peptide- α and peptide- β are indicated by plain and bold lines. Amino acids are numbered underneath. 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]PGE₂ 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]PGE₂ 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 EP₃ subtype of PGE receptor (Armstrong et al. 1990). Hence, we have designated MP660-encoding receptor as EP₃ α (containing the peptide- α), and MP653-encoding one as EP₃ β (containing the peptide- β).

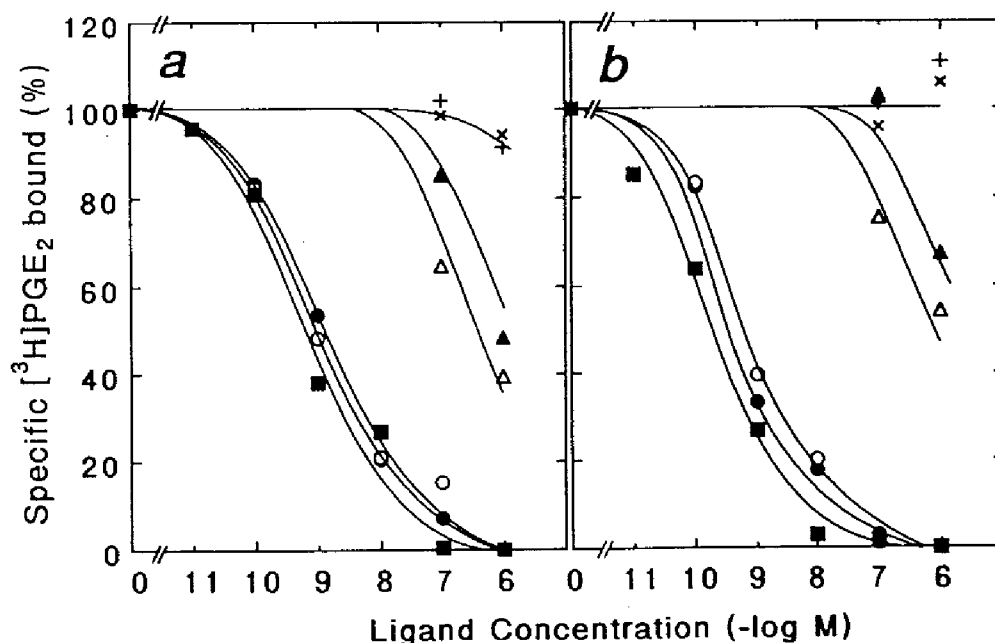


Fig. 3-3. Displacement of [3 H]PGE₂ binding to the two EP₃ isoforms in transfected COS-1 cells by various prostaglandins and PGE subtype-selective ligands. Unlabeled ligands were added to the binding assay mixture at indicated concentrations, and [3 H]PGE₂ binding to MP660-transfected COS-1 cell membranes (a) and MP653-transfected cell membranes (b) was determined. Prostaglandins used are PGE₂ (●), PGE₁ (○), PGF₂ α (Δ), and PGD₂ (▲). Subtype-selective ligands used are SC-19220 (×, for EP₁), butaprost (+, for EP₂), M&B28767 (■, for EP₃).

2. $GTP\gamma S$ conversely affects the binding affinity for $[^3H]PGE_2$ 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 $[^3H]PGE_2$ binding to the two EP_3 isoforms. Figure 3-4 shows the results of Scatchard plot analyses of the specific $[^3H]PGE_2$ binding to each receptor in the presence or absence of $GTP\gamma S$, a nonhydrolyzable GTP analogue. In the absence of $GTP\gamma S$, both receptors displayed indistinguishable binding affinity with a dissociation constant (K_d) of 3.0 nM for $EP_{3\alpha}$, and 2.8 nM for $EP_{3\beta}$. The addition of $GTP\gamma S$ at 100 μM decreased K_d of the $EP_{3\alpha}$ (1.1 nM), while it increased K_d of the $EP_{3\beta}$ (5.9 nM), without any change in B_{max} .

