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Receptor-mediated increases in cytosolic Ca^{2+} in the human erythroleukaemia cell line involve pertussis toxin-sensitive and -insensitive pathways

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The pluripotent human erythroleukaemia cell line, HEL, possesses erythrocytic, megakaryocytic and macrophage-like properties. With respect to signal transduction, HEL cells have been used as a model system for platelets, but little attention has been paid to their phagocytic properties. We studied the effects of various receptor agonists on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in HEL cells. Thrombin, platelet-activating factor (PAF), ATP, UTP, prostaglandins E_1 and E_2 (PGE_1 and PGE_2), the PGE_2 analogue sulprostone and the stable PGI_2 analogues iloprost and cicaprost increased $[Ca^{2+}]_i$. ADP was less effective than ATP, and UDP was unable to increase $[Ca^{2+}]_i$. The increases in $[Ca^{2+}]_i$ induced by thrombin, PAF, ATP, UTP, iloprost and cicaprost were pertussis toxin-insensitive, whereas the increases induced by PGE_2 and sulprostone were completely inhibited by the toxin. The increase in $[Ca^{2+}]_i$ induced by PGE_1 was partially inhibited by pertussis toxin. PGE_2 did not desensitize the increase in $[Ca^{2+}]_i$ induced by iloprost, and *vice versa*. PGE_1 desensitized the response to PGE_2 and iloprost but not *vice versa*. Adrenaline potentiated the iloprost- but not the PGE_2-induced rise in $[Ca^{2+}]_i$. The phorbol ester phorbol 12-myristate 13-acetate completely blocked the rise in $[Ca^{2+}]_i$ induced by ATP and PGE_1, whereas the increases induced by thrombin and PAF were only partially inhibited. Agonists increased $[Ca^{2+}]_i$ through release from internal stores and sustained Ca^{2+} influx. Thrombin stimulated Mn^{2+} influx, which was blocked by Ni^{2+}. Diltiazem, isradipine, gramicidin and 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365) did not affect agonist-induced rises in $[Ca^{2+}]_i$. HEL cells contained substantial amounts of β-glucuronidase which, however, could not be released, and they did not aggregate or generate superoxide. Our data suggest that: (1) HEL cells possess nucleotide receptors with properties similar to those of phagocytes; (2) they possess receptors for PGE_2 and PGI_2, and PGE_1 is an agonist at both receptors; (3) agonist-induced increases in $[Ca^{2+}]_i$ are mediated through pertussis toxin-sensitive as well as -insensitive signal transduction pathways; and (4) agonists increase $[Ca^{2+}]_i$ by mobilization from internal stores and influx from the extracellular space through cation channels with properties similar to those of phagocytes and platelets.

INTRODUCTION

The human erythroleukaemia cell line HEL was derived from a patient with Hodgkin's disease who developed erythroleukaemia in relapse [1]. HEL cells express markers for erythrocytes, megakaryocytes, myelomonocytes and B lymphocytes [1,2]. HEL cells are capable of spontaneous and induced globin synthesis [1]. Phorbol 12-myristate 13-acetate (PMA) and dimethyl sulphoxide induce additional macrophage-like and megakaryocytic properties in HEL cells [3–8]. These cells express various plasma membrane receptors, i.e. for α_2-adrenergic agonists, neuropeptide Y, prostaglandin I_2 (PGI_2), thromboxane A_2, thrombin, platelet-activating factor (PAF) and ADP [8–12].

HEL cells possess α-subunits of guanine-nucleotide-binding proteins (G-proteins) of the G_i family in relative abundances of $G_{i\alpha2} \geqslant G_{i\alpha3} \geqslant G_{i\alpha1}$ and the low-molecular-mass GTP-binding protein rap1 [13,14]. Adrenaline and neuropeptide Y mobilize Ca^{2+} from intracellular stores by pertussis toxin-sensitive mechanisms, suggesting the involvement of G_i-proteins in this process [10,15,16]. Thrombin stimulates phospholipase C-catalysed inositol phosphate generation and inhibits adenylate cyclase in a pertussis toxin-sensitive manner [17]. The stable PGI_2 analogue iloprost stimulates adenylate cyclase and inhibits the thrombin- and PAF-induced generations of inositol phosphates [8,14]. Thrombin stimulates an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through a combination of intracellular Ca^{2+} mobilization and Ca^{2+} influx [17]. In HEL cells, adrenaline, thrombin, PAF and ADP activate phospholipase D, which may be mediated by a rise in $[Ca^{2+}]_i$ [12].

