Biochemical Pharmacology, Vol. 54, pp. 917–926, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00288-8

Prostanoid Receptor with a Novel Pharmacological Profile in Human Erythroleukemia Cells

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ABSTRACT. The purpose of this study was to characterize the prostanoid receptors coupled to intracellular calcium in human erythroleukemia (HEL) cells, a cell line with platelet/megakaryocytic characteristics. Both prostaglandin E_1 (PGE_1) and iloprost increased cyclic AMP (cAMP) in HEL cells, but modulated $[Ca^{2+}]_i$ by different mechanisms. Iloprost (10^{-9} to 10^{-6} M) had no effect on basal $[Ca^{2+}]_i$, but greatly potentiated the increase in $[Ca^{2+}]_i$ produced by thrombin. This effect was mimicked by cholera toxin and other G_s-coupled receptors, and involved calcium influx since iloprost had no effect on $[Ca^{2+}]_i$ in cells incubated in Ca^{2+}-free buffer. Furthermore, iloprost did not increase the generation of baseline or thrombin-induced inositol phosphates at these concentrations. In contrast, PGE_1 (10^{-7} to 10^{-5} M), but not iloprost, increased basal $[Ca^{2+}]_i$ through a pertussis toxin-sensitive mechanism that involved stimulation of inositol phosphate generation and mobilization of intracellular calcium. The order of potencies of other prostaglandins that increased $[Ca^{2+}]_i$ was not consistent with known IP, EP, DP, FP, or TP receptors. 11-Deoxy-16,16-dimethyl PGE_2 was the most potent of the analogs tested (EC_{50} = 28 nM). In summary, at least two prostaglandin receptors are functionally coupled to intracellular calcium in HEL cells: a putative IP receptor coupled to G_s proteins that increases cAMP and enhances calcium influx, and a novel prostanoid receptor that evokes calcium mobilization through stimulation of phospholipase C by a pertussis toxin-sensitive pathway. BIOCHEM PHARMACOL **54**;8:917–926, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. prostanoid receptors; prostacyclin receptors; prostaglandins; human erythroleukemia cells; intracellular free calcium concentration; fura-2

HEL† cells were derived from a patient with Hodgkin's disease who developed erythroleukemia upon relapse [1]. HEL cells express megakaryocytic membrane markers [2], and the initial interest in these cells derived from their potential as a model to study prostanoid receptors. It has been shown that eicosanoids activate several intracellular signaling pathways in HEL cells, adenylate cyclase and intracellular calcium in particular. Current information about the receptors involved, however, is limited and controversial.

It has been shown that iloprost is more potent than PGE_1 in increasing cAMP levels, whereas the reverse is true for increasing intracellular calcium concentrations [3]. Similarly, iloprost modulation of intracellular calcium is pertussis toxin insensitive, whereas the effects of PGE_1 are pertussis toxin sensitive [3, 4]. Little is known about the effects of other eicosanoids on intracellular calcium in HEL cells. Schwaner *et al.* found PGE_2 to be more potent than PGE_1 in increasing intracellular calcium [5], whereas the opposite has been found in other studies [3, 4].

It is clear that these effects cannot be explained by activation of a single prostanoid receptor, but the receptor type(s) has not been identified. The presence of several prostanoid receptors has been documented or inferred in HEL cells. Cell membranes contain binding sites for the prostacyclin analog iloprost [6]. More recent findings have demonstrated the presence of cDNA encoding EP_1 [7] and EP_3 [8] receptors. EP_1 receptors increase intracellular calcium when expressed in *Xenopus* oocytes [7], raising the possibility that EP receptors are responsible for modulation of intracellular calcium in HEL cells.

Previous studies suggest at least two different mechanisms by which eicosanoids modulate intracellular calcium in HEL cells. Iloprost has little if any effect on resting intracellular calcium but greatly potentiates the rise in intracellular calcium induced by other stimuli. In contrast, other eicosanoids, in particular PGE_1, increase resting intracellular calcium. Our purpose, therefore, was to identify the prostanoid receptor(s) and intracellular pathways that explain these disparate actions.

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† *Abbreviations:* HEL, human erythroleukemia; PMA, 12-O-tetradecanoylphorbol-13 acetate; cAMP, cyclic AMP; PGA_2, prostaglandin A_2; PGB_2, prostaglandin B_2; PGD_2, prostaglandin D_2; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PGF_2, prostaglandin F_2; PGJ_2, prostaglandin J_2; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; LTF_4, leukotriene F_4; 5 (*RS*)HETE, 6,8,11,14-eicosatetraenoic acid, 5-hydroxy-, [*RS*-(*E*,*Z*,*Z*,*Z*]-; 11 (S)HETE, 6,8,11,14-eicosatetraenoic acid, 11-hydroxy-, [S-(*E*,*Z*,*Z*,*Z*]-; 12 (S)HETE, 6,8,11,14-eicosatetraenoic acid, 12-hydroxy-, [S-(*E*,*Z*,*Z*,*Z*]-; 15 (S)HETE, 6,8,11,14-eicosatetraenoic acid, 15-hydroxy-, [S-(*E*,*Z*,*Z*,*Z*]-; SQ 29,548, 5-heptenoic acid, 7-[3-[[2-[9-phenylamino]carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl],[1S-[1α,2α(Z),3α,4α]]; I-BOP, 5- heptenoic acid, 7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-,[1S[1α,2α(Z),3β(1E,3S*),4α]]-; U-46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; and M&B 28767, (±)-15α-hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranorprost-13-*trans*-enoic acid.

Received 17 December 1996; accepted 20 May 1997.

In an initial series of studies, we explored the possible interaction between adenylate cyclase activation and the rise in intracellular calcium. Our purpose was to examine if this mechanism can explain the actions of iloprost. Our results suggest that adenylate cyclase activation and potentiation of intracellular calcium are both coupled to G_s proteins, but events distal to that step are parallel and independent. In a second series of studies, we tested various eicosanoids for their ability to increase resting intracellular calcium. These eicosanoids were chosen because of their relative selectivity toward prostanoid receptors. The pharmacological profile found in these studies, however, does not correspond to any of the known prostanoid receptors.

MATERIALS AND METHODS

Cells

HEL cells (TIB 180) obtained from the American Type Culture Collection (Rockville, MD) were maintained in suspension culture at a density between 3 and 9 × 10^5 cells/mL by dilution with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) newborn calf serum, and 2 mM glutamine. Cells were kept in a humidified atmosphere of 5% CO_2 at 37°. Before each experiment, cells were harvested, washed by centrifugation (100 *g* for 10 min), and resuspended to a concentration of 10^7 cells/mL for measurement of cAMP, or 2 × 10^6 cells/mL for measurement of intracellular Ca^{2+} in a buffer, pH 7.4, containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/L D-glucose, 10 mM HEPES-NaOH, and 0.35% bovine serum albumin.

Measurement of cAMP

HEL cells (2 × 10^6/tube) were preincubated in a total volume of 200 μL of buffer, containing the cAMP phosphodiesterase inhibitor papaverine (0.1 mM) for 2 min at 37°. cAMP accumulation in response to prostacyclin receptor activation was measured by the addition of iloprost (2 μL) to the cell suspension. When noted, thrombin (1 μL) was also added together with iloprost to a final concentration of 0.3 U/mL. Cells were then mixed with a vortex mixer and the incubation was allowed to proceed for 2 min at 37°. The reaction was stopped by the addition of 50 μL of 25% trichloroacetic acid. Trichloroacetic acid-treated extracts were washed five times with 10 vol. of water-saturated ether, and cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein derived from bovine muscle that has high specificity for cAMP [9] (Cyclic AMP Assay Kit, TRK.432; Amersham, Arlington Heights, IL).