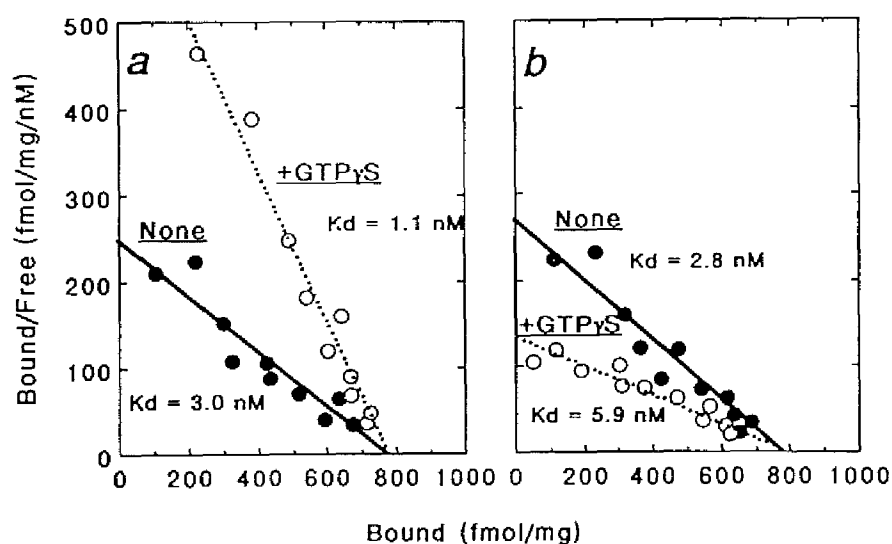


Fig. 3-4. Scatchard plot analyses of the PGE_2 binding to each isoform in COS cell membranes in the presence or absence of $GTP\gamma S$. The specific binding of $[^3H]PGE_2$ (0.5-30 nM) to MP660 ($EP_{3\alpha}$; a) or MP653 ($EP_{3\beta}$; b) in transfected COS-1 cell membranes (50 μg) was determined in the presence (○) or absence (●) of 100 μM $GTP\gamma S$. The Scatchard plot was transformed from the value of specific $[^3H]PGE_2$ binding.

The opposite responses of EP₃ α and EP₃ β to GTP γ S 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 GTP γ S enhanced [³H]PGE₂ binding in EP₃ α -expressing cell membranes. PT treatment also enhanced this binding and additional stimulation by GTP γ S was not observed. On the other hand, the GTP γ S addition reduced [³H]PGE₂ binding in EP₃ β -expressing cells. PT treatment of this membrane also reduced this binding into the same level as that reduced by GTP γ S and abolished the inhibitory effect of GTP γ S. As a result, in either isoform, PT-treatment mimicked the effects of GTP γ S and abolished them in these membranes. This suggests that both EP₃ α and EP₃ β 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 GTP γ S on PGE₂ binding in each membranes (Table 1), indicating that the opposite responses of EP₃ α and EP₃ β to GTP γ S 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]PGE₂ binding to CHO cell membranes expressing the two EP₃ isoforms.

Treatment	Specific PGE ₂ binding (pmol/ng protein)			
	EP ₃ α		EP ₃ β	
	-GTP γ S	+GTP γ S	-GTP γ S	+GTP γ S
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 EP₃ receptor isoforms were cultured in the presence or absence of 20 ng/ml pertussis toxin for 9h. CHO cell membranes (50 μ g) were incubated with or without G_{i2} purified from bovine spleen (10 pmol/mg of the membrane protein) at 0°C for 1 h. PGE₂ binding assays were carried out with 2.0 nM [³H]PGE₂ in the presence or absence of 100 μ M GTP γ S. Values shown are means $\pm$ S. E. for triplicate experiments.

3. Two EP₃ isoforms stimulate GTPase activity and inhibit adenylate cyclase with different efficiency.

Because both EP₃ α and EP₃ β couple to Gi as described and EP₃ α mediates inhibition of adenylate cyclase (described in Section 1), we compared potency of PGE₂ and its analogue in inhibition of adenylate cyclase in the two receptor systems. As shown in Figure 3-5, PGE₂ and M&B28767 dose-dependently inhibited forskolin-induced cAMP formation in EP₃ α -transfected CHO cells, the half maximal concentration for the inhibition being 1×10^{-11} M and 1×10^{-13} M, respectively. PGE₂ and M&B28767 also showed the dose-dependent inhibition in EP₃ β -transfected CHO cells, but their half maximal concentrations for the inhibition (1×10^{-8} M for PGE₂ and 1×10^{-10} M for M&B28767) were three-orders of magnitude higher than those in EP₃ α -transfected CHO cells. EP₃ α - and EP₃ β -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

