Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 263-270

CLONING AND EXPRESSION OF THE EP2 SUBTYPE OF HUMAN RECEPTORS FOR PROSTAGLANDIN E2

Songzhu An*, Jinhong Yang, Menghang Xia and Edward J. Goetzl

Departments of Medicine and Microbiology-Immunology,
University of California, San Francisco, CA 94143-0724

Received October 14, 1993

SUMMARY: Prostaglandin E2 (PGE2) is a potent mediator in many human tissues, that is recognized by three distinct subtypes of receptors, designated EP1, EP2 and EP3. A cDNA from a human lung library encodes a 53 kDa protein of 88% homology with the mouse EP2 receptor. Human EP2 receptors in COS-7 cell transfectants bound [^{3}H]-PGE2 with a mean Kd of 2.2 nM and native specificity, and transduced increases in the intracellular concentration of cyclic AMP, but not of Ca^{++}. That most EP2 receptor mRNA is in lung, kidney, intestinal, glandular and immune tissues, is consistent with functional responses.

© 1993 Academic Press, Inc.

Prostaglandin E2 (PGE2) is a product of the cyclo-oxygenation of arachidonic acid and a specific isomerase, both of which enzymes are widely distributed in mammalian tissues (1). PGE2 is a potent mediator of diverse cardiovascular, pulmonary, renal, endocrine, gastrointestinal, neural and immune responses. Cellular recognition and effects of PGE2 are mediated by three subtypes of high-affinity receptors (Rs), termed EP1, EP2 and EP3, that differ in structure, binding specificity, pathways of signal transduction and physiological activities (1). The recent cloning of subtypes of mouse PGE2Rs (2, 3) has

* To whom correspondence is to be addressed at U-426, Box 0724.

ABBREVIATIONS: PG-prostaglandin, R-receptor, PCR-polymerase chain reaction, PBS^{--}-calcium and magnesium-free phosphate-buffered saline, [Ca++]i-intracellular concentration of calcium, IC50-concentration of a compound at which there is 50% inhibition of binding of a radioligand, G protein-guanine nucleotide-binding protein, PAF-phospholipid platelet-activating factor, MEM^{-}-phosphate-free MEM and PtdIns-phosphatidylinositol.

0006-291X/93 $4.00
Copyright © 1993 by Academic Press, Inc.
All rights of reproduction in any form reserved.

facilitated cloning of a homologous human EP2R for studies of transductional characteristics and tissue distribution.

MATERIALS AND METHODS

Cloning of a Full-Length cDNA Encoding the Human EP2R -- A 0.5 kb cDNA of high homology to those encoding mouse EP3 (3) and human thromboxane A2 (4) Rs was synthesized by polymerase chain reaction (PCR). The template was first-strand cDNAs prepared by oligo-dT-primed reverse-transcription of total RNA from human blood polymorphonuclear leukocytes and the primers were 5'-CCIGG(GC)(TA)CITGGTGCTTC(AC)T, for sequence between putative fourth and fifth transmembrane domains, and 5'-TAIACC(CT)A(GA)G-G(AG)T(CT)CA(GA)GATI(TG)GGTT, for sequence in the seventh transmembrane domain. The PCR consisted of 36 cycles of 1 min each at 95°C, 55°C and 73°C in a Robocycler 40 (Stratagene). The predominant cDNA was subcloned into pCR II (Invitrogen), shown to have a sequence homologous with that of the cDNAs for the two source Rs, labeled with ^{32}P and used to probe a human lung cDNA library in lambda gt10 (Clontech), by employing standard techniques of high-stringency hybridization (5).

Expression of the Human EP2R-- COS-7 and K293 cells were transfected with the 3.0 kb cDNA, that had been excised with Hind III - Xho I and introduced into the pcDNA I expression vector (Invitrogen). Monolayers of cells at 70-80% confluency in 100 mm culture dishes with 15 ml of Dulbecco's minimal essential medium containing 10% fetal bovine serum (Hyclone), 100 U/ml of penicillin and 100 µg/ml of streptomycin (complete medium) were washed twice with 10 ml of Opti-MEM (Gibco-BRL). The washed cells were incubated according to the manufacturer's protocol with a mixture of 50 µl of Lipofectamine (Gibco-BRL) and 10 µg of Qiagen column-purified plasmid DNA in 5 ml of Opti-MEM for 6 hr at 37°C in 5% CO2:95% air. Five ml of complete medium were added to each monolayer of cells and the incubation continued for 18 hr. Then the transfected monolayers were restored to 10 ml of complete medium and the incubation continued for an additional 48 hr. The transfectants were washed once with 10 ml of calcium-and magnesium-free phosphate-buffered saline (PBS^{--}), harvested by agitation in 10 ml of PBS^{--} with 5 mM EDTA for 10 min at 22°C and washed twice at 4°C with PBS^{--}.

