RESEARCH COMMUNICATION

Cloning and expression of a prostaglandin E receptor EP_3 subtype from human erythroleukaemia cells

Satya P. KUNAPULI,*† Guang FEN MAO,‡ Murat BASTEPE,‡ Lee-Yuan LIU-CHEN,‡ Shuixing LI,‡ Peter P. CHEUNG,* Jon K. DERIEL§ and Barrie ASHBY*‡||

*Sol Sherry Thrombosis Research Center, †Department of Physiology, ‡Department of Pharmacology, and §Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.

Prostaglandins inhibit platelet activation by stimulating intracellular cyclic AMP formation. We have postulated that intracellular cyclic AMP levels in platelets are buffered by a distinct prostaglandin receptor that mediates inhibition of cyclic AMP formation. In order to provide evidence for the model, we have cloned the cDNA coding for a prostaglandin receptor EP_3 subtype, which is coupled to inhibition of adenylate cyclase, from the megakaryocytic cell line human erythroleukaemia (HEL) cells. A PCR-generated hybridization probe, produced using primers based on the sequence of the mouse prostaglandin EP_3 receptor published by Sugimoto, Namba, Honda, Hayashi, Negishi, Ichikawa and Narumiya [(1992) J. Biol. Chem. **267**, 6463–6466], was used to screen a λgt11 HEL cell cDNA library. The composite full-length cDNA clone HEP3, generated from the two partial clones pHEP3–7 and pHEP3–5, is 1.6 kb long with an open reading frame coding for 390 amino acids. This clone is 83% identical to the α subtype of the mouse EP_3 receptor. The full-length construct was transfected into COS-1 cells. The cloned receptor exhibited the properties of a prostaglandin EP_3 subtype, inhibiting forskolin-stimulated cyclic AMP formation in response to prostaglandin E_2 (PGE_2) and binding PGE_2 with high specificity and a K_d of 3.2 nM. Radiolabelled PGE_2 could be displaced by prostaglandins in the order PGE_2 = PGE_1 > iloprost = PGD_2. Northern blot analysis revealed that the receptor is also present in human kidney.

INTRODUCTION

Prostaglandins are important modulators of platelet function, since they increase the intracellular level of cyclic AMP and inhibit platelet aggregation and secretion. We have suggested that prostaglandin regulation of cyclic AMP metabolism in platelets involves separate stimulatory and inhibitory prostaglandin receptors linked to adenylate cyclase, giving rise to characteristic patterns of cyclic AMP formation that may buffer the cellular response to localized changes in prostaglandin concentration [1,2]. We have shown similar regulation of cyclic AMP metabolism in human erythroleukaemia (HEL) cells [3], a megakaryocytic cell line possessing several platelet-like features [4] that has been used for cloning of platelet-specific receptors such as the thrombin receptor [5].

In this paper we report the cloning and expression of a prostaglandin receptor from HEL cells that is coupled to inhibition of adenylate cyclase. The cloned receptor exhibited the properties of a prostaglandin EP_3 subtype, inhibiting forskolin-stimulated cyclic AMP formation in response to prostaglandin E_2 (PGE_2) and binding PGE_2 with high specificity. Radiolabelled PGE_2 could be displaced by prostaglandins in the order PGE_2 = PGE_1 > iloprost = PGD_2. The data presented confirm the hypothesis of a two-receptor model of prostaglandin control of cyclic AMP metabolism in platelets.

EXPERIMENTAL

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). Kits for RNA extraction, first-strand synthesis and *pfu* polymerase were from Stratagene (La Jolla, CA, U.S.A.). Sequencing reagents were from United States Biochemical Corp. (Cleveland, OH, U.S.A.). The stable PGI_2 analogue iloprost was a gift from Berlex Laboratories, Inc. (Cedar Knolls, NJ, U.S.A.). [2,8-^{3}H]Adenine, [^{14}C]cyclic AMP, [^{3}H]PGE_2 (154 Ci/mmol) and [^{35}S]dATP were obtained from Du Pont NEN (Boston, MA, U.S.A.). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HEL cells were obtained from the Cell Culture Facility of the University of California, San Francisco. HEL cells were grown in RPMI 1640 medium (Hyclone Laboratories, Logan, UT, U.S.A.) with 10% fetal calf serum and antibiotics in 75 cm^2 culture flasks in an atmosphere of 95% air/5% CO_2 at 37 °C. Cells were grown for 4 days in the presence of 1.25% dimethyl sulphoxide (DMSO) to induce prostaglandin receptors [3]. Sequence comparisons and translations were carried out using the GCG Sequence Analysis Package (University of Wisconsin).