Measurement of Intracellular Calcium

Cytosolic free calcium concentrations were determined by the fluorescent dye technique [10]. HEL cells were loaded with 1 μM fura-2/acetoxymethyl ester in the buffer. After incubation for 1 hr at room temperature, cells were washed to remove excess fura-2/acetoxymethyl ester, and were resuspended in the same buffer at a concentration of 2 × 10^6 cells/mL. Just before each experiment, a 100-μL sample of cell suspension was diluted to 2 mL in albumin-free buffer. HEL cells in suspension were kept in a cuvette at 37° while fluorescence was monitored at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm. Maximal fluorescence was determined by addition of 20 μL of 0.4% digitonin. Minimal fluorescence was determined by the addition of 40 μL of 1 M EGTA. All calcium determinations were done within 2 hr of fura-2 loading. The intracellular calcium concentration was calculated using previously described formulas, assuming a K_d of 224 nM [10]. Fluorescence was measured with a Fluorolog-2 spectrofluorimeter (SPEX Industries, Inc., Edison, NJ).

Measurement of the Formation of [^{3}H]Inositol Phosphates

Formation of inositol phosphates was determined by modification of the previously described procedure [11]. HEL cells, at a concentration of 10^7 cells/mL, were labeled to equilibrium with *myo*-[^{3}H]inositol (5 μCi/mL, DuPont-NEN, Boston, MA) for 24 hr in inositol-free Dulbecco's Modified Eagle's Medium (Gibco-BRL, Gaithersburg, MD). HEL cells were harvested and resuspended in buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$ 1 mM $CaCl_2$, 5 g/L D-glucose, 10 mM HEPES-NaOH, pH 7.4, and 20 mM LiCl to a concentration of 5 × 10^7 cells/mL. After preincubation at room temperature for 10 min, cells (198 μL) were added to tubes containing tested compounds or their corresponding vehicles (2 μL), and the incubation was allowed to proceed for 30 min at 37°. Reaction was stopped by placing tubes into an ice bath, and cells were collected by centrifugation at 12,000 rpm on a microcentrifuge for 30 sec at 4° and resuspended in 200 μL of ice-cold 10 mM formic acid (pH 3). After 1 hr, this solution, containing the extracted inositol phosphates and inositol, was collected by centrifugation at 12,000 rpm on a microcentrifuge for 5 min at 4° and diluted with 800 μL of 5 mM NH_3OH (final pH 8–9). This solution was then applied to columns containing 0.2 mL anion exchange resin (AG 1-X8, formate form, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 mL of H_2O and 1 mL of 40 mM ammonium formate/formic acid, pH 5, respectively. Total inositol phosphates were eluted in a single step with 1 mL of 2 M ammonium formate/formic acid, pH 5, and radioactivity was measured by liquid scintillation counting.

Drugs and Data Analysis

Papaverine, 8-Br-cAMP, and thrombin were obtained from the Sigma Chemical Co. (St. Louis, MO). Fura-2 was purchased from Molecular Probes, Inc. (Eugene, OR). Iloprost and cicaprost, stable analogs of prostacyclin, were gifts from Schering AG (Berlin, Germany) and Berlex

Laboratories, Inc. (Wayne, NJ). M&B 28767 was a gift from Dr. M. P. L. Caton of Rhone Poulenc Rorer (Dagenham, Essex, U. K.). All other eicosanoids were purchased from the Cayman Chemical Co. (Ann Arbor, MI).

Calculation of EC_{50} values from concentration–response curves and curve-fitting analysis by nonlinear regression were done using InPlot 4.0 software (GraphPad Software, San Diego, CA) in a microcomputer. Curve-fitting of monophasic sigmoid curves was performed using standard formulas included in the InPlot software. We used the following equation of nonlinear regression for curve-fitting of a two-site activation model:

$$Y = A + ((B - A)/100 \cdot ((C/(1 + 10^{D}/10^{X})) + ((100 - C)/(1 + 10^{E}/10^{X}))),$$

where A is a minimum, B is a maximum, C is a proportion of high affinity sites, D is EC_{50} for high-affinity sites, and E is EC_{50} for low-affinity sites. Statistical analysis was performed using InStat 2.0 software (GraphPad Software). A two-tailed unpaired t-test was used for single comparisons. The criterion for significance was $P < 0.05$. Results are presented as means ± SEM.

RESULTS

Effect of High Concentrations of Iloprost on Intracellular Calcium Regulation in HEL Cells

The stable prostacyclin analog iloprost produced a concentration-dependent increase in cAMP accumulation and intracellular Ca^{2+} concentration in HEL cells (Fig. 1A). There was, however, a difference of more than two orders of magnitude between the potencies of iloprost to increase cAMP (EC_{50} = 3.3 nM) and to elevate intracellular Ca^{2+} concentration (EC_{50} > 1 μM). Iloprost produced a maximal increase in cAMP at concentrations (30 nM) that had no effect on intracellular Ca^{2+} levels (Fig. 1A). Higher concentrations of iloprost (10 μM) produced a very rapid rise in intracellular Ca^{2+} levels (from 103 to 295 nM) followed by a subsequent decrease in intracellular Ca^{2+} concentration towards control values (data not shown). The reversibility of this effect indicates that the increase in fluorescence was not due to leakage of fura-2 because of cell toxicity. We observed a gradual increase in the magnitude of the calcium response to high concentrations of iloprost over a period of several hours after loading cells with fura-2. Therefore, all interventions used to study this effect were applied in random sequence, and we limited the time of experiments to 2 hr after fura-2 loading.

The effect of high concentration of iloprost on intracellular calcium was not mimicked by incubation of HEL cells with 100 ng/mL cholera toxin, and was not altered by incubation of cells with 500 ng/mL pertussis toxin, indicating that neither G_s nor G_i proteins are involved in this process. The rise in intracellular calcium produced by 10 μM iloprost was decreased if extracellular calcium was

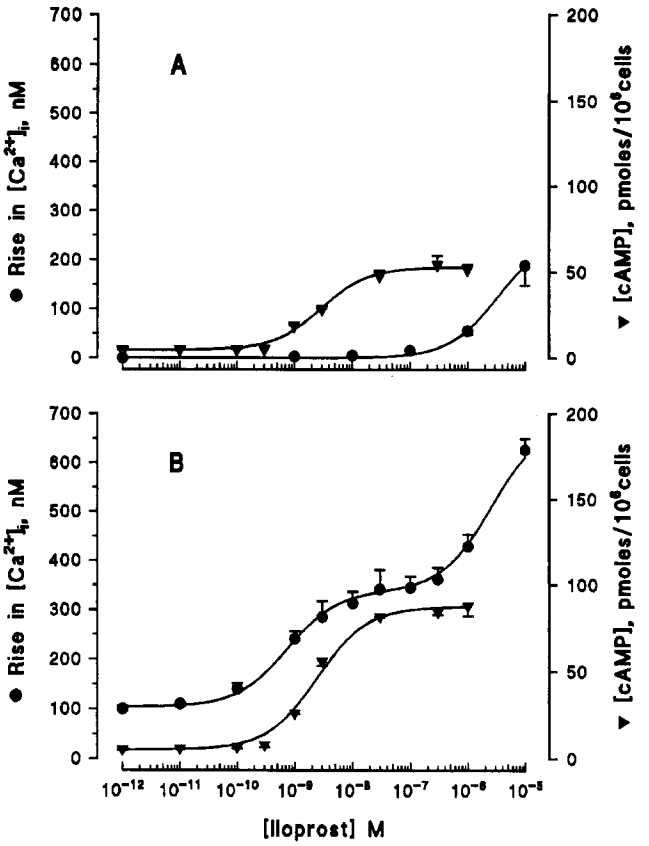

FIG. 1 Effect of increasing concentrations of iloprost (X-axis) on the levels of free intracellular Ca^{2+} (left Y-axis, circles, expressed as the change from unstimulated levels, N = 8) and cAMP (right Y-axis, inverted triangle, N = 3) in the absence (A, upper panel) or in the presence (B, lower panel) of 0.3 U/mL thrombin in HEL cells. Values are means ± SEM.

acutely chelated (68 ± 2 nM compared with 358 ± 10 nM in the presence of extracellular calcium), suggesting that at these concentrations iloprost increases intracellular calcium by mechanisms probably involving both calcium influx and mobilization of internal stores.