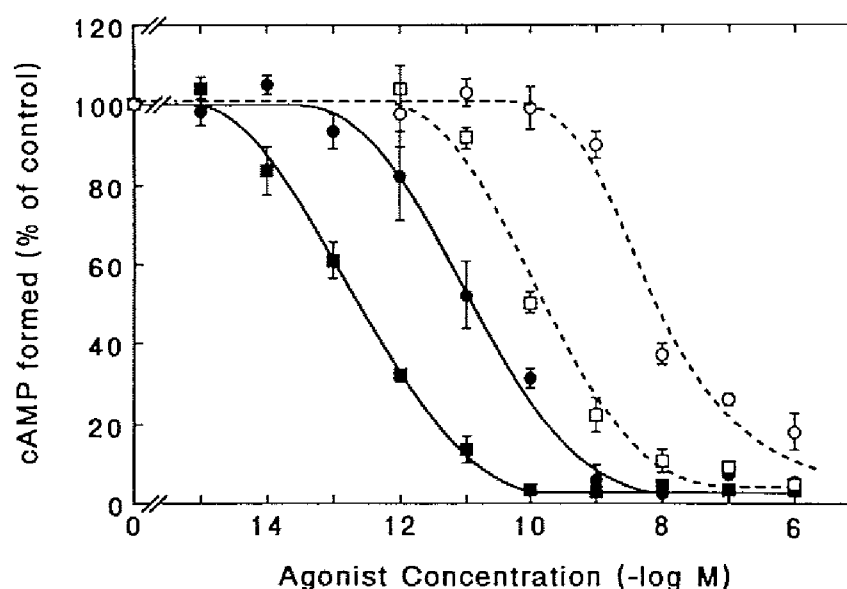


Fig. 3-5. Comparison of adenylate cyclase inhibition by the two EP₃ receptor isoforms permanently expressed in CHO cells. The EP₃ α -CHO cells (closed symbols) or the EP₃ β -CHO cells (open symbols) were incubated at 37°C for 10 min with 1 μ M forskolin in the presence of the indicated concentrations of PGE₂ (●, ○) or M&B28767 (■, □), and cAMP formation was determined. Each point represents the mean $\pm$ S.E. of triplicate determinations. cAMP formed in EP₃ α - and EP₃ β -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 μ M of M&B28767 (data not shown). These results suggest that both EP₃ receptors are engaged in an identical intracellular function, i. e., inhibition of adenylate cyclase, and that the efficiency of signal transduction in EP_{3 α} system is greatly higher than that in EP_{3 β} .

Because the two receptors showed identical affinities to the ligands, the above results suggested that the different efficiency of inhibitory action of the two EP₃ 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, PGE₂ and M&B28767 dose-dependently stimulated the GTPase activity in EP_{3 α} -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