PCR strategy

Sugimoto et al. [6] have cloned a cDNA for a mouse prostaglandin EP_3 receptor coupled to inhibition of adenylate cyclase. The mouse EP_3 receptor cDNA was obtained using PCR primers based on the putative third and sixth transmembrane domains of the human thromboxane A_2 receptor [7] to obtain a PCR product that was used to screen a mouse lung cDNA library. We used a similar strategy to clone the human inhibitory prostaglandin receptor by preparing a PCR product from HEL cell total RNA using primers based on the sequence of the mouse prostaglandin EP_3 receptor.

Total RNA was isolated from 10^8 HEL cells. The first-strand

Abbreviations used: HEL, human erythroleukaemia; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; DMEM, Dulbecco's modified Eagle's medium, IBMX, 3-isobutyl-1-methylxanthine; DMSO, dimethyl sulphoxide.

|| To whom correspondence should be addressed.

cDNA was synthesized from RNA using a first-strand synthesis kit from Stratagene. PCR was performed with *pfu* polymerase in a thermal cycler (Belco Glass, Vineland, NJ, U.S.A.) using primers based on the sequence of the mouse EP_3 prostaglandin receptor containing *Bam*HI and *Hin*dIII sites at their 5′-end, to yield a 644 bp PCR product. Sequences of the PCR primers (with restriction sites underlined were as follows: sense primer, 5′-AGTATGGATCCCGAGAGCAAGCGCAAGAAG-3′; antisense primer, 5′-GACGTAAGCTTACCAACAGACGGACAGCAC-3′.

Isolation of cDNA clones

The PCR product was digested with *Bam*HI and *Hin*dIII, ligated into PUC18 and transfected into DH5α competent cells (Gibco BRL). The PCR product was sequenced using M13 forward and reverse primers using the method of Sanger et al. [8]. The sequence showed 85% identity to that of the mouse EP_3 receptor. Plasmid DNA was purified and the amplified PCR product was released by restriction enzyme digestion, and purified and labelled with [α-^{32}P]dCTP (3000 Ci/mmol) by use of random oligonucleotides as primers and the Klenow fragment of DNA polymerase I. Screening of a λgt11 HEL cell cDNA library was performed according to Benton and Davis [9]. Prehybridization was carried out with 1 × Denhardt's solution, 100 μg/ml salmon sperm DNA and 6 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) at 65 °C for 2 h.

Hybridization was performed with labelled probe in 6 × SSC, 5 × Denhardt's solution and 0.1% sodium pyrophosphate at 65 °C for 18 h. The final wash was conducted with 0.2 × SSC and 0.1% sodium pyrophosphate three times at 60 °C. Two positive clones were isolated. Phage DNA for each clone was purified using Magic Lambda Preps kit (Promega) and the inserts were released by *Eco*RI digestion and subcloned into Bluescript KS for sequence determination [8]. The first clone gave a single *Eco*RI insert of 1.2 kb and was called pHEP3–7. The second clone gave a single *Eco*RI insert of 0.85 kb, and was called pHEP3–5. pHEP3–7 corresponded to the 5′ region of the mouse EP_3 receptor, stretching from the non-coding region to about two thirds into the coding region. pHEP3–5 corresponded to the 3′-region of the mouse receptor. A full-length cDNA clone was constructed from these two clones using a single *Ear*I site in the overlapping region. The full-length cDNA, pHEP3, showed 83.4% sequence identity with the mouse EP_3 receptor and had an open reading frame of 390 amino acids.

Northern blot analysis

Northern blot analysis was performed using a Human Multiple Tissue Northern Blot from Clontech (Palo Alto, CA, U.S.A.), which consists of a nylon membrane with 2 μg of poly(A)$^+$ RNA from each human tissue. The blot was probed with the same ^{32}P-labelled PCR product used to probe the HEL cell library according to the manufacturer's protocol.

