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# Molecular cloning and characterization of the four rat prostaglandin $E_2$ prostanoid receptor subtypes

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**Abstract**

We have characterized the rat prostanoid $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptor subtypes cloned from spleen, hepatocyte and/or kidney cDNA libraries. Comparison of the deduced amino acid sequences of the rat EP receptors with their respective homologues from mouse and human showed 91% to 98% and 82% to 89% identity, respectively. Radioreceptor binding assays and functional assays were performed on EP receptor expressing human embryonic kidney (HEK) 293 cells. The $K_D$ values obtained with prostaglandin $E_2$ for the prostanoid receptor subtypes $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$ were approximately 24, 5, 1 and 1 nM, respectively. The rank order of affinities for various prostanoids at the prostanoid receptor subtypes $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptor subtypes was prostaglandin $E_2$ = prostaglandin $E_1$ > iloprost > prostaglandin $F_{2\alpha}$ > prostaglandin $D_2$ > U46619. The rank order at the prostanoid $EP_1$ receptor was essentially the same except that iloprost had the highest affinity of the prostanoids tested. Of the selective ligands, butaprost was selective for prostanoid $EP_2$, M&B28767 and sulprostone were selective for $EP_{3\alpha}$ and enprostil displayed dual selectivity, interacting with both prostanoid receptor subtypes $EP_1$ and $EP_{3\alpha}$. All four receptors coupled to their predominant signal transduction pathways in HEK 293 cells. Notably, using a novel aequorin luminescence assay to monitor prostanoid $EP_1$ mediated increases in intracellular calcium, both iloprost and sulprostone were identified as partial agonists. Finally, by Northern blot analysis $EP_3$ transcripts were most abundant in liver and kidney whereas prostanoid $EP_2$ receptor mRNA was expressed in spleen, lung and testis and prostanoid $EP_1$ receptor mRNA transcripts were predominantly expressed in the kidney. The rat prostanoid $EP_1$ probes also detected additional and abundant transcripts present in all the tissues examined. These were found to be related to the expression of a novel protein kinase gene and not the prostanoid $EP_1$ gene [Batshake, B., Sundelin, J., 1996. The mouse genes for the $EP_1$ prostanoid receptor and the novel protein kinase overlap. Biochem. Biophys. Res. Commun. 227, 1329–1333]. © 1997 Elsevier Science B.V.

*Keywords:* Prostanoid; HEK 293; Aequorin

## 1. Introduction

Prostaglandin $E_2$ is thought to be an important mediator of the inflammation and pain responses as demonstrated in a number of in vitro and in vivo models (Coleman et al., 1989 and references within). In particular, a recent study by Mnich et al. (1995) showed that neutralizing monoclonal antibodies to prostaglandin $E_2$ were effective in an in vivo mouse model of nociception, implicating prostaglandin $E_2$ as the major prostanoid mediating the pain response.

The physiological and pathophysiological actions of prostaglandin $E_2$ are mediated through interaction with specific G-protein-coupled EP [2] receptors (Coleman et al., 1989; Davies and MacIntyre, 1992) which belong to the superfamily of G-protein coupled receptors. There are four

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[2] Prostanoid receptors are designated following the recommendation of the IUPHAR Commission on Receptor Nomenclature and Classification (Watson and Girdlestone, 1993).

subtypes of the prostaglandin $E_2$ receptor, $EP_1$, $EP_2$, $EP_3$ and $EP_4$, which have been cloned from both human (Funk et al., 1993; Adam et al., 1994; Regan et al., 1994; Bastien et al., 1994) and mouse (Sugimoto et al., 1992; Watabe et al., 1993; Honda et al., 1993; Katsuyama et al., 1995) and which couple to different major signal transduction pathways; namely elevation of intracellular $Ca^{2+}$ ($EP_1$) and stimulation ($EP_2$, $EP_4$) or inhibition of adenylyl cyclase ($EP_3$). Rat prostanoid receptors cloned include the $EP_1$ (Okuda-Ashitaka et al., 1996), $EP_3$ (Takeuchi et al., 1993, 1994; Neuschäfer-Rube et al., 1994) and $EP_4$ (Sando et al., 1994). In addition, several different isoforms of the $EP_3$ subtype have also been identified (Breyer et al., 1994a,b; Schmid et al., 1995), which are produced by alternative splicing and differ only in the length and amino acid composition of their carboxyl-terminal regions.

Rat in vivo models of inflammation and pain are widely used in pharmacology (Chau, 1989). In order to evaluate the true therapeutic utility of prostanoid EP receptor ligands in these models it is necessary to establish the relative potencies of these compounds at the four rat prostanoid receptor EP subtypes. This information can then be correlated with similar results using the human homologues.

We report here, therefore, the molecular cloning of the four rat prostaglandin $E_2$ receptor subtypes, $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$. As a result the prostanoid receptor EP subtypes have been characterized in terms of radioligand binding and functional activities in assays performed under comparable experimental conditions. In addition, expression of prostanoid receptor subtypes, $EP_1$, $EP_2$ and $EP_3$, has been delineated by Northern blot analysis.

## 2. Materials and methods

### 2.1. Chemicals

Prostaglandin $E_2$, prostaglandin $D_2$, prostaglandin $F_{2\alpha}$, prostaglandin $E_1$, 17-phenyl-$\omega$-trinor-prostaglandin $E_2$, 11-deoxy-prostaglandin $E_1$, 19(*R*)-OH-prostaglandin $E_2$, AH23848; [1$\alpha$(*Z*),2$\beta$,5$\alpha$]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid: AH6809; 6-isopropoxy-9-oxoxanthene-2-carboxylic acid: butaprost; 3-hydroxy-2-[4-hydroxy-4-(1-propylcyclobutyl)-1-butenyl]-5-oxo-methyl ester: enprostil; (DL)-9-keto-11$\alpha$,15$\alpha$-dihydroxy-16-phenoxy-17,18,19,20-tetranorprosta-4,5,13-*trans*-trienoic acid methyl ester: GR63799X; [1*R*-[1$\alpha$(*Z*),2$\beta$(*R**),3$\alpha$]-(−)-4-benzoylamino)phenyl-7-[3-hydroxy-3-phenoxypropoxy)-5-oxocyclopentyl]-4-heptenoate: iloprost; 5-[hexahydro-5-hydroxy-4-(3-hydroxy-4-methyl-1-octen-6-ynyl)-2(1H)-pentalenylidene]pentanoic acid: M&B28767; 15*S*-hydroxy-9-oxo-16-phenoxy-$\omega$-tetranorprost-13E-enoic acid: misoprostol; prost-13-en-1-oic acid,11,16-dihydroxy-16-methyl-9-oxo-,methyl ester,(11$\alpha$,13E): SC19220; 1-acetyl-2-(8-chloro-10,11-dihydrodibenz[b,f][1,4]oxazepine-10-carbonyl)hydrazine: sulprostone; 5-heptenamide,7-[3-hydroxy-2-(3-hydroxy-4-phenoxy-1-butenyl)-5-oxocyclopentyl]-*N*-(methylsulfonyl)-[1*R*-[1$\alpha$(*Z*),2$\beta$(1*E*,3*R**),3$\alpha$]]: U46619; 11$\alpha$,9$\alpha$-epoxymethano-15*S*-hydroxy-prosta-5*Z*,13*E*-dienoic acid. AH6809 and GR63799X were kind gifts from Glaxo Group Research and Amersham Life Science, respectively. All other ligands were from BIOMOL Research (Plymouth Meeting, PA) or Cayman Chemical (Ann Arbor, MI).

### 2.2. Cloning of the rat $EP_1$ prostanoid receptor

The rat kidney cDNA library ($1.0–1.2 \times 10^6$ plaques) was probed with two degenerate oligomers, end-labeled with [$^{32}$P] $\gamma$ATP (NEN, Mississauga, Ont.), based on transmembrane domain VII of the prostanoid TP, $EP_1$ and $EP_3$ receptors 5′-ATA (A,C)AC CCA GGG (A,G)TC CA(A,G) GAT CTG (G,A)TT-3′ and the prostanoid $EP_4$ receptor 5′-TA(A,G) ATC CAG GG(A,G) TC(T,C) AGG ATG GG(G,A) TT-3′. The 163 positive phage clones were picked and plated for hybridization with two additional oligomers, derived from the published mouse (Watabe et al., 1993) and human prostanoid $EP_1$ receptor (Funk et al., 1993) sequences, 5′-CTC CAG CAG ATG CAC GAC ACC AC-3′ in transmembrane domain VI and 5′-GGC TGC ATG GTC TTC TTC GGC CTG T-3′ in transmembrane domain III. These clones did not extend far enough 5′ to hybridize to the oligomer in transmembrane domain III and only 4 clones contained the transmembrane domain VI region. Clone 9-2, the longest, was subcloned into bluescript pKS (Stratagene, La Jolla, CA) at the *Eco*RI site and the ends were sequenced on an ABI-373 stretch automated sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA).

To obtain the complete rat prostanoid $EP_1$ receptor, a spleen cDNA library was screened with a rat prostanoid $EP_1$ DNA probe. Rat genomic DNA (Clontech) was used as template for amplification by polymerase chain reaction (PCR), with the oligomers from transmembrane domain III and VI, described above, and Taq DNA polymerase (Boehringer Mannheim), as follows: denaturation at 95°C for 60 s, annealing at 58°C for 30 s and extension at 72°C for 50 s, for 40 cycles on a DNA thermal cycler 480 (Perkin Elmer). The resulting rat prostanoid $EP_1$ receptor DNA fragment was purified with a gel extraction kit (Qiagen, Chatsworth, CA) then labeled with [$^{32}$P]-dCTP by random prime labeling with T7 DNA polymerase using the T7 Quick prime kit following the manufacturer's protocol (Pharmacia, Baie d'Urfé, Que.). Five positive clones were identified, but only one clone (clone 8-1) contained the putative start codon.