Synergism between Iloprost and Thrombin on cAMP Accumulation and Intracellular Calcium in HEL Cells

Whereas low concentrations of iloprost (up to 10^{-7} M) had no effect on basal intracellular Ca^{2+} levels, they potentiated the increase in intracellular Ca^{2+} produced by 0.3 U/mL thrombin and accelerated its rate of increase (Figs. 1B and 2A), with an estimated EC_{50} value of 0.6 nM (Table 1). This effect was observed whether iloprost was added 30 sec before thrombin (as shown in Fig. 2A), or if it was added at the peak response of thrombin (data not shown). Iloprost also acted synergistically with the calcium ionophore A23187, which works through nonreceptor-mediated mechanisms, in increasing intracellular Ca^{2+} concentration; A23187 (10 nM) increased intracellular calcium by 296 ± 15 nM in the absence, and by 382 ± 2 nM in the presence, of 10 nM iloprost (N = 3).

Pretreatment of HEL cells with 100 ng/mL cholera toxin

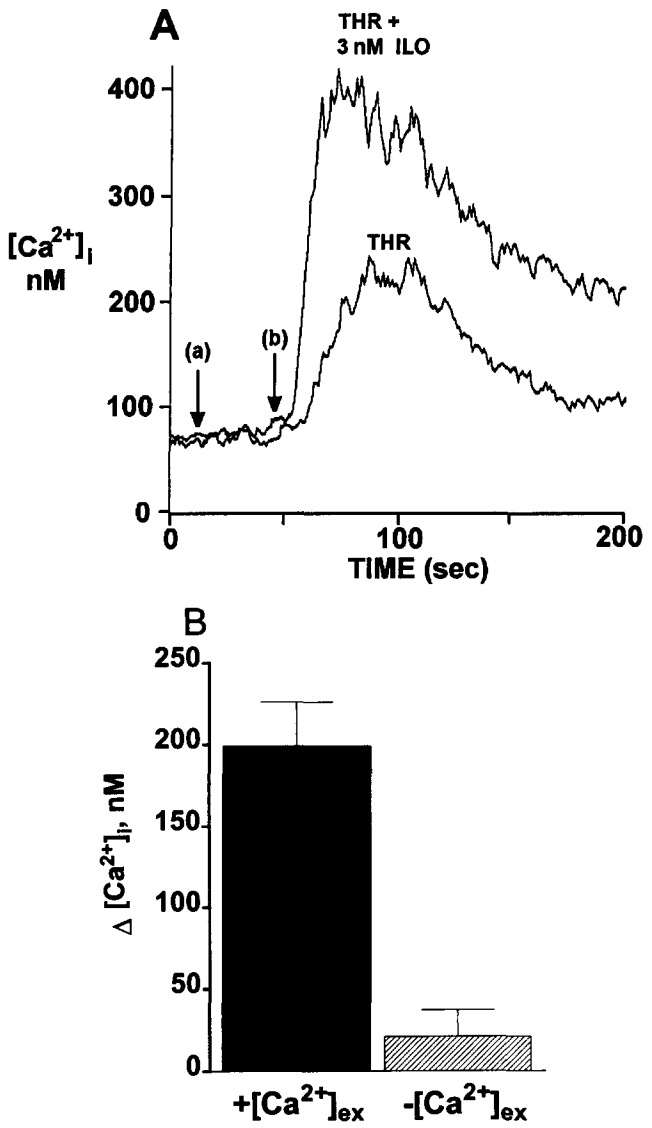

FIG. 2 Effect of iloprost and thrombin on free intracellular Ca^{2+} in HEL cells. (A) Iloprost (ILO) (a), final concentration of 3 nM, was added 30 sec before 0.3 U/mL thrombin (THR) (b) at times indicated by the arrows. (B) Additional rise in free intracellular Ca^{2+} produced by 3 nM iloprost in HEL cells stimulated by 0.3 U/mL thrombin (N = 5). All cells were maintained in a 1 mM $CaCl_2$ buffer and were resuspended immediately before each measurement in a Ca^{2+}-free buffer to reach a final concentration of 50 μM $CaCl_2$. $CaCl_2$ or EGTA was added 15 sec before iloprost to a final concentration of 1 mM to study the effects of iloprost in the presence ($+[Ca^{2+}]_{ex}$) or absence ($-[Ca^{2+}]_{ex}$) of extracellular Ca^{2+}, respectively. Values are means ± SEM.

for 24 hr potentiated the rise in intracellular Ca^{2+} levels produced by thrombin. Addition of low concentrations of iloprost (10^{-10} to 10^{-7} M) to thrombin-stimulated cells produced a further increase in intracellular Ca^{2+} levels, but the maximal effect was similar in cholera toxin-treated and control cells (Fig. 3). The effects of cholera toxin and iloprost, therefore, were not additive but shared the same mechanism. On the other hand, pretreatment of HEL cells for 24 hr with 500 ng/mL pertussis toxin had no effect on

TABLE 1. Potencies of PGE_1 and iloprost in increasing cAMP and intracellular Ca^{2+} levels in HEL cells

	EC_{50} (nM)	
	cAMP	$[Ca^{2+}]_i$
Iloprost	3.3	0.6*
PGE_1	1028	458

EC_{50}, concentration of agonist that produced 50% of the maximal effect. Data for PGE_1 was taken from Ref. 3.

* Potentiation of the increase in $[Ca^{2+}]_i$ produced by thrombin.

basal intracellular Ca^{2+} levels, on the increase in intracellular Ca^{2+} concentration produced by thrombin, or on its potentiation by iloprost (data not shown).

Thrombin produced a 1.6-fold increase in the efficacy of iloprost to raise cAMP (Fig. 1). On the other hand, thrombin did not alter the potency of iloprost; the EC_{50} values in the absence and presence of 0.3 U/mL thrombin were 3.3 ± 0.6 (Table 1) and 2.3 ± 0.3 nM, respectively. These results agree with the findings of Turner *et al.* [12], who showed that thrombin potentiated the maximal increase in cAMP produced by prostacyclin and carbacyclin, without altering its potency.

It could be argued, based on these observations, that cAMP plays a role in the synergistic interaction between thrombin and iloprost on intracellular Ca^{2+}. However, we have demonstrated previously that preincubation of HEL cells with the permeable cAMP analog 8-Br-cAMP, in concentrations ranging from 100 nM to 100 μM, does not potentiate the rise in intracellular Ca^{2+} levels induced by thrombin. At these concentrations, 8-Br-cAMP penetrates HEL cells and activates protein kinase A [3].

Role of Calcium Influx in the Synergistic Interaction between Thrombin and Iloprost in HEL Cells

We used acute chelation of extracellular calcium to determine if calcium influx is involved in the synergistic increase in intracellular calcium by thrombin and iloprost. HEL cells (2×10^6 cells/mL) were kept in buffer containing 1 mM $CaCl_2$ to avoid depletion of intracellular stores. Just before each measurement, 100 μL of cell suspension was diluted to 2 mL in calcium-free buffer to acutely reduce extracellular Ca^{2+} concentrations to 50 μM. Residual extracellular Ca^{2+} was chelated by addition of 10 μL of 200 mM EGTA (final concentration, 1 mM), pH 7.4.