Transfection into COS-1 cells with pSVK3

The full-length construct pHEP3, from clones pHEP3–7 and pHEP3–5, was subcloned into the *Eco*RI site of the eukaryotic expression vector pSVK3 (Pharmacia). COS-1 cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. For transient transfection, for use in binding studies, cells that had reached 30–50% confluency were transfected using Lipofectin reagent (1 mg/ml) (Gibco BRL) according to Felgner et al. [10], with 30 μl of Lipofectin and 10 μg of DNA per 85 mm dish. Cells were incubated overnight, the transfection medium was replaced with fresh medium and the cells were grown for a further 48 h before harvesting for binding studies or for use in cyclic AMP measurements. Mock-transfected cells were subjected to the same protocol but using vector alone.

For stable transfection (for measurement of cyclic AMP), cells were co-transfected with 5 μg of pSVK3-HEP3 DNA and 0.5 μg of pRc/CMV (Invitrogen), which contains a neomycin selection marker, using Lipofectin reagent (1 mg/ml) in 85 mm dishes. Cells were incubated overnight, the transfection medium was replaced with fresh medium and the cells were grown for 6 weeks in the presence of 0.5 mg/ml of the antibiotic G418 (Gibco) to select for transfected cells. Mock-transfected cells were subjected to the same protocol, but using pSVK3 without the prostaglandin receptor and pRc/CMV.

Binding experiments

Cells from 10 dishes each of either cells transfected with EP_3 receptor in pSVK3 or cells mock-transfected with vector alone were lysed in 20 mM Tris/HCl buffer (pH 7.5) and 5 mM $MgCl_2$ by sonication and homogenization. Membranes were prepared by centrifugation at 40000 *g* for 30 min at 4 °C and resuspension of the pellet in 50 mM Tris/HCl buffer (pH 7.5) and 1 mM EGTA. Prostaglandin binding was determined by incubation with [^{3}H]PGE_2 (154 Ci/mmol) at room temperature in 50 mM Tris/HCl containing 1 mM EGTA for 1 h. Non-labelled PGE_2 (5 μM) was used to define non-specific binding. Each binding study was carried out in duplicate with 200 μg of protein/ml. Competition binding studies were performed with 2 nM [^{3}H]PGE_2 and increasing concentrations of non-labelled PGE_2, PGE_1, PGD_2 or iloprost. After incubation, bound and free ligand were separated by rapid filtration over GF/B filters under vacuum using a Brandel Cell Harvester (Gaithersburg, MD, U.S.A.). Filters were washed three times with ice-cold 50 mM Tris/HCl buffer. Radioactivity in the filters was determined by liquid scintillation counting. Protein concentrations were determined by use of the BCA protein assay [11]. Binding data were analysed with the LIGAND program [12].

Determination of cyclic AMP formation

Stably transfected and mock-transfected cells grown in 6-well 35 mm culture dishes were labelled for 2 h with 2 μCi of [^{3}H]adenine (25 Ci/mmol). During this time more than 90% of the [^{3}H]adenine was incorporated into cellular adenine nucleotides. Medium was removed and replaced by fresh medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min. The medium was removed and replaced with fresh medium containing 1 mM IBMX, 10 μM forskolin and 0–10 μM PGE_2 for 30 min at 37 °C. The medium was removed and replaced by a stopping solution containing 1 M HCl and 2000 c.p.m. of [^{14}C]cyclic AMP as recovery standard. The supernatant and cells were removed from the dishes and boiled for 20 min, and cyclic AMP was determined by the two-column method of Salomon [13]. Cyclic AMP is expressed as a percentage of total adenine nucleotides.

RESULTS AND DISCUSSION

In order to demonstrate the presence of a prostaglandin EP_3 receptor coupled to inhibition of adenylate cyclase in the megakaryocytic cell line HEL, two oligonucleotide primers were