The full-length rat prostanoid $EP_1$ receptor coding sequence was constructed by joining the 5′ rat spleen clone 8-1 to the 3′ rat kidney clone 9-2 and by removing the 5′ and 3′ unspliced introns. First the 3′ intron was removed using gene splicing by overlap extension (SOEing) (Horton et al., 1990). For both clones, a DNA fragment adja-

cent to the intron sequence was amplified by PCR using the Pfu DNA polymerase (Stratagene) and the following oligonucleotides; SOE-1s 5′-GTC GCT CTC GAC GTT TCC GAG-3′ and SOE-2a 5′-GAT GGC CAA CAC CAC CAA TAC CAG CAG GGG GCT CCA GCA GAT-3′ for the rat spleen clone 8-1, SOE-3s 5′-GTA TTG GTG GTG TTG GCC ATC-3′ and SOE-4a 5′-GAG GCG AAG CAG TTG GCG CAG CA-3′ for the rat kidney clone 9-2. The PCR protocol was as follows: denaturation at 95°C, 60 s; annealing at 60°C, 60 s; and extension at 72°C, 15 s for 25 cycles. The two fragments were gel purified and mixed for PCR amplification with SOE-1s and SOE-4a. The resulting intronless rat prostanoid $EP_1$ receptor PCR fragment was digested with *Pvu*II and *Pst*I and gel purified. The PCR fragment was subsequently ligated to the 5′ rat spleen $EP_1$ clone 8-1 at the *Pvu*II site and to the 3′ rat kidney prostanoid $EP_1$ receptor clone 9-2 at the *Pst*I site in the bluescript pKS vector. The construct was sequenced on both strands using vector derived primers or primers (Research Genetics, Cambridge, MA) generated from the determined sequence and the ORF sequence was identical to that of the original clones 8-1 and 9-2 above.

Finally, the 5′ intron sequence was removed using the following amplification strategy. The 5′ end of the construct was replaced by an amplified DNA fragment which contained a Kozak consensus sequence (Kozak, 1984) adjacent to the putative rat prostanoid $EP_1$ receptor start codon. The oligomers 5′-ATC TAC GGT ACC CTC TTG GCC GCC ACC ATG AGC CCC TAC GG-3′ and 5′-TCC TGC AGT ATA CAG GCG AAG-3′ were used with the Pfu DNA polymerase and the following PCR protocol: denaturation at 95°C, 60 s; annealing at 55°C, 60 s; extension at 72°C, 20 s for 25 cycles. A unique *Acc*I restriction site in the rat prostanoid $EP_1$ receptor sequence allowed for the ligation of the new 5′ end, digested with *Asp*718I and *Acc*I, to the remaining rat prostanoid $EP_1$ receptor sequence, digested with *Acc*I and *Hin*dIII, in the expression vectors pCEP4 and pcDNA3.1(−) (Invitrogen) previously cut with *Asp*718I and *Hin*dIII. The new fragment was sequenced and found to be correct. The resulting rat prostanoid $EP_1$ receptor constructs were used in transfection experiments for receptor binding and signal transduction assays.

### 2.3. Cloning of the rat prostanoid $EP_2$ receptor

The rat spleen cDNA library ($1.0–1.2 \times 10^6$ plaques) was screened with a mixture of 2 oligonucleotides (mEP2-1F and mEP2-2F) derived from the mouse prostanoid $EP_2$ receptor sequence. The mEP2-1F primer (5′-GAA AGC CCA GCC ATC AGC TC-3′, 20-mer) and the mEP2-2F primer (5′-TGG CGC GCC GCT GGC GTG GGG AC-3′, 23-mer) were both derived from transmembrane domain I of the mouse prostanoid $EP_1$ receptor. These oligonucleotides were end-labeled as above and hybridized to the rat spleen cDNA library yielding two positive clones (λ17.1 and λ22.1) from which the DNA was obtained by the plate lysis method. *Eco*RI digestion of both clones to release the inserted DNA indicated that both phage clones contained 2.5 kb inserts. These were then subcloned into pBluescript KS (17-RI-8, 22-RI-5). Cycle sequencing (as above) revealed that both clones were identical and contained the rat prostanoid $EP_2$ receptor sequence from 43 nt upstream of the start codon up to transmembrane domain VI. They also contained an unspliced intron which is located at the end of transmembrane domain VI.

In order to find a clone containing the 3′ end of the rat prostanoid $EP_2$ receptor, an oligonucleotide derived from the mouse prostanoid $EP_2$ receptor sequence between transmembrane domain VI and transmembrane domain VII (mEP2-4R, TTC CTT TAG GGA AGA GGT TTC AT, 23-mer), was radiolabeled and used to screen the same cDNA library. Two positive clones were obtained and their sequence determined by sequencing the purified phage DNA directly. These 2 clones (λ19A1 and λ21A1) were also identical and contained the rat $EP_2$ receptor sequence from transmembrane domain IV up to 200 nt after the stop codon.

In order to reconstitute the full-length cDNA sequence, the pBS 17-RI-8 clone was first digested with *Sma*I in order to remove the intron sequence present in this clone. One *Sma*I site is located in the transmembrane domain VI sequence with the second *Sma*I site in the polylinker region of pBluescript KS. The resulting plasmid contains the rat prostanoid $EP_2$ receptor sequence 43 nt upstream of the starting ATG up to transmembrane domain VI. Then, a 377 nt *Sma*I–*Dra*I fragment from clone λ19A1, containing the sequence from transmembrane domain VI to 50 nt after the stop codon, was gel-purified and cloned into the *Sma*I-cut pBS 17-RI-8 clone. After verifying the orientation of the inserted 377 nt fragment by PCR using the rat prostanoid $EP_2$ specific primer, mEP2-4R, and the vector-derived KS primer, the complete sequence was obtained on both strands by cycle sequencing (as above) using vector-derived primers as well as sequence-specific primers.

### 2.4. Cloning of the rat prostanoid $EP_{3\alpha}$ receptor

#### 2.4.1. Hepatocyte purification and poly(A)$^+$ RNA preparation

Hepatocytes were isolated from male Wistar rats (200–260 g) according to Meredith (1988) without the use of collagenase by perfusion with $Ca^{2+}$-free Krebs–Henseleit buffer containing 2 mM EDTA as described previously (Püschel et al., 1993). Detritus and non-parenchymal cells were removed by repeated sedimentation of hepatocytes at $50 \times g$ and subsequent centrifugation through a gradient containing 58% Percoll. Poly(A)+ RNA was isolated from these purified hepatocytes by CsCl gradient centrifugation followed by affinity purification on oligo-(dT) beads (Qiagen, Rathingen).

*2.4.2. PCR-amplification of prostanoid $EP_3$ receptor cDNA fragments*

First strand cDNA was synthesized by reverse transcription using oligo-$(dT)_{12-18}$ (Pharmacia, Freiburg) as a primer. PCR was carried out using 1–10 ng first strand cDNA as a template and oligonucleotide primers corresponding to position 769–798 and 1471–1500 (numbers corresponding to GenBank accession number D10204) of the mouse prostanoid $EP_3$ sequence (Sugimoto et al., 1992). Thirty-five cycles of PCR were performed using the following protocol: 40 s 95°C, 40 s 60°C and 1.5 min 72°C. A 640 nt and a 730 nt fragment were amplified and cloned into PUC18 (Pharmacia). Nucleotide sequence analysis was carried out on double stranded templates using the dideoxy chain termination method. To generate digoxigenin-labelled probes for screening a cDNA library, 5% of the dTTP were replaced by 11-digoxygenin-dUTP during the PCR.

*2.4.3. Prostanoid $EP_{3\alpha}$ receptor cDNA cloning*

Rat hepatocyte cDNA was prepared from hepatocyte poly(A) + RNA by an oligo-(dT) priming method using a cDNA synthesis kit (Pharmacia) and inserted into the *Eco*RI site of λgt11 (Gibco-BRL) DNA with *Eco*RI adaptors including an internal *Not*I site (Pharmacia). The $10^6$ clones derived from this library were screened by hybridization with the cloned 640 and 730 bp prostanoid $EP_3$ receptor probes labeled with digoxigenin (see above). Positive plaques were isolated and analyzed with restriction digestion and PCR. The cDNAs of three clones were amplified by PCR using primers flanking the *Eco*RI cloning site, subcloned into PUC18 and sequenced. The sequences of the three clones were found to be identical. A 2.1 kb *Not*I cDNA fragment of clone 15/2 was subcloned into the eukaryotic expression vector pRc/CMV. The insert was then excised by a *Pvu*II-*Not*I double digestion and cloned into pCEP4 cut with *Pvu*II and *Not*I. Both the 5′ end and the 3′ end of this clone were then verified by sequencing using two vector-derived primers (pCEP forward primer and EBV reverse primer).