Thrombin (0.3 U/mL) alone increased intracellular Ca^{2+} levels by 219 ± 8 nM in the absence and by 237 ± 42 nM in the presence of extracellular Ca^{2+}. Acute depletion of extracellular Ca^{2+}, therefore, did not alter the increase in intracellular Ca^{2+} levels produced by thrombin, confirming that this increase is mostly due to mobilization of intracellular Ca^{2+} stores, as previously reported [13, 14]. On the other hand, the potentiation of the thrombin-mediated rise in intracellular Ca^{2+} levels by 3 nM iloprost virtually disappeared in the absence of extracellular Ca^{2+} (Fig. 2B), suggesting that this effect is mediated mostly by Ca^{2+} influx.

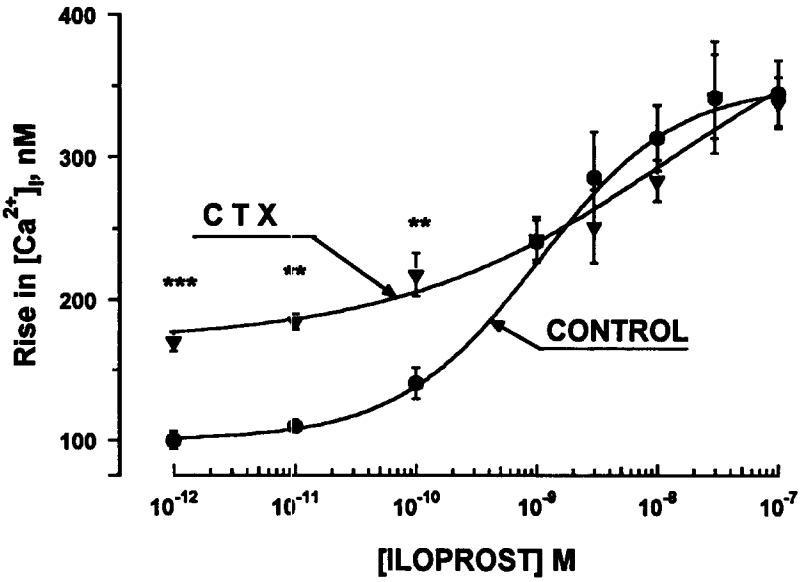

FIG. 3 Effect of increasing concentrations of iloprost (X-axis) on free intracellular Ca^{2+} (Y-axis, expressed as the change produced by thrombin) in HEL cells stimulated by thrombin in the presence and absence of cholera toxin. Cells were studied after incubation with buffer (control, circles) or 100 ng/mL cholera toxin (CTX, inverted triangles) for 24 hr. Iloprost was added 30 sec before 0.3 U/mL thrombin. Asterisks indicate statistical differences compared with control (** = $P < 0.01$ and *** = $P < 0.001$, after ANOVA, N = 8). Values are means ± SEM.

Effect of Eicosanoids on Basal Intracellular Calcium in HEL Cells

We tested several prostanoids, leukotrienes, and fatty acids for their ability to increase resting intracellular calcium. Prostaglandin E_1 produced a concentration-dependent rise in intracellular calcium in HEL cells, with an EC_{50} value of 606 ± 33 nM (Fig. 4). Among all other analogs tested, only 11-deoxy-16,16-dimethyl PGE_2 was more potent than PGE_1 in increasing intracellular calcium (EC_{50}, 28 ± 1 nM, Fig. 4). Both PGE_1 and 11-deoxy-16,16-dimethyl PGE_2 had a similar efficacy, increasing intracellular calcium by as much as 219 ± 19 and 228 ± 22 nM, respectively. This effect was pertussis toxin sensitive because it was abolished after incubation of HEL cells with 500 ng/mL pertussis toxin for 24 hr (Fig. 4, inset). The relatively selective EP_3 agonist M&B 28767 and the prostacyclin metabolite 6-keto PGE_1 were less potent than 11-deoxy-16,16-dimethyl PGE_2, with an EC_{50} value greater than 10 μM. Because a maximal response was not reached at the concentrations tested, we used the maximal effect produced by 11-deoxy-16,16-dimethyl PGE_2 in the estimation of these EC_{50} values. All other compounds tested, including the EP agonists PGE_2, 17-phenyl-trinor PGE_2, sulprostone and misoprostol, the FP agonist $PGF_{2\alpha}$, the DP agonist PGD_2, the IP agonists iloprost and cicaprost, and the TP agonists U-46619 and I-BOP (Fig. 5), were virtually ineffective at concentrations up to 1 μM. We also tested PGA_2, PGB_2, 8-iso PGE_2, 8-epi $PGF_{2\alpha}$, 9α,11β PGF_2, and TXB_2 at a concentration of 1 μM; 13,14-dehydro-15-keto PGD_2, $PGF_{2\beta}$, 11β $PGF_{2\alpha}$, 13,14-dehydro-15-keto $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, 2,3-dinor-6-keto $PGF_{1\alpha}$, 15-deoxy-$\Delta^{12,14}$ PGJ_2, and 11-dehydro TXB_2 at a concentration of 10 μM; LTB_4, LTC_4, LTD_4, LTE_4, *N*-acetyl LTE_4, and LTF_4 at a concentration of 300 nM; and 5 (RS)HETE, 11 (S)HETE, 12 (S)HETE, and 15 (S)HETE at concentrations from 3 to 20 μM. None of these compounds increased intracellular Ca^{2+} in HEL cells.

Effects of 11-Deoxy-16,16-dimethyl PGE_2 on Ca^{2+} Mobilization in HEL Cells

The increase in intracellular calcium produced by 30 nM 11-deoxy-16,16-dimethyl PGE_2 was similar whether cells were incubated in the presence of 1 mM extracellular calcium, or in a calcium-free medium with 1 mM EGTA (130 ± 8 and 116 ± 4 nM, respectively, $P > 0.05$, N = 3). This suggests that 11-deoxy-16,16-dimethyl PGE_2 increases intracellular Ca^{2+} by evoking Ca^{2+} mobilization.

We used an additional approach to determine whether 11-deoxy-16,16-dimethyl PGE_2 increases intracellular calcium by stimulating extracellular calcium influx or by

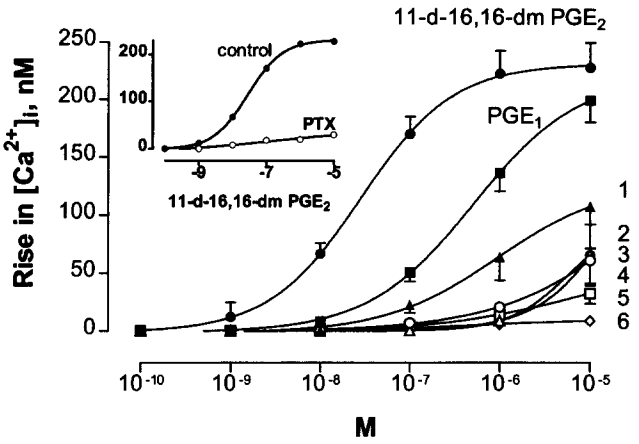

FIG. 4 Effect of increasing concentrations of prostanoid receptor agonists on the levels of free intracellular Ca^{2+} in HEL cells. The following compounds are shown: 11-deoxy-16,16-dimethyl PGE_2 (11-d-16,16-dm PGE_2); prostaglandin E_1 (PGE_1); M&B 28767 (1); 6-keto PGE_1 (2); misoprostol, free acid (3); PGE_2 (4); 17-phenyl-trinor PGE_2 (5); and sulprostone (6). A complete list of compounds tested is presented in the text and in Table 2. Values are means ± SEM of six experiments. *Inset:* Effect of increasing concentrations of 11-deoxy-16,16-dimethyl PGE_2 on the levels of free intracellular Ca^{2+} in control and pertussis toxin-treated (PTX) HEL cells.