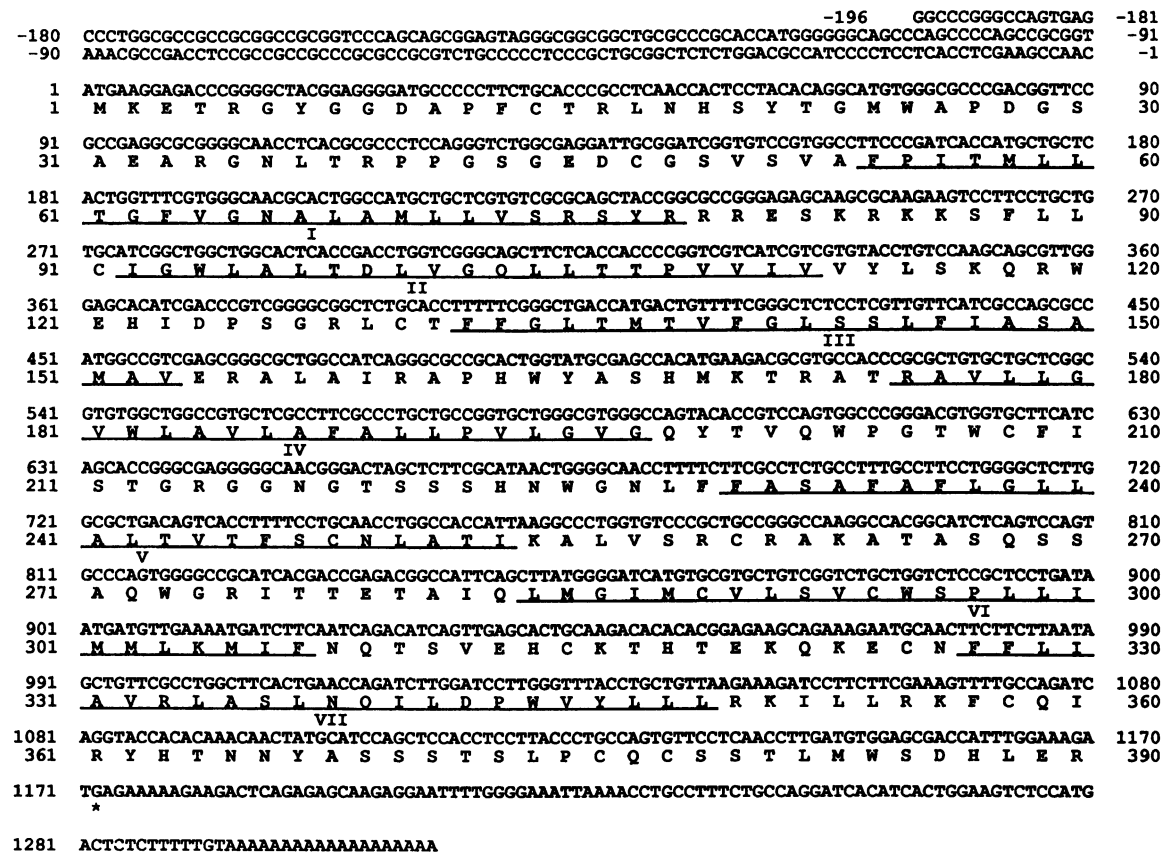

Figure 1 Nucleotide and deduced amino acid sequences of HEP3

The deduced amino acid sequence is shown below the nucleotide sequence in single letter code. Underlined positions indicate the seven putative transmembrane domains of the receptor from hydropathy analysis of the amino acid sequence.

designed based on the published sequence of the mouse prostaglandin EP_3 receptor [6]. Reverse transcriptase PCR was carried out on DMSO-induced HEL cell RNA with these primers and a product of the expected length (644 bp) was obtained. The PCR product was subcloned into PUC 18 and its nucleotide sequence was determined to be 85% identical to a corresponding region of the EP_3 receptor. The PCR product was radiolabelled and used to screen a λgt11 cDNA library from HEL cells. From 500000 plaque-forming units, two positive clones were obtained that were designated pHEP3–7 and pHEP3–5. Sequence analysis revealed that pHEP3–7 corresponded to the 5′ region of the human EP_3 receptor, stretching from the non-coding region to about two-thirds of the way into the coding region. pHEP3–5 corresponded to the 3′-region of the EP_3 receptor, stretching into the 3′ non-coding region. There was a 331 bp overlap of identical nucleotide sequence between the two clones that contained a unique *Ear*I restriction site. The clones were digested with *Ear*I and joined together to yield a full-length human EP_3 receptor cDNA designated HEP3, with an open reading frame of 390 amino acids (Figure 1). The nucleotide sequence of HEP3 is 83% identical with the sequence of the mouse EP_3 receptor [6]. Sugimoto et al. [14] have reported two variants of the mouse EP_3 receptor, designated α and β subtypes, that differ in the last 20–30 amino acids of their C-terminal regions. The HEL receptor sequence that we have cloned corresponds to the α subtype of the mouse EP_3 receptor, and the two receptors are 85.6% identical in terms of amino acid sequence. The HEL cell receptor is also similar to an EP_3 receptor recently cloned from bovine adrenal gland [15], but of the four splice variants of the bovine receptor reported, none corresponds to the C-terminal region of the HEL cell or mouse receptors. The hydropathy profile (not shown) determined by the method of Kyte and Doolittle [16] indicates the presence of the seven hydrophobic domains expected of a G-protein-linked receptor, and the profile is virtually identical to that of the mouse EP_3 receptor [6].