*2.5. Cloning of the rat prostanoid $EP_4$ receptor*

The oligonucleotide probe 5′-CAG ATG GTC ATC TTA CTC ATC GC-3′ based on transmembrane domain VI of the published rat prostanoid $EP_4$ receptor sequence (Sando et al., 1994) was used to screen a rat kidney cDNA library ($1.0–1.2 \times 10^6$ plaques). The 18 positive phage clones were rehybridized with the oligonucleotide 5′-ATG TCC ATC CCC GGA GTC AAC GC-3′ located at the start codon. From the 8 positive clones hybridizing to both probes, clone 3-4 and 7-2 were subcloned into bluescript pKS at the *Eco*RI site and sequenced (as above) on both strands. The clone 3-4 was digested with *Hin*dIII and *Bam*HI, removing about 360 nt of 5′ non-coding, and subcloned into pCEP4 and pcDNA3.

*2.6. Rat EP receptor expression in HEK 293(EBNA) cells and cell membrane preparation*

HEK 293(EBNA) cells were maintained in culture in Dulbecco's modified Eagle's growth medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 u/ml penicillin G, 100 μg/ml streptomycin sulfate and under selection using 250 μg/ml GENETICIN™ (G418) (Life Technologies/BRL, Burlington, Ont.). HEK 293(EBNA) cells were then transfected with pCEP4 expression vectors containing rat EP receptor coding cDNA using LipofectAMINE™ reagent (Life Technologies/BRL) according to the manufacturers' instructions. Transfected cells were either harvested 48–72 h post-transfection (for the radioligand binding and aequorin luminescence assays) or maintained in culture for several weeks with the addition of 200 μg/ml hygromycin B (Boehringer Mannheim) to select for cells expressing the rat prostanoid receptor subtypes, $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptors (used in the cAMP assays). Cells were harvested by incubation in enzyme-free cell dissociation buffer (Life Technologies/BRL), washed in ice-cold phosphate-buffered saline (pH 7.4) and resuspended in 10 mM HEPES/KOH (pH 7.4) containing 1 mM EDTA. Membranes were prepared from harvested cells by differential centrifugation ($1000 \times g$ for 10 min, then $160{,}000 \times g$ for 30 min, all at 4°C) following lysis of the cells by nitrogen cavitation at 800 psi for 30 min on ice in the presence of protease inhibitors (2 mM phenylmethylsulfonylfluoride, 10 μM E-64, 100 μM leupeptin and 0.05 mg/ml pepstatin). The $160{,}000 \times g$ pellets were resuspended in 10

Fig. 1. Amino acid sequence comparisons of the rat, mouse and human EP receptors. The deduced amino acid sequences of the rat, mouse and human prostanoid receptor subtypes (a) $EP_1$, (b) $EP_2$, (c) $EP_{3\alpha}$ and (d) $EP_4$ are shown aligned using GCG Wisconsin DNA software. Non-conserved amino acids compared with the rat sequences are shown. The transmembrane domains I–VII are indicated by overlines. Dashes indicate gaps introduced in the sequences for alignment purposes. The termination codon is indicated by (*). Residues which varied between our predicted amino acid sequences and the data base sequences are as follows: the prostanoid $EP_3$ receptor had an Arg and Ala for Pro at positions 161 and 162 compared with the sequences for rat prostanoid EP3α receptor (GenBank/EMBL Accession number, D14869: Takeuchi et al., 1993) and prostanoid EP3γ receptor (D16443: Takeuchi et al., 1994); the prostanoid $EP_4$ receptor had His (CAC) for Leu (CTC) at position 214 and Gln (CAG) for His (CAC) at 396 compared with the sequence for rat prostanoid $EP_4$ receptor (D28860: Sando et al., 1994). The two amino acid differences are also present in mouse (Honda et al., 1993; D13458), rabbit (Breyer et al., 1994a; L47207) and human (Bastien et al., 1994; L28175) prostanoid $EP_4$ receptors.

**a**

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                                  I                          II
rEP1  MSPYG-LNLSLVDEATTCVTPRVPNTSVVLPTGGNGTSPALPIFSMTLGAVSNVLALALLAQVAGRLRRRRSTATFLLFVASLLAIDLAGHVIPGALVLRLY  101
mEP1     C -     A   A  A   L          D                                  M     A
hEP1     C P     AG     AA W     A P S A---                  L        A         AT          T

                        III                        IV
rEP1  TAGRAPAGGACHFLGGCMVFFGLCPLLLGCGMAVERCVGVTQPLIHAARVSVARARLALALLAAMALAVALLPLVHVGHYELQYPGTWCFISLGPPGGWRQA  203
mEP1                                                                V                R             R
hEP1                                        R  L                   AV  V          AR  R            G

      V                                                                                          VI
rEP1  LLAGLFAGLGLAALLAALVCNTLSGLALLRARWRRRRSRRFRENAGPDDRRRWGSRGLRLASASSASSITSTTAALRSSRGGGSARRVHAHDVEMVGQLVGI  305
mEP1                                        KT              P              A  T
hEP1          S   V                 H   -       PPPAS   S     AH P S         A ASTFFGG  SS     AR

                        VII
rEP1  MVVSCICWSPLLVLVVLAIGGWNSNSLQRPLFLAVRLASWNQILDPWVYILLRQAMLRQLLRLLPLRVSAKGGPTELSLTKSAWEASSLRSSRHSGFSHL*  405
mEP1                                                                                G                 *
hEP1             M    A  V   S T                                V         P  G     AG G  P                L  F*
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**b**

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                                  I                          II
rEP2  MDNSFNDSRRVENCESRQYLLSDESPAISSVMFTAGVLGNLIALALLARRWRGDTGCSAGSRTSISLFHVLVTELVLTDLLGTCLISPVVLASYSRNQTL  100
mEP2     FL    KLM D K    W    G          S
hEP2   G AS    QS- D  T   W PPG           S                    V     R S L           F                A

                        III                        IV                                          V
rEP2  VALAPESRACTYFAFTMTFFSLATMLMLFAMALERYLAIGHPYFYRRRVSRRGGLAVLPAIYGVSLLFCSLPLLNYGEYVQYCPGTWCFIQHGRTAYLQL  200
mEP2         H                                  S  Y      HL          V   A                           R
hEP2                  A                         S  A LLLPAPRLAS   G   V  A           D  Q             R

                                                                  VI
rEP2  YATVLLLLIVAVLGCNISVILNLIRMQLRSKRSRCGLS-GSSLRGPGSRRRGERTSMAEETDHLILLAIMTITFAVCSLPFTIFAYMDETSSRKEKWDLR  299
mEP2     M         A            HR  R       -                                                I            L
hEP2     L       S  A  F         HR  R     P L  GRG   A      V                                        N          Q

      VII
rEP2  ALRFLSVNSIIDPWVFVILRPPVLRLMRSVLCCRTSLRAPEAPGASCSTQ-----QTDLCGQL*                                    357
mEP2                  A                      TQ  QQT     SSASK         *
hEP2         I        A                   I   TQD TQT     SDASK A  *
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**c**

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                                                        I                          II
rEP3α                        MAGVWAPEHSVEAHSNQ---SSAADGCGSVSVAFPITMMVTGFVGNALAMLLVVRSYRRRESKRKKSFLLCIGWLALTDL    77
mEP3α                          SM       A     L---  TT D                          S
hEP3α  MKETRGYGGDAPFCTRLNHSYT M    R A  RG LTRPPGSGED              LL             S

                        III                        IV
rEP3α  VGQLLTSPVVILVYLSQRRWEQLDPSGRLCTFFGLTMTVFGLSSLLVASAMAVERALAIRAPHWYASHMKTRATRAVLLGVWLSVLAFALLPVLGVGRYS  176
mEP3α                                                                                -P
hEP3α         T    V    KQ   HI                        FI                                    A             Q T

                                  V                                                    VI
rEP3α  VQWPGTWCFISTGPAGNETDSAREPGSVAFASAFACLGLLALVVTFACNLATIKALVSRCRAKAAASQSSAQWGRITTETAIQLMGIMCVLSVCWSPLLI  276
mEP3α                  P                                                  V
hEP3α               RG  G SSSHNW NLF      F      T   S                  T

                        VII
rEP3α  MMLKMIFNQMSVEQCKTQMGKEKECNSFLIAVRLASLNQILDPWVYLLLRKILLRKFCQIRDHT-NYASSSTSLPCPGSSVLMWSDQLER*            366
mEP3α                                                                  -             A        *
hEP3α           T   H   HTE Q    F                                   Y  N          Q   T     H   *
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**d**

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                                                  I                          II
rEP4                                MSIPGVNASFSSTPERLNSPVTIPAVMFIFGVVGNLVAIVVLCKSRKEQKETTFYTLVCGLAVTDLLGTLLVSPVTIAT    79
mEP4  MAEVGGTIPRSNRELQRCVLLTTTI
hEP4                                  T    S A LS D

      _          III                        IV
rEP4  YMKGQWPGDQALCDYSTFILLFFGLSGLSIICAMSIERYLAINHAYFYSHYVDKRLAGLTLFAVYASNVLFCALPNMGLGRSERQYPGTWCFIDWTTNVTAYAA  183
mEP4                                                                I
hEP4         G P  E          S          V                                        S RL   D             H

      V                                                                                    VI
rEP4  FSYMYAGFSSFLILATVLCNVLVCGALLRMHRQFMRRTSLGTEQHHAAAAAAVASVACRGHAAASPALQRLSDFRRRRSFRRIAGAEIQMVILLIATSLVVLIC  287
mEP4                                                                G
hEP4  Y                                                 ---    S   P      P

      _____                VII
rEP4  SIPLVVRVFINQLYQPSVVKDISRNPDLQAIRIASVNPILDPWIYILLRKTVLSKAIEKIKCLFCRIGGSGRDGSAQHCSESRRTSSAMSGHSRSFLSRELREI  391
mEP4                  N                                                     S                          A   K
hEP4           V       LEREV K                                          R ER G    D Q                I   K

rEP4  SSTSQTL---LYLPDLTESSLGGKNLLPGTHGMGLTQADTTSLRTLRISETSDSSQGQDSESVLLVDEVSGSQREEPASKGNSLQVTFPSETLKLSEKCI*      488
mEP4         ---             R     S                                          H                          *
hEP4         LPD S    S NG   R     VP    A E                                 A   G AG  P  S          N      *
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mM HEPES/KOH (pH 7.4) containing 1 mM EDTA at approximately 5–10 mg/ml by Dounce homogenization (Dounce A; 10 strokes), frozen in liquid nitrogen and stored at −80°C.