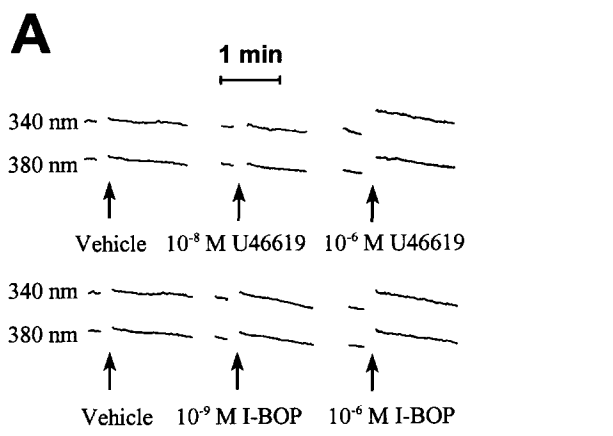

FIG. 5 Effect of the thromboxane receptor agonists U-46619 and I-BOP on fura-2 fluorescence (A) and cytosolic free calcium concentration (B) in HEL cells. (A) Fura-2 fluorescence intensity was monitored at emission wavelength 510 nm and excitation wavelengths 340 nm (upper traces) and 380 nm (lower traces). Arrows indicate time of addition of compounds or their vehicle. Parallel changes in both wavelengths were observed, so that the ratio of fluorescence intensities F_{340}/F_{380} remained unchanged after addition of the thromboxane analogs. (B) Cytosolic free calcium levels were calculated from the I-BOP traces shown in panel A. No differences were found between I-BOP (1 nM or 1 μM) or its vehicle (added at the time indicated by the arrow).

evoking intracellular calcium mobilization. Mn^{2+} and Ca^{2+} have been shown to share the same channel for entry into cells. At an excitation wavelength of 360 nm and an emission wavelength of 500 nm, fluorescence is selectively quenched by influx of Mn^{2+} and is unaltered by changes in Ca^{2+}. On the other hand, mobilization of Ca^{2+} from intracellular stores can be assessed simultaneously by monitoring fluorescence at an excitation wavelength of 340 nm in cells incubated in a calcium-free medium [15]. For these studies, Mn^{2+} was added into Ca^{2+}-free buffer just before each measurement, to a final concentration of 100 μM. The addition of 100 nM 11-deoxy-16,16-dimethyl PGE_2 to the incubation medium produced an increase in Ca^{2+} signal without a significant alteration in the rate of fura-2 signal quenching by Mn^{2+} (Fig. 6, upper panel). We used 500 nM ionomycin as a positive control to demonstrate calcium influx using this technique (Fig. 6, lower panel). Taken together, these results indicate that 11-deoxy-16,16-dimethyl PGE_2 increased intracellular calcium through release of internal stores rather than influx of extracellular calcium.

To ensure that thromboxane receptors do not contribute to or modulate the effects of 11-deoxy-16,16-dimethyl PGE_2, we repeated these studies in the presence of the TXA_2 antagonist SQ 29,548. At 10 nM, 11-deoxy-16,16-dimethyl PGE_2 increased intracellular calcium by 59 ± 6 and 48 ± 5 nM in the absence and presence of 100 nM SQ 29,548, respectively ($P = 0.2$, $N = 7$).

Effect of 11-Deoxy-16,16-dimethyl PGE_2 and Iloprost on the Formation of Inositol Phosphates in HEL Cells

Stimulation of phospholipase C and subsequent phosphoinositide hydrolysis is considered to be a major pathway for calcium mobilization from internal stores. To elucidate the role of this pathway in the effects of 11-deoxy-16,16-dimethyl PGE_2 and iloprost, we measured accumulation of total inositol phosphates in the presence of 20 mM LiCl. A 1 μM concentration of 11-deoxy-16,16-dimethyl PGE_2 considerably increased the accumulation of inositol phosphates (from 14,810 ± 600 to 25,500 ± 1,560 dpm/tube, $N = 4$, $P < 0.001$, Fig. 7). On the other hand, 3 nM iloprost had no effect on inositol phosphate accumulation (to 15,480 ± 690 dpm/tube, $N = 4$, $P = 0.5$) but higher concentrations (10 μM) produced a significant increase (to 18,760 ± 500 dpm/tube ($N = 4$, $P < 0.01$).

Thrombin at 0.3 U/mL, a concentration that approximates its EC_{50} for increase in intracellular calcium in HEL cells [3], increased the accumulation of inositol phosphates (to 21,270 ± 930 dpm/tube, $N = 4$, $P < 0.01$), in agreement with previous reports [13]. Iloprost at 3 nM did

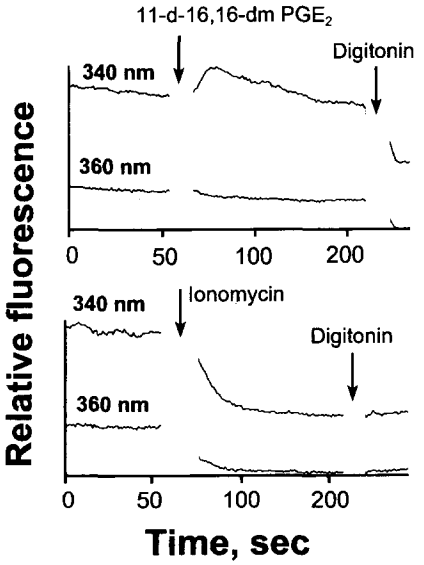

FIG. 6 Effect of 11-deoxy-16,16-dimethyl PGE_2 on calcium influx and mobilization in HEL cells. The upper panel shows the effect of 1 μM 11-deoxy-16,16-dimethyl PGE_2 on calcium influx and mobilization from internal stores. Calcium mobilization was determined in the presence of 1 mM $MnCl_2$, by monitoring fura-2 fluorescence at an excitation wavelength of 340 nm (upper trace). Calcium influx was monitored at an excitation wavelength of 360 nm (lower trace). The lower panel shows the effect of 500 nM ionomycin using the same conditions as described above. The calcium influx induced by ionomycin is shown as a positive control.

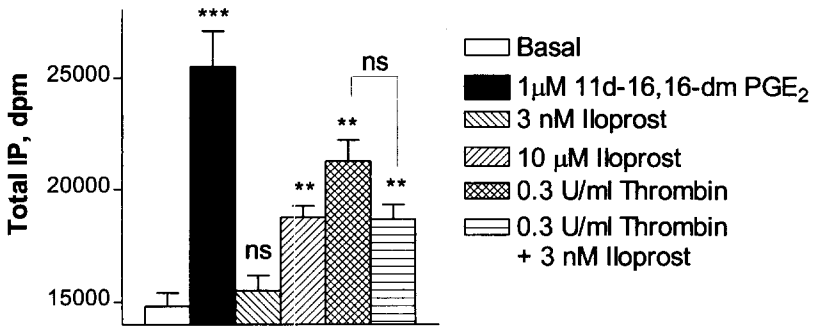

FIG. 7 Accumulation of total inositol phosphates in unstimulated HEL cells (basal), and in cells stimulated with 1 μM 11-deoxy-16,16-dimethyl PGE_2 (11-d-16,16-dm PGE_2), 3 nM iloprost, 10 μM iloprost, 0.3 U/mL thrombin and 3 nM iloprost in the presence of 0.3 U/mL thrombin. Values are means ± SEM of four experiments. Asterisks indicate statistical differences when treated cells were compared with basal: (**) $P < 0.01$ and (***) $P < 0.001$, after ANOVA; "ns" indicates non-significant differences. Iloprost, 3 nM, in the presence of 0.3 U/mL thrombin was also compared to 0.3 U/mL thrombin alone ($P = 0.06$, $N = 4$).

not potentiate the thrombin-induced accumulation of inositol phosphates, but rather tended to inhibit it to 18,670 ± 650 dpm/tube ($N = 4$, $P = 0.06$). Similar inhibitory actions of prostacyclin have been reported by Brass and Woolkalis [16]. These findings agree with our conclusion that iloprost potentiates the thrombin-induced rise in calcium by facilitating calcium influx rather than through an increase in calcium mobilization.