Binding of [^{3}H]-PGE2 by COS-7 Cells Expressing Human EP2Rs-- Replicate 100 ul suspensions of 2 x 10^5 COS-7 cell transfectants in Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 1 mg/ml of recrystallized ovalbumin were incubated for 60 min at 4°C with pM-nM [5, 6, 8, 11, 12, 14, 15- ^{3}H]-PGE2 ([^{3}H]-PGE2, Amersham, 184 Ci/mmol) without and with 1 µM nonradioactive PGE2 for saturation binding analyses or with 30,000 cpm of [^{3}H]-PGE2 and pM-µM nonradioactive PGE2, PGE1, PGF2alpha and PGD2 (The Upjohn Co.) for studies of relative affinity. Bound and unbound [^{3}H]-PGE2 were resolved by filtration of each COS-7 suspension through a GF/C glass fiber filter (Whatman), that was washed twice with 3 ml of ice-cold PBS^{--} and added to scintillation fluid for quantification of bound [^{3}H]-PGE2. Saturation and competitive binding data were analyzed as described (6).

Biochemical Responses to PGs of Transfectants Expressing Human EP2Rs-- To assess EP2R-mediated increases in intracellular concentration of cyclic AMP, duplicate 100 µl suspensions of 2 x

10^5 COS-7 cell transfectants in HBSS with 1 mg/ml of recrystallized ovalbumin, 10 mM HEPES (pH=7.3), and 1 mM isobutyl methylxanthine (IBMX, Calbiochem) were preincubated for 10 min at 37°C, received 10^{-12} M to 10^{-6} M PGE2 or buffer alone and were incubated for 10 min at 37°C. The responses were stopped by addition of cold ethanol to 70% and cyclic AMP in the 5,000 xg supernatants was quantified by radioimmunoassay, according to the manufacturer's protocol (Dupont-New England Nuclear). To measure EP2R-mediated increases in intracellular concentration of calcium ([Ca^{++}]i), replicate 2 ml suspensions of 2 x 10^6 COS-7 cell transfectants were incubated in the dark for 30 min at 37°C with 2.5 µM Fura 2-AM (Calbiochem) in HBSS containing 1 mg/ml of ovalbumin and 25 mM HEPES (pH=7.3). For a positive control, COS-7 cells were transfected identically with a human cDNA in pcDNA I, that encodes the human R for phospholipid platelet-activating factor (PAF) (7) and stimulated with 0.5 µM PAF (Sigma). The Fura 2-loaded transfectants were washed, resuspended at 2 x 10^6/2 ml in PBS and warmed to 37°C. Fluorescence was quantified from 0 to 4 min after the addition of 1 µM PGE2 or 1 µM ionomycin in a stirred and 37°C-controlled cell of a Perkin Elmer model LS 50B fluorimeter, as described (6). After recording the response, maximum and minimum fluorescence were determined by the respective additions of 20 µl of 1g/100ml of Triton X-100 in distilled water and then 80 µl of 0.25 M EGTA-0.25 M Tris-HCl (pH=7.5). The values of [Ca^{++}]i were calculated as described (6). EP2R-mediated increases in turnover of phosphatidylinositol (PtdIns) were determined by non-equilibrium labeling, as described (8), using transfected K293 cell monolayers in six-well plates. The transfectants in each well were washed twice with 5 ml of phosphate-free-MEM (MEM$^-$), layered with 2 ml of MEM$^-$ containing 200 uCi of [^{32}P]-orthophosphate, preincubated 30 min at 37°C and incubated for 10 min at 37°C with 0.1 uM eicosanoid. Phospholipids then were extracted from the cells and resolved by chromatography on oxalate-impregnated silica plates (8), in parallel with standard synthetic PtdIns-2-[^{3}H] (Amersham), PtdIns(4)P-[^{3}H] and PtdIns(4,5)P2-[^{3}H] (New England Nuclear-Dupont) for recovery and quantification of the [^{32}P]-PtdIns species by beta-scintillation counting. Fifty-percent inhibitory (IC50) concentrations were calculated by a standard computer program (Cricket Graph,Apple).