### 2.7. Radioligand binding assays

Rat EP receptor equilibrium competition binding assays were performed in a final incubation volume of 0.2 ml in 10 mM MES/KOH (pH 6.0) containing 1 mM EDTA, 10 mM $MgCl_2$ (3 mM $MgCl_2$ for $EP_{3\alpha}$) and [$^3$H]prostaglandin $E_2$ [1 nM for $EP_1$ and 0.5 nM for $EP_2$, $EP_{3\alpha}$ and $EP_4$ (185 Ci/mmol; Amersham Life Science, Oakville, Ont.)]. Prostanoid $EP_{3\alpha}$ assays also contained 100 $\mu$M GTP$\gamma$S. The reaction was initiated by addition of membrane protein (approximately 62 $\mu$g for $EP_1$, 27 $\mu$g for $EP_2$, 2 $\mu$g for $EP_{3\alpha}$ and 13 $\mu$g for $EP_4$) from the 160,000 $\times g$ fraction. Ligands were added in dimethylsulfoxide ($Me_2SO$) which was kept constant at 1% (v/v) in all incubations. Non-specific binding was determined in the presence of 1–10 $\mu$M of prostaglandin $E_2$. Incubations were conducted for 60 min at 30°C and terminated by rapid filtration through a 96-well GF/C Unifilter (Canberra Packard, Ont.) prewetted in cold 10 mM MES/KOH (pH 6.0). The Unifilters were washed with 3–4 ml of the same buffer, dried for 90 min at 55°C and the residual radioactivity bound to the individual filters determined by scintillation counting with addition of 50 $\mu$l of Ultima Gold F (Canberra Packard). Specific binding was calculated by subtracting non-specific binding from total binding and accounted for 90–95% of the total binding and was linear with respect to the concentrations of radioligand and protein used. Total binding represented 5–10% of the radioligand added to the incubation media.

### 2.8. cAMP assays in HEK 293(EBNA) cells expressing the rat EP receptors

HEK 293(EBNA) cells were harvested at approximately 80% confluence by incubation in enzyme-free cell dissociation buffer, washed once with Hanks' Balanced Salt Solution (HBSS) and resuspended in HBSS. The cAMP generation assay was performed in 0.2 ml HBSS containing 100 $\mu$M of the phosphodiesterase type IV inhibitor RO-20-1724 (BIOMOL Research, Plymouth Meeting, PA) to prevent hydrolysis of cAMP. Assays were performed in both the absence and presence of 12.5 $\mu$M forskolin to measure both stimulation ($EP_2$ and $EP_4$) and inhibition ($EP_{3\alpha}$) of cAMP production, respectively. RO-20-1724, forskolin and test ligands were added to the incubation mixture in $Me_2SO$ or ethanol to a final vehicle concentration 1% (v/v) which was kept constant in all samples. The reaction was started by addition of $1 \times 10^5$ cells ($EP_2$, $EP_4$) or $2 \times 10^5$ cells ($EP_{3\alpha}$) per incubation. Samples were incubated at 37°C for 10 min and the reaction terminated by immersing the samples in boiling water for 3 min. Cell viability was always $\geq$ 96% as determined by trypan blue exclusion. Measurement of cAMP was performed by scintillation proximity assay using [$^{125}$I]cAMP (Amersham).

### 2.9. Aequorin luminescence assay in HEK 293 cells expressing the rat prostanoid $EP_1$ receptor

HEK 293 cells which stably express apo-aequorin, AEQ-17 HEK 293 cells (24), were transiently transfected with rat prostanoid $EP_1$ in pcDNA3.1 using the lipofectAMINE™ transfection protocol as described above. Two days after transfection, the cells were charged by incubation for 4 h at 37°C in Ham's F12 medium (with 0.1% fetal bovine serum, 25 mM Hepes at pH 7.3) (GIBCO BRL, Missisauga, Ontario) containing 30 $\mu$M of reduced glutathione (Sigma, St. Louis, MO) and 8 $\mu$M of coelenterazine cp (Molecular Probes, Eugene, OR). After charging the harvested cells were rinsed once and resuspended in Ham's F12 medium at $5 \times 10^5$ cells/ml. Test compounds were diluted in Hank's balanced salt solution (HBSS) containing 25 mM Hepes at pH 7.3 (GIBCO BRL, Missisauga, Ont.) and 100 $\mu$l aliquots dispensed into a 96-well plate. The 96-well plate was then inserted into a Luminoskan luminometer (Labsystems, Needham Heights, MA) and $5 \times 10^4$ cells (in 100 $\mu$l volume) were injected into each well, starting with well A1. Light emission was recorded for 30 s (Peak 1), after which 100 $\mu$l of 0.3% (v/v) Triton X-100 in HBSS was added to solubilize the cells and the light emission was measured for another 10 s (Peak 2). Fractional luminescence was determined at each ligand concentration by dividing the area under Peak 1 by the sum of the area under Peak 1 plus Peak 2.

### 2.10. Northern blot analysis

A rat Multiple Tissue Northern (MTN) blot (Clontech, Palo Alto, CA) containing 8 different tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis) was hybridized with the following probes: (1) a 350 nt PCR probe obtained by amplifying the rat prostanoid $EP_1$ receptor in pCEP4 with a forward primer covering the ATG start codon (5′-ATC TAC GGT ACC CTC TTG GCC GCC ACC ATG AGC CCC TAC GG-3′) and a reverse primer spanning an *Acc*I site, 350 nt 3′ to the start codon (5′-TCC TGC AGT ATA CAG GCG AAG-3′). (2) A 5′-intron probe (termed intron 1 probe) prepared by random priming a 160 nt PCR fragment obtained by amplifying a cDNA clone (clone 8.1 in pBluescript KS), which contained an unspliced 5′-intron, using the vector-derived SK forward primer and an intron-specific reverse primer (5′-CTG CGT AGA TAA AAA GAG GGC AC-3′). (3) A 3′-intron probe (410 nt) (termed intron 2 probe) obtained using two 3′-intron specific primers, rat prostanoid $EP_1$ receptor (3′intron) F (5′-GCG CAG TCG CTC CTC AAG

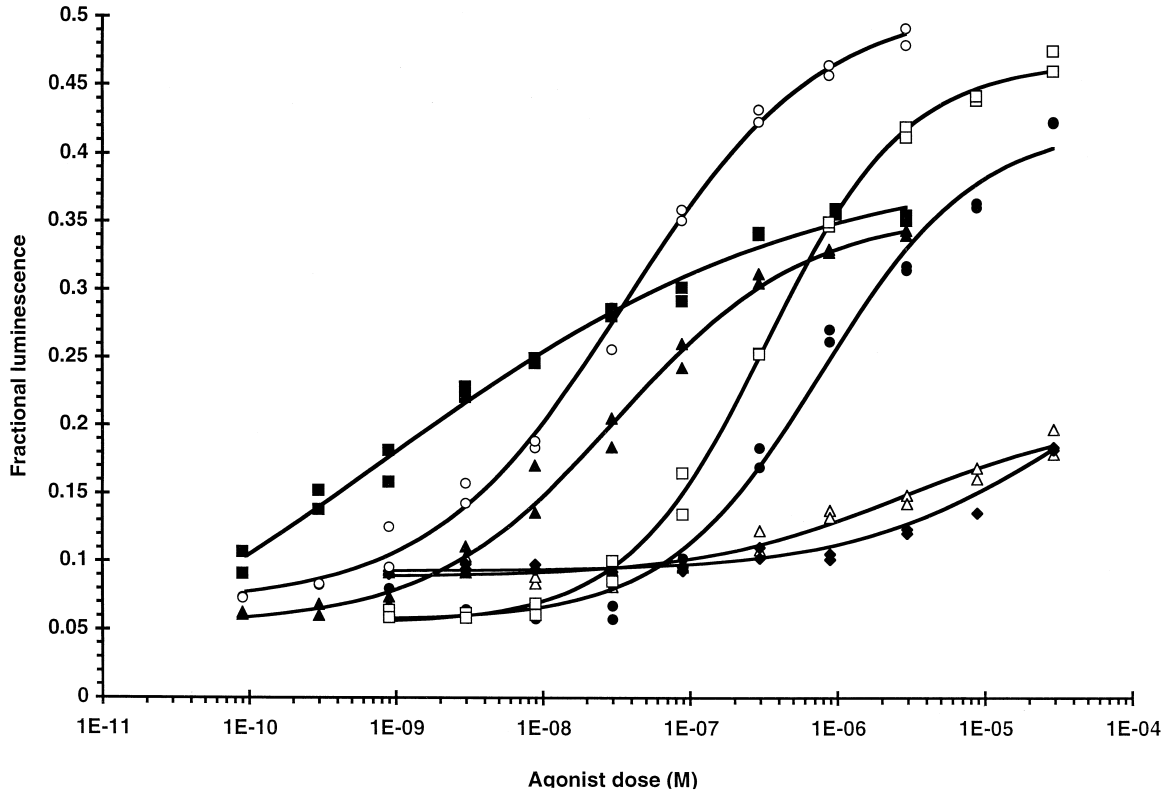



Fig. 2. Activation of aequorin luminescence in AEQ17-HEK293 cells transiently expressing the rat prostanoid $EP_1$ receptor. The fractional luminescence responses to prostaglandin $E_2$ (○), iloprost (■), sulprostone (▲), prostaglandin $E_1$ (□), prostaglandin $F_{2\alpha}$ (●), U46619 (△) and prostaglandin $D_2$ (◆) of pcDNA3.1-r$EP_1$ transfected AEQ17-HEK293 cells are plotted as a function of their concentrations. Duplicates for each sample are shown.