Northern blot analysis using a human Multiple Tissue Northern Blot revealed a positive species at 2.3 kb, close to the expected size of the receptor cDNA, and this was strongly apparent in kidney (Figure 2), which contains a high level of EP_3 receptor [6]. A species with a weaker hybridization signal was apparent at about 7.0 kb. A similar pattern of two species was observed with the mouse EP_3 receptor [6]; the identity of the 7.0 kb species in each case is unknown.

Membranes of COS-1 cells transfected with pSVK3-HEP3 exhibited high specific binding of $[^3H]PGE_2$ which was saturable, with K_d and $B_{max.}$ values calculated to be 3.2 ± 0.2 nM and 1.1 ± 0.2 pmol/mg of protein respectively ($n = 2$). The K_d for binding of $[^3H]PGE_2$ is very close to the value of 2.9 nM reported for the mouse EP_3 receptor [6]. Membranes from mock-transfected cells showed no detectable binding of $[^3H]PGE_2$. $[^3H]PGE_2$ binding to pSVK3-HEP3-transfected cells could be displaced by non-labelled prostaglandins (Figure 3) in the order $PGE_2 = PGE_1 >$ iloprost $= PGD_2$, which is characteristic of a receptor of the EP subtype of prostaglandin receptors [17].

Forskolin stimulated cyclic AMP formation in stably transfected COS-1 cells from 0.1% (basal) to 1.5% cyclic AMP,

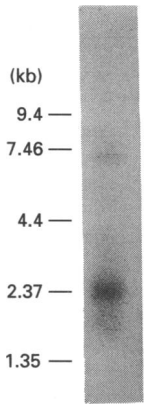

Figure 2 Northern blot analysis using a human Multiple Tissue Northern Blot from Clontech

Following prehybridization at 65 °C in 10 × Denhardt's solution, 5 × SSPE, 100 μg/ml denatured, sheared salmon sperm DNA, 50% diethyl pyrocarbonate-treated water and 2% SDS for 3 h, the blot was probed with a ^{32}P-labelled 644 bp PCR fragment of the HEL cell EP_3 receptor for 21 h at 65 °C. The initial wash was at room temperature with 2 × SSC and 0.05% SDS, followed by a final wash at 50 °C with 0.1 × SSC and 0.1% SDS.

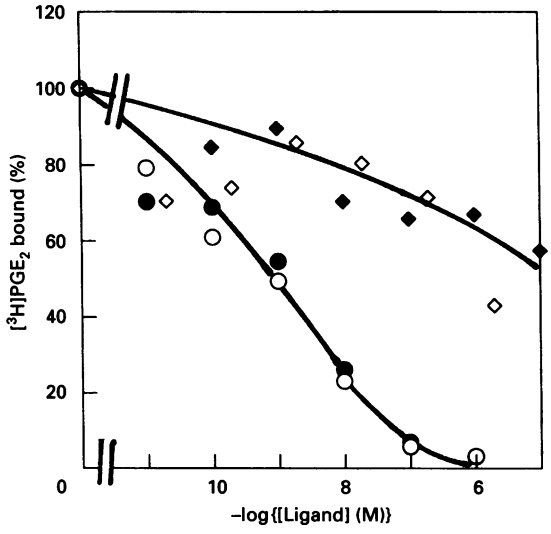

Figure 3 Displacement of bound [^{3}H]PGE_2 from pSVK3-HEP3-transfected COS-1 membranes