Northern Blot Analysis of EP2R mRNA-- Nylon membranes, on which had been blotted the electrophoretic patterns of 2 µg of poly(A+) RNA from each of multiple human tissues (Clontech), were probed with the [^{32}P]-labeled 1.4 kb EP2 cDNA under high-stringency conditions.

RESULTS AND DISCUSSION

The screening of 10^6 plaques of a human lung cDNA library, using a PCR-derived 500 bp cDNA probe homologous with the fourth to seventh transmembrane domains of both the mouse EP3R and human thromboxane A2 R, permitted isolation of 10 clones with overlapping sequences. One of these clones had a 3.0 kb cDNA insert with a 1464 bp open-reading frame, that encodes a protein

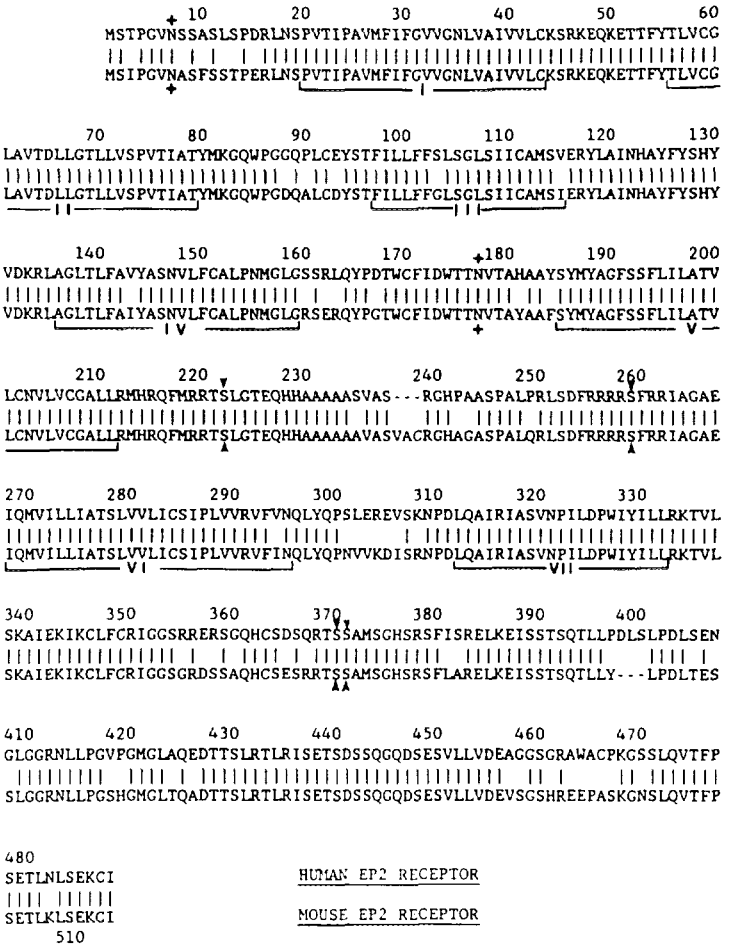

Figure 1. **Amino acid sequences deduced from the base sequences of the human (top) and mouse (bottom) EP2-subtype of PGE2R.** The putative transmembrane segments are shown by I-VII, + = potential sites of N-linked glycosylation and ▲ = potential sites of phosphorylation by cyclic AMP-dependent protein kinase. The base sequence of the human EP2R has been deposited in the GenBank database (accession no. L25124).

of 488 amino acids, seven putative transmembrane domains typical of guanine nucleotide-binding (G) protein-associated Rs and 88% homology with the mouse EP2-subtype of PGE2R (Fig. 1). The human EP2R also is nearly equally homologous with the mouse EP3 and human thromboxane A2 Rs at 30% and 27%, respectively. Northern blot analysis of RNA from many different human tissues, developed with a [^{32}P]-labeled cDNA encoding the human EP2R, revealed a single 3.8 kb transcript that was most strongly