CCA A-3′) and rat prostanoid $EP_1$ receptor (3′intron) R (5′-GCG AGA GAA GCA GGT GTG AGC C-3′) used to amplify the cDNA clone 9.2 which contained an unspliced 3′intron. (4) A 748 nt rat novel protein kinase (25) PCR specific probe obtained from a rat spleen cDNA library after 2 rounds of PCR amplification using a forward novel protein kinase-1F primer (5′-CCT GAT GTG TGA GAA GCG GAT-3′) and a reverse novel protein kinase-2R primer (5′-GAA CTC CTC ATC GAA GTT GCT G-3′). This PCR fragment amplifies the DNA corresponding to amino acids 660 to 908 of the novel protein kinase protein. (5) a 1.2 kb full-length rat prostanoid $EP_2$ receptor probe. The rEP2(KS)-41 clone was digested with *Asp*718 and *Not*I to release the complete rat prostanoid $EP_2$ receptor DNA. (6) A rat $EP_3$ receptor probe prepared by digesting the r$EP_{3\alpha}$-pRC/CMV plasmid with *Hin*dIII to isolate the full-length receptor plus 200 nt of the 5′ and 3′-UTR (1.5 kb).

All fragments were labeled with [$^{32}$P]-dCTP by random priming (as described above) and added to the blot which was pre-hybridized for 1 h at 68°C in Express Hybrid solution (Clontech, Palo Alto, Ca). The incubation was carried out at the same temperature for 2 h. The blot was then washed once in 2× SSC/0.05% SDS for 30 min at room temperature and then at 50°C for 30 min in 0.1× SSC/0.1% SDS. Exposure was carried out on a Kodak XAR film with intensifying screens for 4–7 days at −80°C.

## 3. Results

### 3.1. Cloning of the rat EP receptors [3]

#### 3.1.1. Rat prostanoid $EP_1$ receptor cloning

To construct the open reading frame (ORF) of the rat prostanoid $EP_1$ receptor two clones were used, the 5′ clone (clone 8-2) derived from a spleen cDNA library and the 3′ clone (clone 9-1) derived from a kidney cDNA library, with both the 5′ and the 3′ introns removed (see Section 2 for details). The ORF is 1215 nt and encodes for a 405 amino acid protein with a predicted relative molecular mass of 43,053 Da. There are 3 potential N-glycosylation sites (Asn 7, 24 and 34), 6 potential protein kinase C phosphorylation sites (Ser 241, 281, 287, 374, 393, and

[3] The nucleotide sequences for the rat $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptors have been submitted to the GenBank/EMBL Data Bank with the accession numbers U68037, U94708, X83855 and U94709, respectively.

396) and two potential protein kinase A phosphorylation sites (Ser 71 and Thr 72). The rat prostanoid $EP_1$ receptor shares 96% and 84% amino acid identity with the mouse and human prostanoid $EP_1$ receptor, respectively (Fig. 1a).

### 3.1.2. Rat prostanoid $EP_2$ receptor cloning

Two overlapping clones were identified from a rat spleen cDNA library which together spanned the full length ORF of the rat prostanoid $EP_2$ receptor. The two clones were then spliced together to reconstitute the full length ORF (see Section 2 for details) with 43 nt of 5′-UTR and 37 nt of 3′-UTR. The ORF is 1017 nt and encodes for a 357 amino acid protein with a predicted relative molecular mass of 39,776 Da. There is one potential N-glycosylation site (Asn 6) and 5 potential protein kinase C phosphorylation sites (Ser 150, 230, 241, 247 and 335). Rat prostanoid $EP_2$ receptor shares 91% and 82% amino acid identity with mouse and human prostanoid $EP_2$ receptor, respectively (Fig. 2b).

### 3.1.3. Rat prostanoid $EP_{3\alpha}$ receptor cloning

Three cDNA clones were isolated from a rat hepatocyte cDNA library in λgt11 using mouse prostanoid $EP_3$ receptor PCR derived probes (see Section 2 for details). Clone 15/2 contained an open reading frame of 1098 nt and could be translated into a 366 amino acid protein. The protein displayed 97% and 85% sequence identity with the mouse prostanoid $EP_{3\alpha}$ receptor and human prostanoid $EP_{3\text{-}I}$ receptor, respectively (Fig. 1c).

The sequence-deduced molecular mass was 40,059 Da. The C-terminal domain was more hydrophilic than in the rat or mouse prostanoid $EP_{3\beta}$ receptor (Neuschäfer-Rube et al., 1994). Protein kinase A (PKA) phosphorylation sites (Ser 59 and 64) and N-glycosylation sites (Asn 16 and 194) described for the mouse mastocytoma prostanoid $EP_{3\beta}$ receptor and the rat kidney $EP_{3\alpha}$ receptor (Takeuchi et al., 1994) were preserved. The carboxyl-terminal domain of rat prostanoid $EP_{3\alpha}$ receptor contains 7 Ser and 2 Thr residues that might be potential phosphorylation sites. None of these sites, however, correspond to the consensus sequences for protein kinase A or protein kinase C.

### 3.1.4. Prostanoid $EP_4$ receptor cloning

The rat prostanoid $EP_4$ receptor cDNA (clone 3-4), cloned from a rat kidney cDNA library (see Section 2 for details), contained approximately 600 nt of 5′-UTR, a 1464 nt ORF and only a short 6 nt 3′-UTR. The ORF encodes for a 488 amino acid protein with a predicted relative molecular mass of 53,373 Da. There is one potential N-glycosylation site (Asn 7), nine potential protein kinase C phosphorylation sites (Ser 45, 262, 357, 369, 430, 460, 484 and Thr 433 and 480) and an additional two potential protein kinase A phosphorylation sites (Ser 222 and 373). The rat protanoid $EP_4$ receptor shares 98% and 89% amino acid identity with mouse and human prostanoid $EP_2$ receptor, respectively (Fig. 1d).

## 3.2. Expression of rat EP receptors in HEK 293(EBNA) cells

### 3.2.1. Receptor binding studies

Saturation analysis was performed to determine the affinity of prostaglandin $E_2$ for the rat EP receptors ($K_D$) as well as the level of receptor expression attained in the HEK 293(EBNA) cell line, as defined by maximum number of detectable prostaglandin $E_2$ specific binding sites ($B_{max}$) (Table 1). Prostaglandin $E_2$ displayed the highest affinity for the prostanoid $EP_{3\alpha}$ and $EP_4$ receptors with $K_D$ values of approximately 1 nM. The $K_D$ values obtained for prostanoid $EP_1$ receptor and prostanoid $EP_2$ receptor were approximately 25-fold and 5-fold higher, respectively. These data are comparable to the values obtained for the human EP receptors in this cell line (Abramovitz et al., submitted to Biochem. Biophys. Acta). In every case the data conformed to a single-site binding model except for prostanoid $EP_2$ receptors where a second site of lower affinity was also detected. The highest level of high affinity receptor expression was obtained for the prostanoid $EP_{3\alpha}$ receptor with a $B_{max}$ of 22 pmol/mg protein. Similar levels of high affinity expression were obtained for the prostanoid $EP_1$ receptor and prostanoid $EP_2$ receptor at approximately 3 pmol/mg protein with the lower affinity site observed for the prostanoid $EP_2$ receptor being 10-fold more abundant. The level observed for prostanoid $EP_4$ receptor was somewhat lower at 0.9 pmol/mg protein. These results are also similar to those obtained for the expression of the human EP receptors in HEK 293(EBNA) cells (see above). Membranes from wild type and mock-transfected (vector alone) HEK 293(EBNA) cells were tested for their ability to bind [$^3$H] prostaglandin $E_2$ and were negative in both cases (data not shown).

Equilibrium competition binding assays were performed to determine the affinities of prostanoids and related analogs at the rat prostanoid EP receptors (Table 2). In

Table 1
Saturation analysis of [$^3$H]prostaglandin $E_2$ specific binding to HEK 293(EBNA) cell membranes expressing rat $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptors

| Rat prostanoid receptor | $K_D$ (nM) | | $B_{max}$ (pmol/mg protein) | |
|---|---|---|---|---|
| | one-site | two-site | one-site | two-site |
| $EP_1$ | 24±2 | nd | 3.3±1.3 | nd |
| $EP_2$ | 5.3±2.3 | 233±33 | 3.1±1.6 | 30±20 |
| $EP_{3\alpha}$ | 0.83±0.16 | nd | 22±5 | nd |
| $EP_4$ | 1.1±0.6 | nd | 0.9±0.5 | nd |

Saturation analyses were conducted using the binding assay protocol described in Section 2 and using a radioligand concentration ranging 10-fold above and below the determined $K_D$. Non-specific binding was determined with 1000-fold excess of prostaglandin $E_2$. Non-linear transformation of the deduced specific binding saturation isotherms was performed using Accufit Saturation Two-Site data analysis software (Beckman Instruments, CA). Values are mean±s.e.m. where $n=3$ with nd indicating not detected under these experimental conditions.