Unlabelled prostaglandins were added to the binding mixture at the indicated concentrations. Specific binding of [^{3}H]PGE_2 was determined as described in the Experimental section. ●, PGE_2; ○, PGE_1; ◆, PGD_2; ◇, iloprost.

measured as a percentage of the total adenine nucleotides, and the level of cyclic AMP increased as a function of PGE_2 concentration in mock-transfected cells, indicating the presence of low levels of endogenous PGE_2 receptors coupled to stimulation of adenylate cyclase (Figure 4). In cells stably transfected with pSVK3-HEP3, cyclic AMP levels also increased with PGE_2 concentration, but levels were 30% lower than those in mock-transfected cells (Figure 4), indicating expression of a functional receptor of the EP_3 subtype coupled to inhibition of adenylate cyclase.

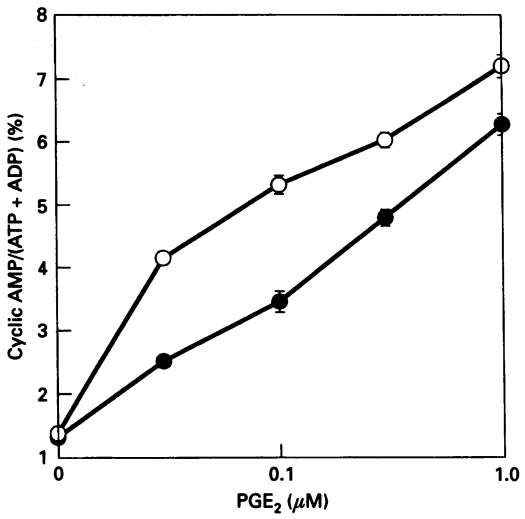

Figure 4 Intracellular cyclic AMP accumulation in G418-selected pools of COS-1 cells

The effect of PGE_2 on forskolin-stimulated cyclic AMP formation is shown in pSVK3-HEP3-transfected and mock-transfected COS-1 cells stimulated for 30 min by 10 μM forskolin in the presence of 1 mM IBMX and the indicated concentrations of PGE_2. ●, pSVK3-HEP3-transfected cells; ○, mock-transfected cells. The values are means ± S.E.M. of six determinations, and the experiment was repeated twice with similar results. Forskolin in the absence of PGE_2 gave identical levels of cyclic AMP, whereas in the presence of PGE_2 levels of cyclic AMP attained with HEP3-transfected cells were significantly lower than those in mock-transfected cells ($P < 0.01$) at all concentrations.

Taken together, our results show that HEL cells are capable of expressing receptors of the EP_3 prostaglandin receptor subtype which are coupled to inhibition of adenylate cyclase. The result is significant because in earlier work [3] we have suggested that HEL cells possess both stimulatory and inhibitory receptors coupled to adenylate cyclase. The presence of a stimulatory receptor is obvious, since prostaglandins stimulate cyclic AMP formation in HEL cells. The presence of a co-localized inhibitory receptor was inferred from the kinetics of cyclic AMP formation: prostaglandins induced a time-dependent inhibition of their own rapid stimulation of cyclic AMP formation that showed a different prostaglandin-concentration-dependence from stimulation. The current work confirms that an inhibitory prostaglandin receptor is present in HEL cells.

HEL cells are megakaryocytic cells that provide a model for certain aspects of platelet function. In platelets, the major effect of a rise in the steady-state level of cyclic AMP is to inhibit platelet aggregation and secretion. We have postulated that cyclic AMP levels in platelets are controlled by distinct prostaglandin receptors coupled to stimulation and inhibition of adenylate cyclase [1,2]. The two-receptor model provides a homeostatic control mechanism. Prostaglandins are autocrine or paracrine hormones involved in localized feedback, and may reach transiently high levels at their sites of action. The presence of an inhibitory receptor would buffer against rapid variations in agonist concentration, maintaining cellular responsiveness within reasonable bounds. Hence, while circulating PGI_2 may be important in maintaining platelets in a non-thrombogenic state, the ability to buffer against excessive prostaglandin-stimulated cyclic AMP formation caused by inflammatory agents and other stimuli would prevent platelets from becoming refractory to appropriate stimulatory challenges. Demonstration of the presence of an EP_3 receptor in HEL cells supports our two-receptor

model and points to an important role for the EP_3 receptor in the control of cyclic AMP metabolism in autocrine and paracrine responses.

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