Effect of Prostaglandins on Intracellular Calcium in Differentiated HEL Cells

Phenotypic differentiation of HEL cells can be induced by incubation with phorbol esters or DMSO. It has been shown that differentiation of HEL cells with 160 nM PMA for 2 days increases the expression of prostacyclin (IP) receptors [6] and EP_1 receptors [7]. Given the potential changes in phenotypic expression produced by differentiation of HEL cells, we determined if this process alters the modulation of intracellular calcium by prostaglandins.

HEL cells were exposed to 160 nM PMA for 2 days. Under these conditions, HEL cells underwent the well characterized change in morphology and increased adherence. Neither 11-deoxy-16,16-dimethyl PGE_2, nor the specific EP_1 agonist 17-phenyl-trinor PGE_2 at concentrations up to 10 μM had any effect on intracellular Ca^{2+} in PMA-differentiated HEL cells. Likewise, high concentrations of iloprost (3 μM), which increased intracellular Ca^{2+} in undifferentiated HEL cells, had no effect on intracellular Ca^{2+} in differentiated cells. On the other hand, thrombin (0.3 U/mL) increased intracellular Ca^{2+} in differentiated HEL cells by 41 ± 2 nM, and iloprost (100 nM) potentiated this increase to 142 ± 5 nM ($N = 5$). The synergism between thrombin and iloprost, therefore, was intact after differentiation of HEL cells.

cDNA encoding EP_3 receptors have been cloned from a DMSO-differentiated HEL cell library [8]. To determine whether DMSO produced a different phenotypic expression than phorbol esters, HEL cells were exposed to 1.25% DMSO for 4 days. As with phorbol ester, HEL cells changed their morphology and became adhesive with DMSO treatment. In contrast to our finding in PMA-induced differentiation, the increase in intracellular calcium produced by 11-deoxy-16,16-dimethyl PGE_2 was preserved in DMSO-differentiated HEL cells. At 1 μM, 11-deoxy-16,16-dimethyl PGE_2 increased intracellular Ca^{2+} levels by 188 ± 11 nM ($N = 3$). Thrombin (0.3 U/mL) increased intracellular Ca^{2+} by 687 ± 60 nM in DMSO-differentiated HEL cells. Iloprost (10 nM) potentiated this increase to 1487 ± 173 nM ($N = 5$).

DISCUSSION

Our results indicate the functional expression of at least two prostanoid receptors that modulate intracellular calcium in HEL cells. We will review the pharmacological characteristics of these receptors and their intracellular pathways.

Both iloprost and PGE_1 activate adenylate cyclase in HEL cells [3], but iloprost is several-fold more potent in increasing cAMP than PGE_1 (Table 1). This order of potencies is consistent with an IP receptor [17]. Iloprost had no effect on intracellular calcium at concentrations ranging from 10^{-9} to 10^{-6} M, but interacted synergistically with other agents that raised intracellular calcium, such as thrombin or calcium ionophore. These observations agree with the previous finding that the prostacyclin analog carbacyclin potentiates the increase in intracellular Ca^{2+} levels produced by thrombin [12].

The potentiation of intracellular calcium by iloprost is cholera toxin sensitive, indicating the involvement of G_s in this process. Also, iloprost potentiated intracellular Ca^{2+} and increased cAMP levels with similar potencies (Table 1). It could be postulated, therefore, that the potentiation of intracellular Ca^{2+} levels produced by iloprost is mediated by cAMP. However, 8-Br-cAMP, at concentrations known to activate protein kinase A in HEL cells, did not increase resting intracellular Ca^{2+} levels or potentiate the rise in intracellular Ca^{2+} levels induced by thrombin [3]. Similarly, it has been reported that dibutyryl cAMP does not increase resting intracellular Ca^{2+} levels or potentiate the rise in intracellular Ca^{2+} induced by iloprost or thrombin in HEL cells [5]. We conclude, therefore, that iloprost potentiates intracellular Ca^{2+} levels through a mechanism that is coupled to G_s, but is parallel and independent of cAMP.

This potentiation of intracellular Ca^{2+} by iloprost is not observed in the absence of extracellular Ca^{2+} and, therefore, is probably due to facilitation of Ca^{2+} influx. In support of this conclusion, iloprost (3 nM) did not potentiate the thrombin-induced formation of inositol phos-

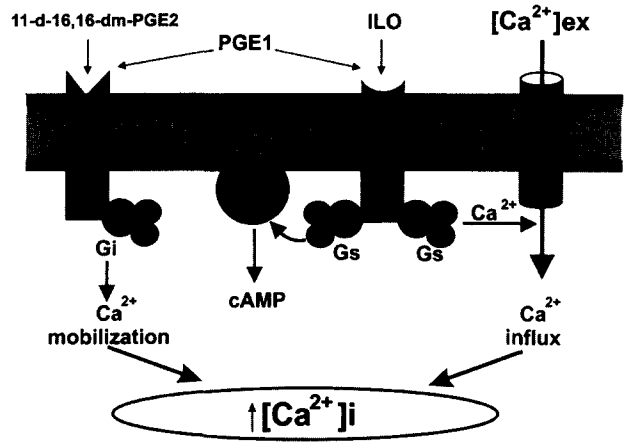

FIG. 8 Proposed scheme for prostanoid receptors coupled to intracellular Ca^{2+} in HEL cells. See text for details. Abbreviations: 11-d-16,16-dm PGE_2, 11-deoxy-16,16-dimethyl PGE_2; PGE_1, prostaglandin E_1; ILO, iloprost; IP, prostacyclin receptor; XP, putative prostanoid receptor; and AC, adenylate cyclase.

phates, as would be expected if iloprost potentiated calcium mobilization. This process is observed only if intracellular Ca^{2+} is increased simultaneously, whether this increase is mediated by receptor- (e.g. thrombin) or nonreceptor- (e.g. calcium ionophore) mechanisms. This suggests that iloprost-induced facilitation of calcium influx involves a calcium-dependent process. A similar phenomenon has been described for adenosine A_{2B} receptors in these cells [3], suggesting that this intracellular signaling pathway is common to receptors that are coupled to G_s in HEL cells.

Based on these results, we speculate that iloprost activates a receptor, most likely an IP receptor, that is coupled through G_s protein to a calcium channel, whose opening requires calcium (Fig. 8). Similar modulation of calcium channels, i.e. involving G_s proteins but unrelated to adenylate cyclase, has been documented with β-adrenergic receptors in cardiac myocytes [18] and skeletal muscle [19], and ATP receptors in cardiac myocytes [20]. Activation of this IP receptor by PGE_1 was evident in pertussis toxin-treated HEL cells. Under these conditions, PGE_1 mimicked the effects of iloprost, potentiating the increase in intracellular calcium induced by thrombin [3].

At high concentrations (from 1 to 10 μM) iloprost produced a significant and reversible increase in resting intracellular Ca^{2+}. This process probably involves calcium mobilization, as well as calcium influx, because 10 μM iloprost induced the formation of inositol phosphates (Fig. 7), and, on the other hand, the rise in intracellular calcium was reduced in the absence of extracellular calcium. The increase in intracellular calcium induced by a high concentration of iloprost was not mimicked by cholera toxin, and was pertussis toxin insensitive as reported previously [5]. We have not further explored the intracellular pathways that mediate this effect because of doubtful physiological significance given the high concentrations of iloprost required to elicit it.