Table 2
Competition for [$^3$H]prostaglandin $E_2$ specific binding to HEK 293(EBNA) cell membranes expressing rat $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$ receptors by prostanoids and related analogs

| Ligand | $K_i$ (nM) | | | |
|---|---|---|---|---|
| | $EP_1$ | $EP_2$ | $EP_{3\alpha}$ | $EP_4$ |
| prostaglandin $E_2$ | 22 | 6.8 | 0.9 | 1.1 |
| prostaglandin $E_1$ | 95 | 10 | 1.1 | 0.66 |
| prostaglandin $D_2$ | 5680 | 4800 | 1280 | 1240 |
| prostaglandin $F_{2\alpha}$ | 423 | 2360 | 213 | 570 |
| iloprost | 11.5 | 1410 | 47 | 277 |
| U46619 | 6750 | 7220 | 16200 | 2330 |
| AH6809 | 1300 | 522 | 40700 | > 100000 |
| SC19220 | 26400 | > 100000 | > 100000 | > 100000 |
| butaprost (free acid) | 38700 | 65 | 11815 | 15700 |
| butaprost (methyl ester) | > 100000 | 2580 | 39650 | 40400 |
| M&B28767 | 508 | 1370 | 0.3 | 24 |
| sulprostone | 94 | > 100000 | 0.7 | 43600 |
| GR63799X | 978 | > 100000 | 73 | 632 |
| enprostil | 192 | > 20000 | 74 | > 20000 |
| misoprostol | 38300 | 15200 | 1090 | 26300 |
| AH23848(racemic) | 33300 | > 100000 | 4080 | 9380 |
| AH23848(−) | 23500 | > 100000 | 12000 | 8010 |
| 17-phenyl-$\omega$-trinor-prostaglandin $E_2$ | 25 | 874 | 4.3 | 54 |
| 11-deoxy- prostaglandin $E_1$ | 2980 | 30 | 3.2 | 1.1 |
| 19($R$)-OH-prostaglandin $E_2$ | > 1350 | 148 | 150 | 30 |

Rat EP receptor equilibrium competition binding assays were performed as described in Section 2. Non-specific binding was determined using 1 $\mu$M prostaglandin $E_2$. Specific binding was calculated by subtracting non-specific binding from total binding. Sigmoidal equilibrium competition curves were constructed by plotting percentage maximum specific binding at each competing ligand concentration and analyzed by a validated custom designed software employing a simplex driven non-linear least-squares curve fitting routine based on a four parameter equation to determine the inflection point (InPt) of the curve. Equilibrium inhibition constants ($K_i$) were derived from the equation $K_i = \mathrm{InPt}/1 + ([\mathrm{radioligand}]/K_D)$ where $K_D$ is the equilibrium dissociation constant. In cases where the InPt could not be determined the concentration of ligand required to inhibit 50% of the maximum specific binding ($IC_{50}$) was substituted.

general, the rank order of affinities for prostanoids (with iloprost and U46619 employed as mimetics of the unstable prostaglandin $I_2$ and $TXA_2$, respectively) was as previously shown (Coleman et al., 1994) for the prostanoid EP receptor subtypes with prostaglandin $E_2$ = prostaglandin $E_1$ > iloprost > prostaglandin $F_{2\alpha}$ > prostaglandin $D_2$ >

Table 3
Activation of rat EP receptor signal transduction pathways by selected prostanoids and related analogs

| Ligand | $EC_{50}$ (nM) | | | |
|---|---|---|---|---|
| | $EP_1$ | $EP_2$ | $EP_{3\alpha}$ | $EP_4$ |
| prostaglandin $E_2$ | 37 | 2.1 | 0.41 | 0.22 |
| prostaglandin $E_1$ | 333 | 3.4 | 0.45 | 0.17 |
| prostaglandin $D_2$ | > 25000 | 30000 | 13 | 2300 |
| prostaglandin $F_{2\alpha}$ | 790 | 7050 | 4.2 | 1110 |
| iloprost | 1.6 (72%)[a] | 1130 | 0.63 | 1200 |
| U46619 | > 25000 | 21400 | 149 | 8120 |
| butaprost (free acid) | | 68 | | > 100000 |
| M&B28767 | | 3680 | 0.55 | |
| sulprostone | 32 (72%)[a] | | 0.42 | |
| enprostil | | | | |
| 11-deoxy- prostaglandin $E_1$ | | 25 | | 0.32 |
| 19($R$)-OH-prostaglandin $E_2$ | | 51 | | 30 |

cAMP generation assays were performed using HEK 293(EBNA) cells in the absence and presence of 12.5 $\mu$M forskolin to measure both stimulation ($EP_2$ and $EP_4$) and inhibition ($EP_{3\alpha}$) of cAMP production, respectively, at each ligand concentration. Aequorin luminescence assays ($EP_1$) were performed using HEK 293 cells which stably express apo-aequorin. In this case, fractional luminescence was determined at each ligand concentration by dividing the area under Peak 1 by the sum of the area under Peak 1 plus Peak 2. Sigmoidal concentration–response curves were analyzed as described for the radioligand binding assay in Table 2. The concentration of agonist required to produce a half-maximal response for that agonist has been defined as $EC_{50}$.
[a]The maximal response for a given agonist has been calculated as a percentage of the maximal response elicited by 1 $\mu$M prostaglandin $E_2$ except as indicated by where agonists produced 72% of the maximal response obtained with 3 $\mu$M prostaglandin $E_2$.

U46619. The main exception to this rank order, was the affinity of iloprost for the prostanoid $EP_1$ receptor which was similar to prostaglandin $E_2$. This has also been shown in other prostanoid $EP_1$ receptor tissues and cell lines (Funk et al., 1993; Coleman et al., 1994). Several more selective ligands were also evaluated and their affinities are shown in Table 2. In summary, butaprost is selective for the prostanoid $EP_2$ receptor, M&B28767 and sulprostone are selective for the prostanoid $EP_{3\alpha}$ receptor, being equipotent with prostaglandin $E_2$, but retain reasonable affinity for the prostanoid $EP_4$ receptor and the prostanoid $EP_1$ receptor, respectively, and, finally, enprostil has dual selectively interacting with both the prostanoid $EP_1$ receptor and protanoid $EP_{3\alpha}$ receptor. In the case of butaprost, although it is routinely synthesized as the methyl ester this ligand is 40-fold more potent at prostanoid $EP_2$ receptor in the free acid form. The additional ligands tested displayed differential affinities at the four EP receptors without being markedly selective for a particular subtype.

### 3.2.2. Functional studies

Second messenger assays monitoring the predominant signal transduction pathways (calcium mobilization ($EP_1$) and stimulation ($EP_2$ and $EP_4$) or inhibition ($EP_{3\alpha}$) of cAMP production) demonstrated that the rat EP receptors were functional and efficiently coupled when expressed in the HEK 293(EBNA) cell line. The $EC_{50}$ values obtained for selected ligands in these assays are given in Table 3 for all four EP receptors and follow the profile observed in competition binding assays. Of note is the activity of butaprost which has an $EC_{50}$ of 68 nM in cAMP stimulation assays using the prostanoid $EP_2$ receptor, but is inactive at the prostanoid $EP_4$ subtype at a concentration up to 100 $\mu$M.

Using an aequorin luminescence assay, in which the rat prostanoid $EP_1$ receptor was transiently transfected into AEQ17-HEK 293 cells stably expressing apo-aequorin (Button and Brownstein, 1993), the $EC_{50}$ for prostaglandin $E_2$ was 37 nM, prostaglandin $F_{2\alpha}$ was 25-fold less potent and prostaglandin $D_2$ and U46619 were weak agonists at 30 $\mu$M (Fig. 2, Table 3). Iloprost and sulprostone behaved like potent partial agonists with $EC_{50}$ values of 1.6 and 32 nM, respectively, and percent maximal stimulation of 72% for both compounds relative to prostaglandin $E_2$ (Fig. 2).

Second messenger responses were also measured in non-transfected and mock-transfected wild type HEK 293(EBNA) cells challenged with prostaglandin $E_2$, prostaglandin $F_{2\alpha}$, prostaglandin $D_2$, U46619 and iloprost. In the aequorin assay prostaglandin $E_2$ (1 $\mu$M) alone gave rise to a small increase in luminescence. This peak of luminescence was delayed compared with receptor-mediated responses. Similarly, only challenge with prostaglandin $E_2$ resulted in stimulation of cAMP production in these cells. The magnitude of the response was very low ($< 5\%$) compared with that obtained for prostanoid $EP_2$ receptor or prostanoid $EP_4$ receptor expressing cells and the $EC_{50}$ was considerably higher at 300 nM. This endogenous response, therefore, did not compromise the results obtained with receptor expressing cells. These prostanoids were negative under the conditions used to measure inhibition of cAMP production (data not shown).