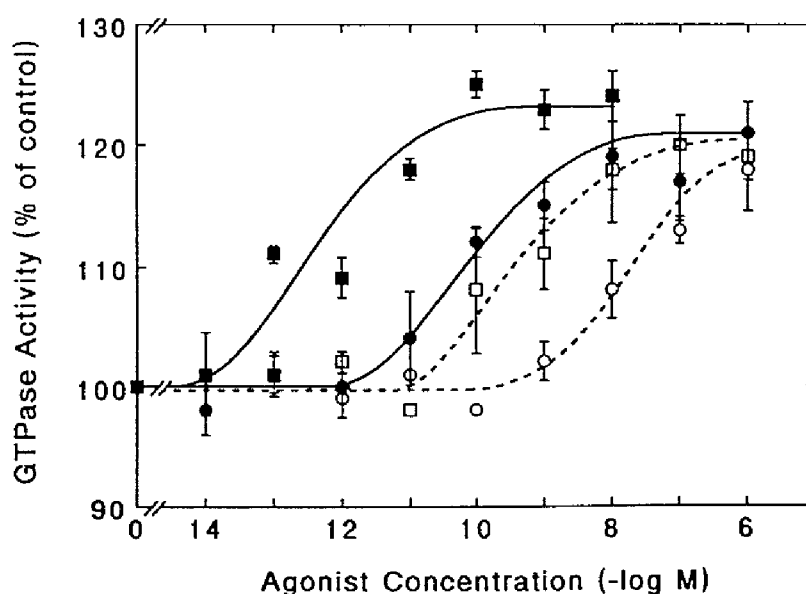


Fig. 3-6. Comparison of agonist-induced stimulation of the GTPase activity in CHO cell membranes expressing the two EP₃ receptor isoforms. GTPase activity of EP_{3 α} -CHO (closed symbols) or EP_{3 β} -CHO (open symbols) cell membranes was measured in the presence of the indicated concentrations of PGE₂ (●, ○) or M&B28767 (■, □). Each point represents the mean $\pm$ S.E. of triplicate determinations. The basal GTPase activity of EP_{3 α} - and EP_{3 β} -CHO cell membranes was 16.6 ± 0.09 and 14.5 ± 0.18 pmol/min/mg of protein, respectively.

stimulated the GTPase activity in EP₃ β -transfected CHO cell membranes, but the half maximal concentrations (5×10^{-8} M for PGE₂ and 1×10^{-9} M for M&B28767) were about three-orders of magnitude higher than those in EP₃ α -transfected CHO cell membranes. The maximal increase in GTP hydrolysis by PGE₂ and M&B28767 in EP₃ α -transfected CHO cell membranes was about 20% above the control and was the same as that in EP₃ β -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 $EP_{3\alpha}$ and $EP_{3\beta}$, respectively. In any tissue expressing EP_3 , $EP_{3\alpha}$ was dominantly expressed over $EP_{3\beta}$. In uterus and ileum, the $EP_{3\alpha}$ isoform was expressed at 3~4-fold higher levels than the $EP_{3\beta}$. In brain, very little amount of expression of $EP_{3\beta}$ could be detected under this PCR condition in spite of significant amount of expression of $EP_{3\alpha}$. In P-815 cells and stomach, on the other hand, the mRNA ratio for $EP_{3\alpha}$ / $EP_{3\beta}$ was about 1.5, which is in accordance with the results of our P-815 cDNA library screening. While the same amount of $EP_{3\alpha}$ expression was observed in uterus and P-815 (Fig. 3-7), apparently the much higher level of expression of $EP_{3\beta}$ was detected in P-815 cells.

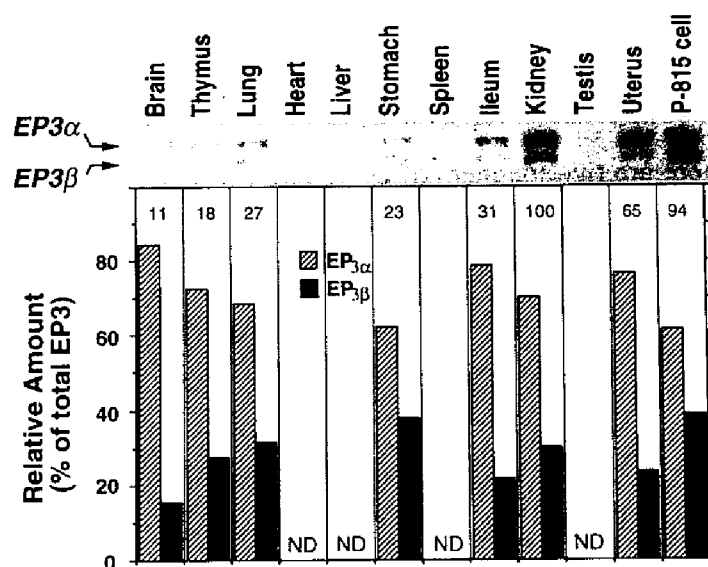


Fig. 3-7. Relative abundance of $EP_{3\alpha}$ and $EP_{3\beta}$ in various tissue. Aliquots (3 μ l) of each PCR product were resolved in 1.5% agarose gel. Arrows point to the 732-bp (upper; $EP_{3\alpha}$) and 643-bp (lower; $EP_{3\beta}$) DNA fragments, ascertained by sequencing. The relative expression 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 EP₃ 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 EP₃-mediated actions are believed to be mediated by inhibition of adenylate cyclase (Lu et al. 1988; Sonnenburg and Smith 1988), the concentrations of PGE₂ to inhibit cAMP formation varied among tissues, the IC₅₀ values of PGE₂ 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.