Our results also suggest that PGE_1 activates a different receptor, not sensitive to iloprost, that increases basal intracellular calcium. This process is abolished in cells preincubated with pertussis toxin, as has been reported previously [3, 4]. This receptor, therefore, is probably coupled to a GTP-binding protein of the G_i family. This rise in intracellular calcium is probably due to calcium mobilization since it is observed even in cells incubated in a calcium-free medium, and this process does not induce influx of Mn^{2+}, a cation that shares the same influx mechanisms as calcium [15]. Furthermore, we have found that this receptor stimulates the formation of inositol phosphates, implicating a βγ-mediated activation of phospholipase C through dissociation of G_i proteins, one of the major pathways for calcium mobilization.

The nature of the prostanoid receptor that mediates this effect remains to be established. Of all the analogs tested, 11-deoxy-16,16-dimethyl PGE_2 activated this receptor with the greatest potency. This compound is marketed as a preferential EP_2 agonist. However, from a review of the literature it is evident that the selectivity of 11-deoxy-16,16-dimethyl PGE_2 has not been tested sufficiently, probably because this analog was developed before prostanoid receptors were adequately classified. Furthermore, other prostaglandins known to act on EP_2 receptors, such as PGE_2 and misoprostol, had no effect on intracellular calcium in HEL cells. The lack of efficacy of iloprost excludes the possibility that this effect is mediated by an IP receptor. Similarly, none of the prostaglandin analogs that specifically activate EP_1, EP_2, EP_3, EP_4, DP, FP or TP receptors showed any activity on this receptor (Table 2) [21]. This pharmacologic profile, therefore, does not correspond to any of the known prostanoid receptors. A prostanoid receptor, positively coupled to adenylate cyclase and with a unique pharmacological profile distinct from all four known subtypes of EP receptor, has been reported recently in Jurkat cells [22]. This receptor and the prostanoid receptor that we are describing in HEL cells have in common the lack of responsiveness to EP subtype-selective agonists, but they are different in their sensitivity to PGE_2. The prostanoid receptor in Jurkat cells is activated by nanomolar concentrations of PGE_2, whereas the receptor in HEL cells is not sensitive to PGE_2 in concentrations up to 1 μM. These receptors differ also in their coupling to G proteins. In Jurkat cells the prostanoid receptor stimulates adenylate cyclase, implying coupling to G_s protein, whereas in HEL cells we are describing a pertussis toxin-sensitive prostanoid receptor coupled to Ca^{2+} mobilization, presumably through G_i protein.

It should be noted that previous studies in HEL cells have described an increase in intracellular Ca^{2+} produced by PGE_2 [5], and TP receptor agonists U-46619 and I-BOP [23, 24]. We have not been able to reproduce these findings in the present study. One potential explanation for this discrepancy may be differences in the source of PGE_2. Throughout this study we have used eicosanoids obtained from the Cayman Chemical Co., whereas previous studies used PGE_2 from the Sigma Chemical Co. [5]. We, therefore, compared the effects of PGE_2 purchased from Cayman

TABLE 2. Receptor sensitivity of the prostaglandin analogs tested

Compound	IP	EP_1	EP_2	EP_3	EP_4	DP	FP	TP
Iloprost	+	+						
Cicaprost	+							
PGE_1	+	+	+	+	+			
PGE_2	+	+	+	+	+			
17-Phenyl-trinor PGE_2		+		+				
Sulprostone		+		+				
Misoprostol			+	+				
M&B 28767				+	+			
PGD_2						+		
$PGF_{2\alpha}$							+	
U-46619								+
8-epi $PGF_{2\alpha}$								+

The "+" signifies that the compound activates the receptor. The effects of these analogs on the levels of intracellular calcium in HEL cells are shown in Fig. 4. The selectivity of 11-deoxy-16,16-dimethyl PGE_2 has not been determined. Other analogs tested included PGA_2, PGB_2, 8-iso PGE_2, 9α,11β PGF_2, and TXB_2 at a concentration of 1 μM; 13,14-dehydro-15-keto PGD_2, $PGF_{2\beta}$, 11β $PGF_{2\alpha}$, 13,14-dehydro-15-keto $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, 2,3-dinor-6-keto $PGF_{1\alpha}$, 15-deoxy$\Delta^{12,14}$ PGJ_2, and 11-dehydro TXB_2 at a concentration of 10 μM; LTB_4, LTC_4, LTD_4, LTE_4, *N*-acetyl LTE_4, and LTF_4 at a concentration of 300 nM; 5 (*RS*)HETE, 11 (S)HETE, 12 (S)HETE, and 15 (S)HETE at concentrations from 3 to 20 μM. None of these compounds increased intracellular Ca^{2+} in HEL cells.

(catalog No. 14010, Lot 202926n) and from Sigma (catalog No. P4172, Lot 71H3795, minimum 98% TLC). We found that a high concentration of PGE_2 (1 μM) purchased from Sigma increased resting intracellular Ca^{2+} levels by 60 nM in HEL cells (average of two experiments), whereas 1 μM PGE_2 purchased from Cayman had no effect on intracellular Ca^{2+}. It is possible, therefore, that the putative effects of PGE_2 are due to impurities present in some preparations.

These apparent contradictory results can also be explained by methodological differences in the measurement of intracellular Ca^{2+} by the fura-2 technique. In previous studies, intracellular Ca^{2+} was measured by monitoring fura-2 fluorescence at only one excitation wavelength of 340 nm [5, 23, 24], whereas we monitored the ratio of fura-2 fluorescence at excitation wavelengths of 340 and 380 nm. This ratio provides a more accurate calculation of intracellular Ca^{2+} concentrations [10], and is less affected by optical properties of tested compounds that may induce non-specific increases in fluorescence. This indeed seems to explain the apparent increase in intracellular calcium produced by U-46619 and I-BOP. As seen in Fig. 5, the TP receptor agonists U-46619 and I-BOP increased fluorescence monitored at 340 nm. These findings replicate previous results [23, 24], but do not indicate a real increase in calcium concentrations, since the 340/380 ratio remained unchanged. We believe, therefore, that there is no real discrepancy between our findings and previously published results because the apparent differences can be explained by limitations in the methodology of calcium measurements used in previous studies. Our results do not imply that TP receptors are not present in HEL cells, but suggest that they are not functionally coupled to pathways regulating intracellular calcium. This conclusion is also supported by the lack of effect of the TP receptor antagonist SQ 29,548.

We speculate that results of this study provide functional evidence for a novel prostanoid receptor (designated "XP" in the scheme presented in Fig. 8). The potential relevance of this putative receptor remains to be determined. To our knowledge, no receptor with similar characteristics has been described in other cell systems. We have not been able to determine the endogenous ligand for this receptor. Of the naturally occurring prostaglandins tested, only PGE_1 was effective in activating this receptor, but with a relatively low potency. PGE_1 can be found in high concentrations, e.g. in seminal vesicles, but its physiological role in this circumstance is not known.

cDNA encoding for EP_1 receptors has been cloned recently from a library obtained from PMA-differentiated HEL cells [7]. This human EP_1 receptor increases intracellular Ca^{2+} when expressed in *Xenopus* oocytes, but the G protein and second messenger involved have not been characterized in this expression system. Signal transduction of EP_1 receptors is not well understood, but it appears to be mediated by a pertussis toxin- and cholera toxin-insensitive G protein, that is able to increase intracellular Ca^{2+} (for review see Refs. 25 and 26). The prostanoid receptor described in this paper stimulates mobilization of intracellular Ca^{2+} presumably by dissociation of G_i protein and activation of β-phospholipase C by βγ subunits, as would be expected for a pertussis toxin-sensitive increase in intracellular Ca^{2+} (for review see Ref. 27). Furthermore, sulprostone and PGE_2 activate EP_1 receptors with higher affinity than PGE_1 [7, 28]. These analogs in concentrations up to 1 μM had no effect on intracellular calcium in PMA-differentiated or undifferentiated HEL cells. Similarly, a cDNA encoding an EP_3 receptor has been cloned in a library obtained from DMSO-differentiated HEL cells [8]. This receptor inhibited forskolin-stimulated adenylate cyclase when expressed in COS-1 cells. However, none of the EP_3 selective agonists that we tested modulated intracellular calcium. We, therefore, found no evidence for the functional coupling of EP_1 or EP_3 receptors to intracellular calcium in HEL cells. It is possible that these receptors are expressed in HEL cells but are not coupled to intracellular calcium.