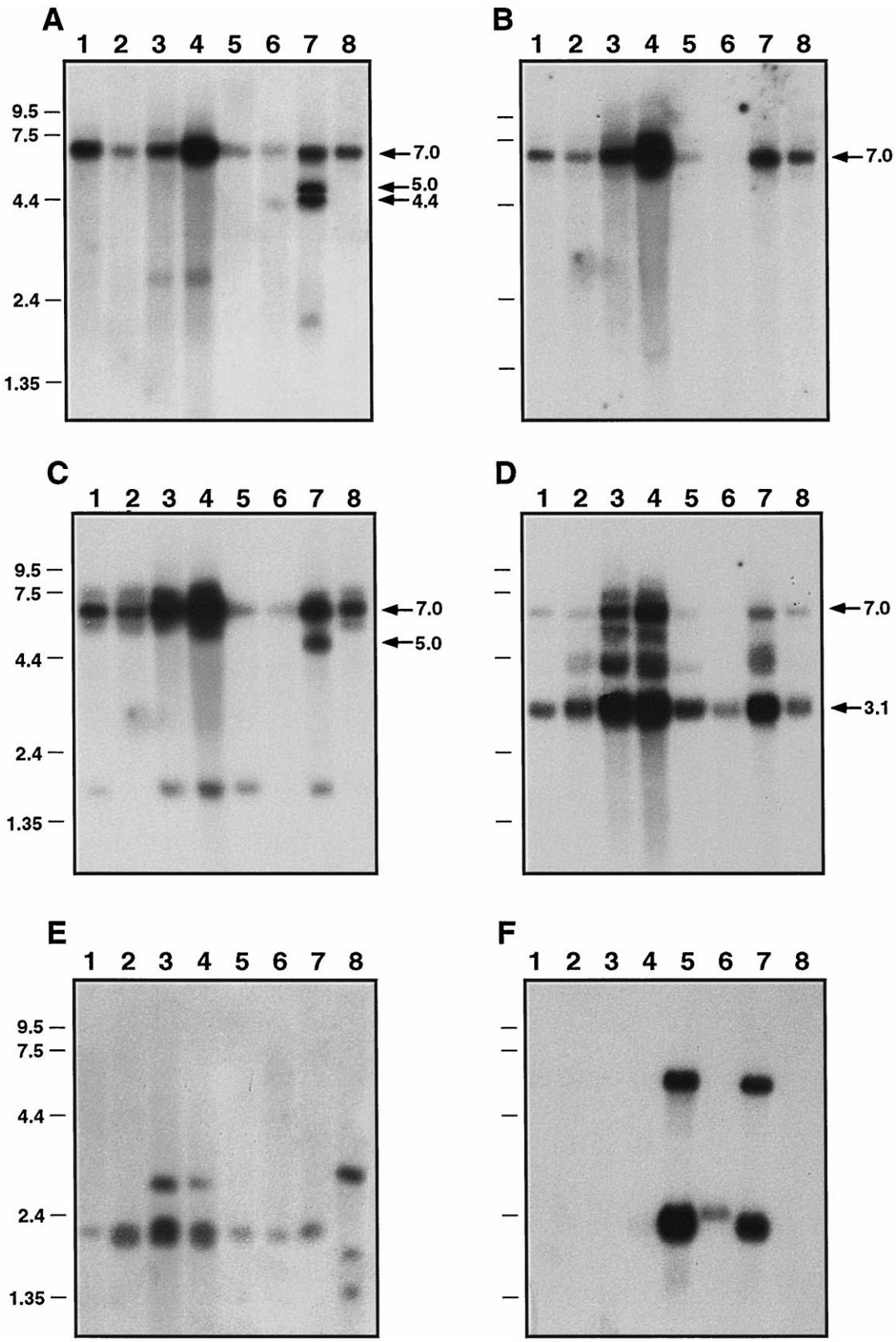
## 3.3. Northern blot analysis

### 3.3.1. Rat prostanoid $EP_1$ receptor

Poly A+ RNA from eight different rat tissues was probed with a rat prostanoid $EP_1$ receptor cDNA fragment (ORF probe) (Fig. 3A). Five transcripts of different sizes were detected: three high molecular weight bands, including one prominent transcript of approximately 7.0 kb which was expressed in all eight tissues and two additional transcripts, approximately 5.0 and 4.4 kb which were seen only in the kidney. In order to account for these high molecular weight bands and in light of the fact that all of the cDNA clones isolated contained unspliced introns we wanted to determine which of the bands detected represented putative processed transcripts. The blot was, therefore, reprobed alternatively with the 5′ intron, located just upstream of the start codon (intron 1 probe) and the 3′ intron, located near the C-terminal end of transmembrane domain VI (intron 2 probe) of rat prostanoid $EP_1$ receptor. The hybridization patterns obtained with the intron 1 and intron 2 probes was compared with that using the ORF probe. The intron 1 probe (Fig. 3B.) did not detect the two smaller molecular weight species (5.0 and 4.4 kb) seen in the kidney probed with the ORF probe (Fig. 3A). The intron 2 probe, however, did detect the 5.0 kb but not the 4.4 kb kidney transcript (Fig. 3C). In addition, the intron 2 probe hybridized to another band of approximately 1.8 kb, which was detected in most tissues, but did not cross react with either the ORF probe or the intron 1 probe.

During these experiments, Batshake and Sundelin (1996) reported the tail to tail arrangement of the mouse prostanoid $EP_1$ receptor gene and the novel protein kinase gene (Mukai and Ono, 1996) showing that the long 3′ UTR of the novel protein kinase gene completely overlapped the prostanoid $EP_1$ receptor gene. We, therefore, probed the same Northern blot with a coding region fragment (amino

Fig. 3. Northern blot analysis of eight different rat tissues probed with $EP_1$, novel protein kinase, $EP_2$ and $EP_{3\alpha}$. The blot containing poly(A)$^+$ RNA (2 $\mu$g) from eight different tissues: (1) heart; (2) brain; (3) spleen; (4) lung; (5) liver; (6) skeletal muscle; (7) kidney; (8) testis; was hybridized successively using $^{32}$P-labeled cDNA fragments (as described in Section 2 as follows: (A) rat $EP_1$ ORF probe; (B) rat $EP_1$ intron 1 probe; (C) rat $EP_1$ intron 2 probe; (D) rat novel protein kinase probe; (E) rat $EP_2$ cDNA probe; (F) rat $EP_3$ cDNA probe. The positions of the RNA markers (kb) on the gel are indicated on the right side and other specific bands (kb) (see text for details) on the left side.


A
1 2 3 4 5 6 7 8
9.5
7.5
4.4
2.4
1.35
7.0
5.0
4.4
B
1 2 3 4 5 6 7 8
7.0
C
1 2 3 4 5 6 7 8
9.5
7.5
4.4
2.4
1.35
7.0
5.0
D
1 2 3 4 5 6 7 8
7.0
3.1
E
1 2 3 4 5 6 7 8
9.5
7.5
4.4
2.4
1.35
F
1 2 3 4 5 6 7 8

acid 660 to 908) of the novel protein kinase gene (see Section 2). As shown in Fig. 3D the pattern observed was similar to that seen with prostanoid $EP_1$ receptor based probes, with two additional bands, one approximately 4.0 kb and another prominent band of approximately 3.1 kb, detected in all tissues.

*3.3.2. Rat prostanoid $EP_2$ receptor*

Expression of the rat prostanoid $EP_2$ receptor was detected in spleen, lung and testis (Fig. 3E). Multiple transcripts can be seen in each lane ranging in size from approximately 3.0 kb down to approximately 1.6 kb. The smallest band was apparent only in the testis.

*3.3.3. Rat prostanoid $EP_3$ receptor*

Northern blot analysis using the entire rat prostanoid $EP_{3\alpha}$ receptor cDNA as a probe identified abundant expression in kidney and in liver, with very weak expression in lung and skeletal muscle and barely detectable expression in heart and brain (Fig. 3F). Two major transcripts were apparent in kidney and liver (7.0 and 2.2 kb), with an additional minor band of size 1.6 kb seen only in liver. The 2.4 kb band detected in skeletal muscle was unique to this tissue. Of note, this probe would not discriminate between the three alternatively spliced forms of the rat prostanoid $EP_3$ receptor, $EP_{3\alpha}$, $EP_{3\beta}$ or $EP_{3\gamma}$.

## 4. Discussion

In the present study all four rat prostanoid EP receptor subtypes have been cloned and characterized with respect to both radioligand binding and functional properties using the same heterologous expression system. Northern blot analysis has been used to examine tissue distribution of prostanoid receptor subtypes $EP_1$, $EP_2$ and $EP_{3\alpha}$.

Comparison of the deduced amino acid sequences of EP receptor homologues from rat, human and mouse (see Fig. 1) revealed that, in general, rat receptors are more related to mouse receptors ($>$ 90% amino acid identity) than they are to human receptors (82%–89% amino acid identity). It is of note, however, that the rat and human prostanoid $EP_4$ receptors are similar with respect to the initiating methionine which differs in the mouse being located 30 amino acids further upstream. Additional differences between the sequences occur mainly outside of the seven transmembrane domains with transmembrane domain VII being the most highly conserved, not only between species homologues but also between all eight prostanoid receptors (Boie et al., 1995). Most of the putative post-translational modification sites (N-glycosylation, protein kinase C and protein kinase A phosphorylation) have been conserved across species.

Transient expression of the rat EP receptors in HEK 293(EBNA) cells was very efficient, with $B_{max}$ values all in the pmol/mg protein range. The rank order of potencies of prostanoids, at the rat EP receptors, as assessed in radiobinding assays was similar to that obtained with the mouse and human EP homologues. As observed for the human receptors, prostanoid $EP_3$ and $EP_4$ receptors consistently displayed higher affinities for prostaglandin $E_2$ as compared to prostanoid $EP_1$ receptor and prostanoid $EP_2$ receptor (Abramovitz et al., submitted).