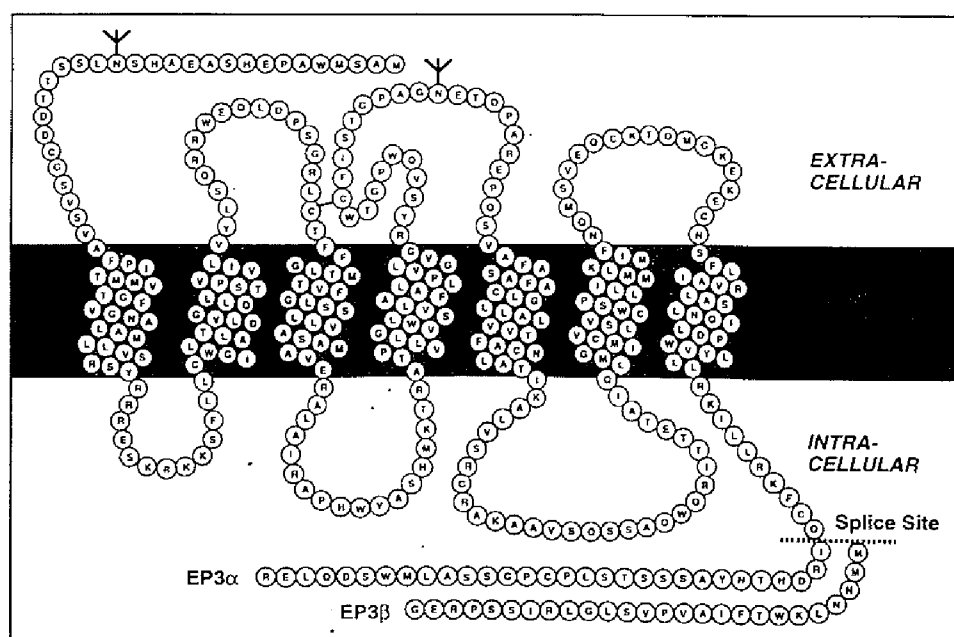


Fig. 3-8. Putative structures of the two EP₃ isoforms.

These differences in efficiency to agonists as well as responses to GTP of the EP₃ receptor implicate heterogeneity of this type of receptor.

Here two isoforms of EP₃ receptor represented by MP660 (EP₃ α) and MP653 (EP₃ β) was identified (Fig. 3-8). The two isoforms show identical ligand binding specificity, couple to the same G protein, probably G_i, 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 D₂ 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

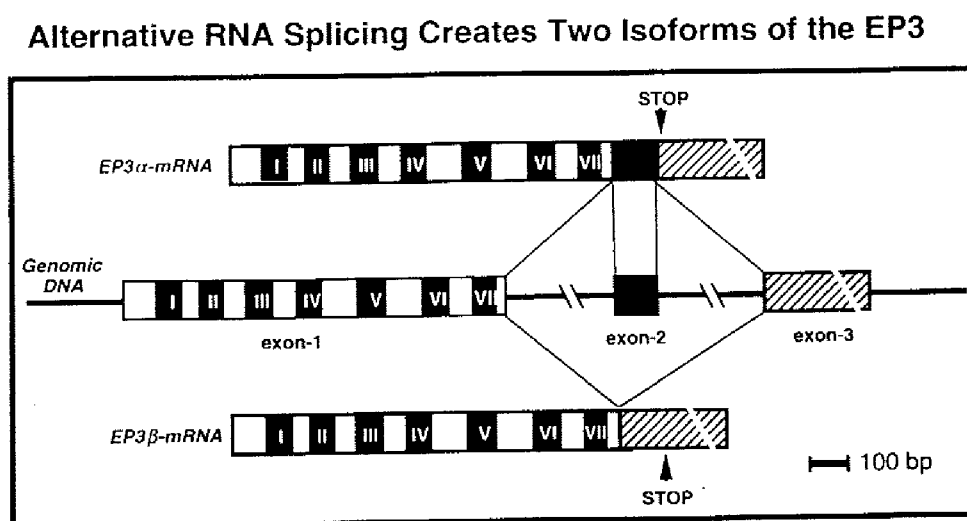


Fig. 3-9. Model system for alternative RNA splicing creating the two isoforms of the mouse EP₃ receptor.