With respect to signal transduction, HEL cells have been used as a model system for platelets, but little attention has been paid to their phagocytic properties [7,8,11–17]. This prompted us to study the effects of a broad variety of receptor agonists on $[Ca^{2+}]_i$ in HEL cells. We report here that ATP, UTP, PAF, thrombin and E- and I-type prostaglandins increase $[Ca^{2+}]_i$ through mobilization of intracellular stores and stimulate Ca^{2+} influx from the extracellular space. The effects of E-type prostaglandins, but not the other agonists studied, involve pertussis toxin-sensitive signal transduction pathways.

MATERIALS AND METHODS

Materials

1-{β-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SK&F 96365) was a gift from Dr. D. Arndts (Boehringer Ingelheim, Ingelheim, Germany); sulpro-

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Ca^{2+}_e, extracellular Ca^{2+}; EC_{50}, concentration causing 50% of maximal stimulation; G-protein, guanine-nucleotide-binding protein; G_i, G-protein that inhibits adenylate cyclase; G_s, G-protein that stimulates adenylate cyclase; HEL cells, human erythroleukaemia cells; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); PGE_1, PGE_2, E-type prostaglandins; PGI_2, prostaglandin I_2 (prostacyclin); U46619, 9,12-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; SK&F 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride; PMA, phorbol 12-myristate 13-acetate.

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stone, iloprost and cicaprost (ZK 96 480) were gifts from Dr. E. Schillinger and Dr. K.-H. Thierauch (Schering, Berlin, Germany); pertussis toxin was a gift from Dr. M. Yajima (Kyoto, Japan). PGE_1, PGE_2, PGD_2 and $PGF_{2\alpha}$, U46619, thrombin, collagen and adrenaline were obtained from Sigma Chemie (Deisenhofen, Germany). Sources of other materials have been described elsewhere [18,19].

Cells and cell culture

HEL cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in RPMI 1640 medium containing fetal calf serum (10%, v/v), non-essential amino acids (1%, v/v), L-glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were maintained at a density of (0.5–1) $\times 10^6$ cells/ml. In some experiments, pertussis toxin (500 ng/ml) or its vehicle (control) were added to the culture medium, and the cells were incubated for additional 24 h. Under these conditions, the toxin completely ADP-ribosylates pertussis toxin substrates in HEL cells [17]. Pertussis toxin did not affect cell viability, as assessed by Trypan Blue exclusion (results not shown).

Measurements of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed according to the protocol described recently for HL-60 cells with modifications [19]. Briefly, HEL cells were centrifuged (250 *g*, 10 min) and then resuspended in buffer containing NaCl (138 mM), KCl (6 mM), $MgSO_4$ (1 mM), Na_2HPO_4 (1 mM), $NaHCO_3$ (5 mM), glucose (5.5 mM), Hepes/NaOH (20 mM), pH 7.4, and BSA (0.1%, w/v) at 10^7 cells/ml. Fura-2/acetoxymethyl ester was added to a final concentration of 4 μM, and cells were incubated for 10 min at 37 °C, protected from light. Thereafter, cells were diluted with the same buffer to 5×10^6 cells/ml and were further incubated for 45 min at 37 °C. Subsequently, cells were diluted 10-fold with the above buffer, then centrifuged (250 *g*, 10 min) and finally resuspended in fresh buffer at a concentration of 10^6 cells/ml and stored at room temperature for up to 1 h. During this period, experiments were performed. HEL cells (10^6 cells) were suspended in 2 ml of the above buffer in the absence or the presence of various compounds and were incubated for 2 min at 37 °C. Basal fluorescence was measured for 1 min prior to the addition of stimuli. Experiments were performed under constant stirring (1000 rev./min) using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.) at excitation and emission wavelengths of 340 nm and 500 nm respectively. Quenching of fura-2 fluorescence by Mn^{2+} was performed as described [20]. Unless stated otherwise, all experiments were performed in the presence of 1 mM-$CaCl_2$. Basal $[Ca^{2+}]_i$ in HEL cells ranged from 120 to 230 nM (results not shown).

Assay of β-glucuronidase release

HEL cells [(3–4) $\times 10^6$ cells/tube] were suspended in 500 μl of buffer containing NaCl (138 mM), KCl (6 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM), glucose (5.5 mM) and Hepes/NaOH (20 mM), pH 7.4, in the presence or absence of cytochalasin B (5 μg/ml) [19]. After preincubation of the HEL cells for 5 min at 37 °C, stimuli were added. After 10 min of incubation, reactions were terminated by placing the tubes on to crushed ice. Reaction mixtures were centrifuged at 1000 *g* for 10 min at 4 °C, and the activities of β-glucuronidase and lactate dehydrogenase in the supernatant fluids and cell lysates were determined as described [21]. The release of lactate dehydrogenase amounted to < 5.0% of total cellular content, indicating that none of the agonists studied caused cell damage (results not shown).