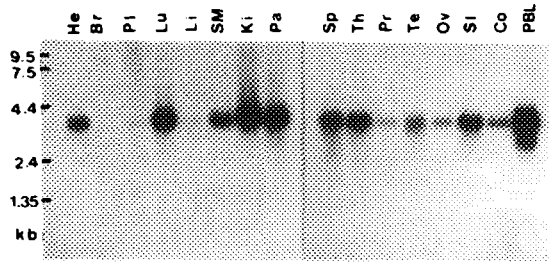

Figure 2. **Northern blot analysis of the expression of mRNA for the EP2-subtype of PGE2R in human tissues.** The principal transcript detected is 3.8 kb. Tissue sources of RNA, from left to right, are: He=heart, Br=brain, Pl=placenta, Lu=lung, Li=liver, SM=skeletal muscle, Ki=kidney, Pa=pancreas, Sp=spleen, Th=thymus, Pr=prostate, Te=testis, Ov=ovary, SI=small intestine, Co=colon, and PBL=peripheral blood leukocytes.

expressed in lungs, kidneys, pancreas, small intestine, immune organs and blood leukocytes (Fig. 2).

COS-7 cells, that had been transfected with EP2R cDNA in pcDNA I, bound [^{3}H]-PGE2 saturably and specifically to one apparent class of receptors with a mean Kd of 2.2 nM (Fig. 3). Untransfected COS-7 cells had less than 5% of the binding activity of the transfectants. Competitive displacement of the

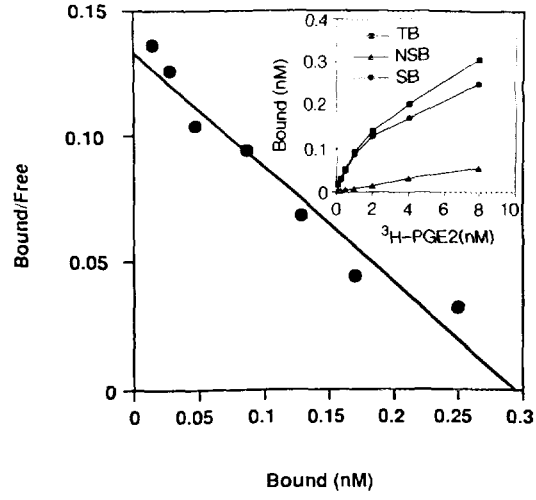

Figure 3. **Saturation binding and Scatchard analysis of binding of [^{3}H]-PGE2 by COS cell transfectants expressing human EP2Rs.** The principal figure depicts the Scatchard analysis and the inset presents the saturation binding data. Specific binding (SB, ●) is the difference between total binding (TB, ■) and non-specific binding (NSB, ▲) with 1 μM nonradioactive PGE2.

Table 1. Specificity of Recombinant Human EP2 Receptors

a. [^{3}H]-PGE2 Binding (COS-7 Cells) **IC50** (nM, Mean±S.D., n=3)

PGE2	PGE1	PGF2alpha	PGD2
0.7±0.1	0.8±0.1	110±11	976±117

b. Increase in [Ca^{++}]i (COS-7 Cells) **nM**, Mean±S.D., n=3

		Ionomycin (1 μM)	PAF (0.5 μM)	PGE2 (1 μM)
Control Cells		626±318*	ND	24±4
Transfectants	EP2	591±69*	ND	27±6
	PAFR		125+	ND

The mean±S.D.(n=3) [Ca^{++}]i of COS-7 control cells prior to stimulation with ionomycin and PGE2, respectively, were 138±24 and 130±2 nM and of COS-7 cell transfectants before ionomycin, PAF and PGE2 were 152±8, 180 and 143±4. For the studies of Ca^{++}, COS-7 cells were harvested in 250 mg of trypsin and 20 mg of EDTA per 100 ml. *= $p<0.05$ by a t test, pairing to unstimulated values. += mean of 2. ND= not done.

c. PtdIns Turnover(K293 Cells) **Fold-Increase** (Mean[Range], n=2)

	PGE2	PGE1	PGF2alpha
PtdIns	3.2[1]*	2.7[0.7]*	1.4[0.3]
PtdIns(4)P1	1.2[0.5]	1.1[0.3]	ND
PtdIns(4,5)P2	1.4[0.3]	1.2[0.4]	ND

A mean of 118, 239 and 60 x 10^3 cpm, respectively, were recovered in [^{32}P]-PtdIns, [^{32}P]-PtdIns(4)P1 and [^{32}P]-PtdIns(4,5)P2 from COS-7 cell transfectants incubated in MEM without a stimulus (n=4). *= $p<0.05$ compared to unstimulated cells by a paired t test. ND= not done.

binding of [^{3}H]-PGE2 to EP2Rs on COS cell transfectants by non-radioactive related eicosanoids revealed a mean IC50 of 0.7 nM for PGE2 and a rank-order of relative affinities similar to that observed with native receptors (Table 1a).