end peptides of the EP₃ 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 GTP γ S increased K_d for PGE₂ of EP₃ β , and decreased that of EP₃ α . PT treatment, by losing the ability of G_i protein to bind to a receptor (reviewed by Gilman 1987), mimicked the effect of GTP γ S. 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 EP₃ isoforms couple to the same G protein, the EC₅₀ values of PGE₂ and M&B28767 for G protein activation were different by three orders between the two EP₃ systems and this difference was reflected in the different IC₅₀ values of the agonists to inhibit adenylate cyclase. Although the different EC₅₀ may be partly attributed to the difference in the K_d 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 EP₃ 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 EP₃ 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 (Cys³³⁴) in common at the ninth position out of the TM VII. An additional cysteine residue (Cys³⁵¹) 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 PGE₂ and M&B28767 showed almost equal binding affinity. As for both agonists, the K_d in EP₃ α system is about 1×10^{-9} M. On the other hand, the IC₅₀ of agonists for inhibition of adenylate cyclase were lower (1×10^{-10} M for PGE₂; 1×10^{-12} M for M&B28767) than the K_d, indicating that agonist binding to a part of EP₃ α 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 IC₅₀ and EC₅₀ than PGE₂ in both EP₃ receptor systems. This suggests that the M&B28767-receptor complex couples to the G protein and, hence, to adenylate cyclase more efficiently than the PGE₂-receptor complex.

The result in the amplification of RNA (Fig. 3-7) demonstrated that in every tissue expressing EP₃, EP₃ α is dominantly expressed but the two EP₃ mRNA coexist with

different relative abundance. The pattern of effect of GTP γ S observed in EP 3α 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 3α appears to be responsible for the GTP sensitivity in these tissues. Considering EP 3α and EP 3β 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, EP₁, EP₂ and EP₃, were characterized.
- (2) The mRNAs for the three PGE receptors show different tissue distribution and distinct cellular localization in the kidney.
- (3) In the EP₃ receptor, there exist functionally different molecular forms with different C-terminal structures.

The actions of PGE₂ are mediated by multiple receptor subtypes and signal transductions according to its sites of actions (Fig. 4-1). The existence of multiple EP₃ isoforms different in their functions is supported by more recent studies, also in humans, and contributes to a variety of PGE₂ 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 PGE₂, but also development of more subtype-specific PGE analogues for therapeutic purposes.

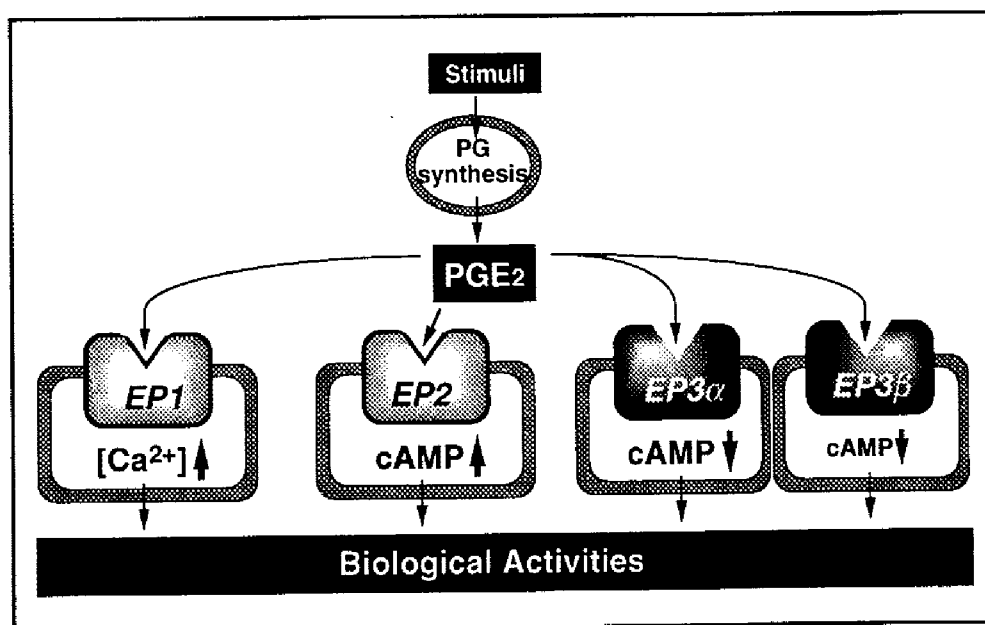


Fig. 4-1. Prostaglandin E receptors (subtypes and isoforms).