The majority of compounds tested behaved in a similar manner across species. AH6809 was the only example found of a compound which might display species differences with respect to inhibition of receptor binding, even though the rat prostanoid $EP_1$ receptor is 96% and 84% identical to the mouse and human prostanoid $EP_1$ receptor, respectively, at the amino acid level. This putative prostanoid $EP_1$ receptor antagonist has $IC_{50}$ values against the rat, human (Funk et al., 1993), and mouse (Watabe et al., 1993) prostanoid $EP_1$ receptors of 1300 nM, 500 nM and $> 10\ \mu M$, respectively. However, the $IC_{50}$ value for prostaglandin $E_2$ at the mouse prostanoid $EP_1$ receptor (Watabe et al., 1993) was also higher than determined for the rat and human.

The free acid form butaprost was found to be 40-fold more active than the methyl ester form in the equilibrium binding assays. The free acid form is probably the hydrolysis product that is active in in vivo and in in vitro whole cell/tissue assay systems. This has also been found to be the case for an IP receptor agonist, SM-10906, which was found to be much more potent than the methyl ester form SM-10902 (Oka et al., 1994). It has previously been suggested that other methyl ester compounds such as misoprostol act as pro-drugs which are converted to their active forms by esterases (Tsai et al., 1991). These results suggest that the $\alpha$-carboxyl group on these compounds is an important determinant for binding. The conserved arginine residue in transmembrane domain VII, which occurs in all prostanoid receptors, has been implicated in the binding of prostanoid receptor ligands by hydrogen bonding with the $\alpha$-carboxyl group (Sugimoto et al., 1993).

All four EP receptors were shown to be functional when expressed in HEK (EBNA) 293 cells. The effectiveness of the ligands tested in stimulating ($EP_2$ and $EP_4$) or inhibiting ($EP_{3\alpha}$) intracellular cAMP production was as predicted from previous reports employing recombinant receptors and from pharmacological studies (Coleman et al., 1994). In general, the potencies in functional assays paralleled the affinities determined in the receptor binding assays. All the ligands tested were full agonists in these systems of high receptor reserve. These data also confirmed that butaprost is highly selective for the prostanoid $EP_2$ receptor versus the prostanoid $EP_4$ receptor subtype and that sulprostone is a potent full agonist at the prostanoid $EP_{3\alpha}$ receptor.

Previously, we have used the photoprotein aequorin in *Xenopus* oocytes to identify the human prostanoid $EP_1$ (Funk et al., 1993) and FP (Abramovitz et al., 1994) receptors. Recently, an aequorin luminescence assay for G-protein coupled receptors using mammalian cells has

been described by Button and Brownstein (1993). This assay was used to assess the functional coupling of the rat prostanoid $EP_1$ receptor to the mobilization of intracellular $Ca^{2+}$. AEQ17-HEK 293 cells which constitutively express apo-aequorin in the cytoplasm of the cell were used as a very sensitive indicator of intracellular $Ca^{2+}$ fluctuations (Button and Brownstein, 1993). The rank order of potencies of prostanoids in this assay was as predicted (Coleman et al., 1989, 1994). Iloprost, however, behaved like a potent partial agonist. This result is in agreement with those of Dong et al. (1986) who showed in three different contractile models, bullock iris sphincter, rat stomach fundus strip and guinea-pig tracheal, that iloprost behaved as a partial agonist. They also found in the same study that prostaglandin $I_2$ was much less potent than iloprost. Although iloprost is used extensively as a prostaglandin $I_2$ analogue at the $EP_1$ receptor these data should be interpreted with caution since its agonist properties are probably modulated by the receptor reserve present in individual tissues/cells. Interestingly, sulprostone which is a selective prostanoid $EP_1/EP_3$ agonist, and a full agonist in the prostanoid $EP_3$ receptor cAMP inhibition assay used here, was also a partial agonist in the aequorin assay. Sulprostone had an $EC_{50}$ of 32 nM which is comparable to that for prostaglandin $E_2$, but the maximal stimulation was the same as that achieved with iloprost. Sulprostone is not reported to act as a partial agonist. This compound, however, has never been previously tested at the prostanoid $EP_1$ receptor in a functional assay containing exclusively the prostanoid $EP_1$ receptor. This is important, since other compounds, heretofore assumed to be full agonists at the individual prostanoid receptors, may in fact be partial agonists.

When a Northern blot of eight different rat tissues was probed with the rat prostanoid $EP_1$ receptor coding region, a major transcript of 7.0 kb was detected in most of the tissues in addition to two minor species, one just above and one just below the 7.0 kb band. The kidney was the only tissue for which unique bands were seen. Interestingly, Watabe et al. (1993) only detected transcripts in the mouse kidney by Northern blot when they used an antisense riboprobe. Since the prostanoid $EP_1$ receptor is known to be expressed in the mouse (Watabe et al., 1993) and human (Abramovitz et al., 1995) kidney we wanted an explanation for these ubiquitous higher molecular weight bands. The same higher molecular weight bands were also visualized upon reprobing the blot successively with intron 1 and intron 2 of the rat prostanoid $EP_1$ receptor gene. The only band which clearly did not hybridize with either of these probes was a 4.4 kb band unique to the kidney and representing the processed transcript. The most probable explanation for the occurrence of these higher molecular weight bands comes from a recent study by Batshake and Sundelin (1996). They reported that the mouse prostanoid $EP_1$ receptor gene (Batshake et al., 1995) is completely overlapped by a novel protein kinase gene in a tail-to-tail manner. Two novel protein kinase transcripts have been detected as widely expressed in rat tissue, a 3.1 kb or short form and a 6.8 kb or long form (Mukai and Ono, 1996). However, only the long form of novel protein kinase would be detected using any one of the three rat prostanoid $EP_1$ receptor probes described above. The long form would, therefore, encompass both rat prostanoid $EP_1$ receptor intron 1 and 2 as well as the coding region. The high molecular weight bands were indeed confirmed as novel protein kinase transcripts by reprobing the same Northern blot with a coding region fragment of the novel protein kinase gene. Under these conditions the long and the short forms of novel protein kinase can be readily detected in all tissues (Fig. 3D). This would also help to explain why the potential prostanoid $EP_1$ receptor clones we have isolated from various mouse and rat cDNA libraries almost always contained putative protanoid $EP_1$ receptor introns since they were most likely novel protein kinase related clones.

The only transcript which cannot be rationalized by the presence of novel protein kinase mRNA is the 5 kb band detected in the kidney with both the ORF and intron 2 probes but not the intron 1 probe. This transcript legitimately represents a rat prostanoid $EP_1$ receptor mRNA species containing an unspliced intron 2. It has been recently suggested by Okuda-Ashitaka et al. (1996) that this transcript is expressed as a variant form of the prostanoid $EP_1$ receptor which has ligand binding but no functional properties.

This overlap of prostanoid $EP_1$ receptor and novel protein kinase transcripts does not appear to occur in the human as it does in both rat and mouse. Using a human Northern blot, only a 3.1 kb novel protein kinase transcript can be detected (Mukai and Ono, 1996) and for human prostanoid $EP_1$ receptor the major transcript is 1.6 kb (Funk et al., 1993; Boie and Abramovitz, unpublished observations). The gene for human $EP_1$ is on chromosome 19p13.1 (Duncan et al., 1995) and, although plausible, it has yet to be determined if the tail to tail configuration for the prostanoid $EP_1$ receptor and novel protein kinase genes in human is as in mouse and rat. The physiological relevance of this configuration is not known, but certainly it would become important if both genes are transcribed within the same cell where they could play a role in regulation (Batshake and Sundelin, 1996, and references within). This would also have implications in making a prostanoid $EP_1$ receptor knockout mouse since the knockout vectors would have to be constructed to leave the novel protein kinase gene intact.

The prostanoid $EP_2$ receptor was principally detected in spleen, lung and testis. This limited expression is in marked contrast to the distribution of the prostanoid $EP_4$ receptor, which is the most ubiquitously expressed of all the prostanoid receptors (Honda et al., 1993; An et al., 1993; Sando et al., 1994; Boie and Abramovitz, unpublished observations). Although the major signal transduction pathway for both receptors is through elevation of intra-

cellular cAMP, their tissue distribution patterns suggests that the two receptors may play very different roles.

Abundant expression of the rat prostanoid $EP_3$ receptor was limited to liver and kidney, with minor expression in lung and skeletal muscle. The expression in the liver was unexpected since the prostanoid $EP_3$ receptor was barely detectable in mouse (Sugimoto et al., 1992) or human liver (Schmid et al., 1995; Abramovitz et al., 1995) by Northern blot analysis. The prostanoid $EP_3$ receptor seems to be involved in a negative feedback inhibition loop that restrains glucagon-stimulated glycogenolysis in hepatocytes via a glucagon-elicited prostanoid release from non-parenchymal cells (Hespeling et al., 1995). The physiological role of the $EP_3$ receptor on hepatocytes has so far been studied only in rat models.

In conclusion, we have cloned and characterized the four rat prostaglandin $E_2$ prostanoid receptor subtypes, $EP_1$, $EP_2$, $EP_{3\alpha}$ and $EP_4$. Although their sequences are highly conserved with those of mouse and human, the amino acid differences could be important when comparing the potencies of compounds, especially novel antagonists, across species. Having the four rat EP receptors expressed in the same cell line as their human counterparts will allow a direct comparison of both receptor binding and functional properties. This will support the understanding of pharmacological models in the rat and allow the predictions from preclinical models to be extrapolated into the clinical environment.

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