In summary, we report that prostaglandins modulate intracellular Ca^{2+} through two independent mechanisms

in HEL cells (Fig. 8). At low concentrations, iloprost evokes calcium influx through a cholera toxin-sensitive mechanism independent of cAMP. This effect requires the concomitant increase in intracellular Ca^{2+}, regardless of whether this increase is produced by a receptor-mediated (i.e. thrombin) or non-receptor-mediated (i.e. calcium ionophore) mechanism. We speculate that this effect is mediated by an IP receptor coupled to a calcium channel through G_s proteins. In addition, PGE_1 and 11-deoxy-16,16-dimethyl PGE_2 increase intracellular calcium with a pharmacologic profile that does not correspond to any of the known prostanoid receptors. This novel receptor is coupled to phosphoinositide-specific phospholipase C through a pertussis toxin-sensitive G_i protein and triggers mobilization of intracellular calcium.

The authors would like to thank Drs. Colin Funk and Alan Brash (Division of Clinical Pharmacology, Vanderbilt University) for useful discussions regarding the design of experiments and interpretation of results. Dr. Feoktistov is the recipient of an American Lung Association Research Grant Award, an Asthma and Allergy Foundation of America Investigator Grant Award, and a James A. Shannon Director's award (R55HL55596) from NIH. The work also was supported by NIH Grant RR00095.

References

1. Martin P and Papayannopoulou T, HEL cells: A new human erythroleukemia cell line with spontaneous and induced globin expression. *Science* **216:** 1233–1235, 1982.
2. Papayannopoulou T, Raines E, Collins S, Nakamoto B, Tweeddale M and Ross R, Constitutive and inducible secretion of platelet derived growth factor analogs by human leukemic cell lines coexpressing erythroid and megakaryocytic markers. *J Clin Invest* **79:** 859–866, 1987.
3. Feoktistov I, Murray JJ and Biaggioni I, Positive modulation of intracellular Ca^{2+} levels by adenosine A_{2b} receptors, prostacyclin, and prostaglandin E_1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Mol Pharmacol* **45:** 1160–1167, 1994.
4. Wu H, James-Kracke MR and Halenda SP, Direct relationship between intracellular calcium mobilization and phospholipase D activation in prostaglandin E-stimulated human erythroleukemia cells. *Biochemistry* **31:** 3370–3377, 1992.
5. Schwaner I, Seifert R and Schultz G, Receptor-mediated increases in cytosolic Ca^{2+} in the human erythroleukemia cell line involve pertussis toxin-sensitive and -insensitive pathways. *Biochem J* **281:** 301–307, 1992.
6. Murray R, Furci L and FitzGerald GA, Induction of prostacyclin receptor expression in human erythroleukemia cells. *FEBS Lett* **255:** 172–174, 1989.
7. Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M and Metters KM, Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. *J Biol Chem* **268:** 26767–26772, 1993.
8. Kunapuli AP, Fen Mao G, Bastepe M, Liu-Chen L, Li S, Cheung PP, DeRiel JK and Ashby B, Cloning and expression of a prostaglandin E receptor EP_3 subtype from human erythroleukaemia cells. *Biochem J* **298:** 263–267, 1994.
9. Gilman AG, A protein binding assay for adenosine 3′,5′-monophosphate. *Proc Natl Acad Sci USA* **67:** 305–312, 1970.
10. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
11. Feoktistov I and Biaggioni I, Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest* **96:** 1979–1986, 1995.
12. Turner JT, Camden JM, Kansra S, Shelton-James D, Wu H and Halenda SP, Potentiation of cyclic adenosine monophosphate production by thrombin in the human erythroleukemia cell line HEL. *J Pharmacol Exp Ther* **263:** 708–716, 1992.
13. Brass LF, Manning DR, Williams AG, Woolkalis MJ and Poncz M, Receptor and G protein-mediated responses to thrombin in HEL cells. *J Biol Chem* **266:** 958–965, 1991.
14. Lazarowski ER, Winegar DA, Nolan RD, Oberdisse E and Lapetina EG, Effect of protein kinase A on inositide metabolism and rap 1 G-protein in human erythroleukemia cells. *J Biol Chem* **265:** 13118–13123, 1990.
15. Merritt JE, Jacob R and Hallam TJ, Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J Biol Chem* **264:** 1522–1527, 1989.
16. Brass LF and Woolkalis MJ, Dual regulation of cyclic AMP formation by thrombin in HEL cells, a leukaemic cell line with megakaryocytic properties. *Biochem J* **281:** 73–80, 1992.
17. Namba T, Oida H, Sugimoto Y, Kakizuka A, Negishi M, Ichikawa A and Narumiya S, cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla. *J Biol Chem* **269:** 9986–9992, 1994.
18. Cavalié A, Allen TJA and Trautwein W, Role of the GTP-binding protein G_s in the β-adrenergic modulation of cardiac Ca channels. *Plfugers Arch* **419:** 433–443, 1991.
19. Hamilton SL, Codinas J, Hawkes MJ, Yatani A, Sawada T, Strickland FM, Froehner SC, Spiegel AM, Toro L, Stefani E, Birnbaumer L and Brown AM, Evidence for direct interaction of $G_{s\alpha}$ with the Ca^{2+} channel of skeletal muscle. *J Biol Chem* **266:** 19528–19535, 1991.
20. Scamps F, Rybin V, Puceat M, Tkachuk V and Vassort G, A G_s protein couples P_2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. *J Gen Physiol* **100:** 675–701, 1992.
21. Breyer RM and Breyer MD, Renal prostanoid receptors. In: *Molecular Biology of the Kidney in Health and Disease* (Eds. Schlondorf D and Bonventre J), pp. 201–213. Marcell Dekker, New York, 1995.
22. De Vries GW, Guarino P, McLaughlin A, Chen J, Andrews S and Woodward DF, An EP receptor with a novel pharmacological profile in the T-cell line Jurkat. *Br J Pharmacol* **115:** 1231–1234, 1995.
23. Mayeux PR, Mais DE, Carr C and Halushka PV, Human erythroleukemia cells express functional thromboxane A_2/prostaglandin H_2 receptors. *J Pharmacol Exp Ther* **250:** 923–927, 1989.
24. Matsuda K, Mathur RS, Duzic E and Halushka PV, Androgen regulation of thromboxane A_2/prostaglandin H_2 receptor expression in human erythroleukemia cells. *Am J Physiol* **265:** E928–E934, 1993.
25. Hébert RL, Cellular signalling of PGE_2 and its selective receptor analogue sulprostone in rabbit cortical collecting duct. *Prostaglandins Leukot Essent Fatty Acids* **51:** 147–155, 1994.
26. Negishi M, Sugimoto Y and Ichikawa A, Molecular mechanisms of diverse actions of prostanoid receptors. *Biochim Biophys Acta* **1259:** 109–120, 1995.
27. DeVivo M and Iyengar R, G protein pathways: Signal processing by effectors. *Mol Cell Endocrinol* **100:** 65–70, 1994.
28. Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S and Ichikawa A, Cloning and expression of cDNA for a mouse EP_1 subtype of prostaglandin E receptor. *J Biol Chem* **268:** 20175–20178, 1993.