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]PGE₂ (185 Ci/mmol) were obtained from Du Pont-New England Nuclear. PGE₁, PGE₂, PGD₂, PGF₂ α 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 PGE₂ and 11-deoxy PGE₁ 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, USA), 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 EP₃ 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 TXA₂ receptor cDNA (HPL). Using these primers, a cDNA fragment (LT3; a fragment of the mouse TXA₂ 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 EP₃ 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 EP₃.

Mouse lung cDNA prepared by an oligo(dT) priming method was size-selected (> 1.8 kilobase) and inserted into the EcoRI site of λ ZAPII 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 λ ZAPII 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 EP₁ 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 EP₃, 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 EP₁.

2-4) cDNA cloning of the mouse prostanoid EP₂ receptor.

Previously, it was demonstrated that high level of PGE₂ binding is seen in mouse mastocytoma P-815 cells, neoplastic mast cells, and PGE₂ strongly stimulates adenylate cyclase (Yatsunami et al. 1981; Hashimoto et al. 1990), suggesting that the mastocytoma cells express EP₂ receptor. For isolation of a cDNA for the EP₂ receptor, we chose this cell line as a cDNA source, and performed homology screening using the partial cDNA fragment of the EP₃ (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 EP₁, EP₃, 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 EP₂.

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

The EcoRI inserts of the cloned cDNAs were individually subcloned into pcDNA1, 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 MgCl₂, 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 MgCl₂, 1 mM EDTA (buffer A). The 50 µg of membrane protein was used for [³H]PGE₂ 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 EP₃, each cell membranes were incubated with various concentrations (Scatchard plot analyses) or 2.5 nM (displacement experiments) of [³H]PGE₂ 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 PGE₂ 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 dT30. Poly (A)⁺ RNAs (5-10 μ g) from each tissue were separated by electrophoresis on a 1.2% agarose gel, transferred onto nylon membranes (Hybond-N, Amersham), and hybridized with a ³²P-labeled fragment of the appropriate cDNA clone. For Northern hybridization for EP₁ mRNA, an antisense RNA probe was prepared with T7 RNA polymerase (Toyobo, Osaka, Japan), in the presence of ³²P-UTP. Hybridization was carried out at 68°C in 6 $\times$ SSC for 12-16 h, and filters were washed twice at 68°C in 2 $\times$ 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 ($[\text{Ca}^{2+}]_i$) 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. $[\text{Ca}^{2+}]_i$ 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 EP₃ α - and EP₃ β -CHO cell membranes was described as follows;. The final reaction mixture contained 10 μ g of each CHO cell membranes, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mM 3-isobutyl-1-methylxanthine, 0.1 mM phenylmethanesulfonyl fluoride, 0.25 mM ATP, 0.5 mM AppNHp, indicated concentrations of PGE₂ or M&B28767, and 0.25 mM [γ -³²P]GTP (0.3 μ Ci) in 100 μ l 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 $\times$ g at 4°C, and the ³²Pi in 300 μ l of each supernatant was counted in scintillation fluid.

9. Reconstitution of the EP₃ receptor with Gi₂ in pertussis toxin-treated membranes

Reconstitution of the EP₃ receptor with Gi₂ in PT-treated membranes was performed according to the method of Asano et al. (1985). CHO cells stably expressing EP₃ receptor isoforms were cultured in the presence or absence of 20 ng/ml PT for 9h. CHO cell membranes (50 μ g) were incubated with or without Gi₂ purified from bovine spleen (10 pmol/mg of the membrane protein) at 0°C for 1 h. PGE₂ binding assays were carried out with 2.0 nM [³H]PGE₂ in the presence or absence of 100 μ M GTP γ S.

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 ³²P-labeled PCR II (0.3 pmol; 1.0×10⁶ 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 EP₃ 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 [³⁵S]CTP to a specific activity of 1.0 × 10⁹ 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 EP₂ and EP₁ 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 EP₃ and EP₁. 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 receptors¹. 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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