Signal transduction by EP2Rs was studied first in COS-7 cell transfectants. PGE2 stimulated significant increases in the intracellular concentration of cyclic AMP in the COS-7 cell transfectants, in a PGE2 concentration-dependent relationship consistent with the binding affinity of the recombinant PGE2R (Fig.4). PGE2 and PGE1, but not PGF2alpha, elicited increases in the non-equilibrium rate of turnover of PtdIns in K293 cell transfectants, without altering significantly the turnover of the 4- and 4,5- phosphate derivatives of PtdIns (Table 1c). In

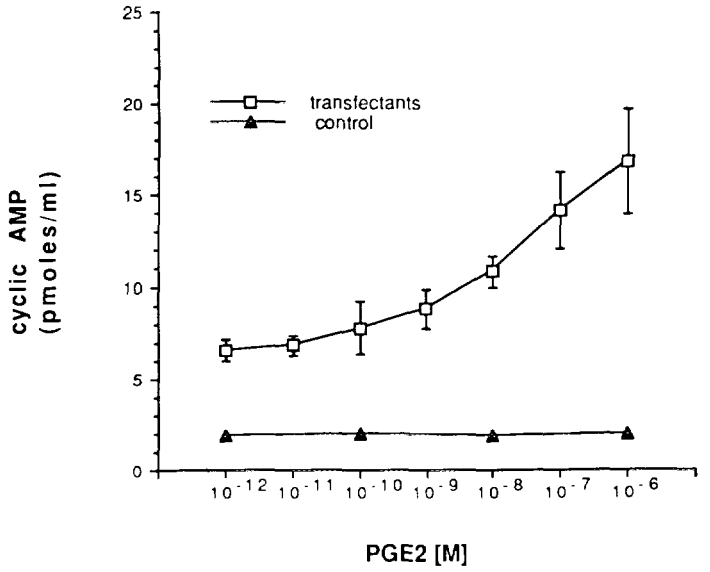

Figure 4. Stimulation by PGE2 of increases in the intracellular concentration of cyclic AMP in COS cells expressing EP2R. The points and brackets depict the mean ± S.D. of the results of three different studies of the effects of PGE2 on transfectants (□) and the mean of two studies of untransfected COS-7 cells(▲).

one experiment, 0.1 uM PGE2 raised the rate of PtdIns turnover only a mean of 1.3-fold in untransfected K293 cells, whereas a mean increase of 3.6-fold was observed in transfectants. In contrast, PGE2 had no significant effect on the [Ca^{++}]i of COS-7 cell transfectants bearing EP2Rs, in studies where PAF increased the [Ca^{++}]i of COS-7 cells transfected with PAF receptors and the response to ionomycin was the same in control and transfected COS-7 cells (Table 1b). EP2Rs thus transduce cellular signals preferentially by cyclic AMP-dependent pathways.

ACKNOWLEDGMENTS-- We thank Dr. Craig Gerard of Harvard Medical School for providing the human PAF receptor cDNA. The studies were supported by NIH grant HL-31809.

REFERENCES

1. Coleman, R.A., Kennedy, I., Humphrey, P.P.A., Bunce, K., and Lumley, P. (1990) In Comprehensive Medicinal Chemistry: Membranes and Receptors(J.C. Emmett, Ed.), vol.3, pp.643-714. Pergamon Press, NY

2. Honda, A., et al. (1993) J. Biol. Chem. 268, 7759-7762
3. Sugimoto, Y., et al. (1992) J. Biol. Chem. 267, 6463-6466
4. Hirata, M., et al. (1991) Nature 349, 617-620
5. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, NY
6. Goldman, D.W., Chang, F.-H., Gifford, L.A., Goetzl, E.J., and Bourne, H.R. (1985) J. Exp. Med. 162, 145-156
7. Nakamura, M., et al. (1991) J. Biol. Chem. 266, 20400-20405
8. Ross, T.S., Wang, F.-P., and Majerus, P.W. (1992) J. Biol. Chem. 267, 19919-19923