Continuous assay for superoxide formation

HEL cells [(2.5 or 5) $\times 10^6$ cells/cuvette] were suspended in 500 μl of the buffer used for determination of β-glucuronidase release, supplemented with ferricytochrome *c* (100 μM) with or without cytochalasin B (5 μg/ml) [18]. After preincubation of the HEL cells for 3 min at 37 °C, stimuli were added and the superoxide dismutase-inhibitable reduction of ferricytochrome *c* was measured continuously at 550 nm for 10 min using an Uvikon 810 spectrophotometer (Kontron, Eching, Germany).

Aggregation assay

Aggregation of HEL cells was measured by turbidometry as described recently, with modifications [18]. Briefly, 0.5×10^7 or 1×10^7 cells were suspended in 1 ml of the buffer used for determination of β-glucuronidase release. After incubation for 5 min at 37 °C in the presence of cytochalasin B (5 μg/ml) and subsequent addition of the stimulus, aggregation was measured under constant stirring (1000 rev./min) using an Uvikon 810 spectrophotometer.

Calculations and statistics

Calculations and curve plotting of the data shown in Figs. 1–4 were performed using GraphPAD v. 3.00, and non-linear regression analysis was used to fit the curves and to calculate the EC_{50} values (concns. causing 50% of maximal stimulation). Data shown in Figs. 1–4 are the means of assay triplicates. The S.D. values of the experiments were generally < 10% of the means. Similar results were obtained in at least three experiments performed with different batches of HEL cells. Data shown in Figs. 5–9 are representative of results obtained in at least three independent experiments with different batches of HEL cells.

RESULTS

First, the effects of various purine and pyrimidine nucleotides on $[Ca^{2+}]_i$ were studied. Both ATP and UTP increased $[Ca^{2+}]_i$ in a concentration-dependent manner, with EC_{50} values of about 2 μM and a plateau at 10–100 μM (Fig. 1). Pertussis toxin did not affect the rises in $[Ca^{2+}]_i$ induced by ATP and UTP. Adenosine 5′-[γ-thio]triphosphate and ADP (10 μM each) increased $[Ca^{2+}]_i$ with an effectiveness amounting to 65% and 45% respectively

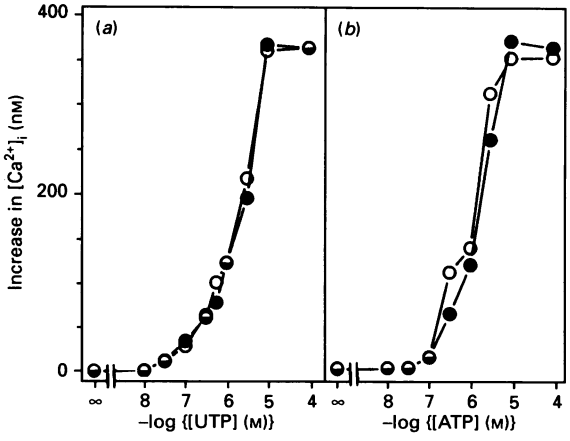

Fig. 1. Concentration–response curves of (*a*) UTP- and (*b*) ATP-induced increases in $[Ca^{2+}]_i$ in HEL cells

HEL cells were treated with pertussis toxin (○, 500 ng/ml) or its vehicle (●, control) for 24 h. Thereafter cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by UTP and ATP at various concentrations were assessed.

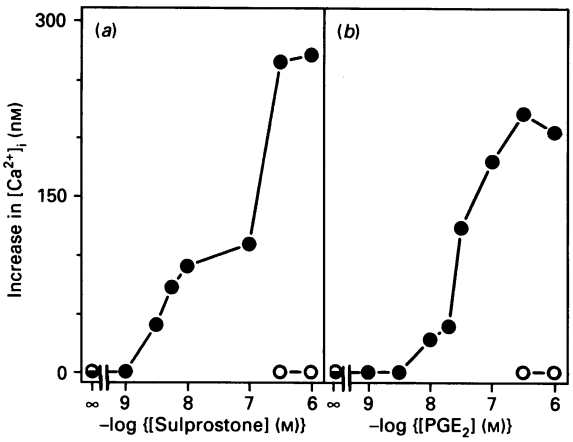

Fig. 2. Concentration–response curves of (*a*) sulprostone- and (*b*) PGE_2-induced increases in $[Ca^{2+}]_i$ in HEL cells

HEL cells were treated with pertussis toxin (○, 500 ng/ml) or its vehicle (●, control) for 24 h. Thereafter cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by sulprostone and PGE_2 at various concentrations were assessed.

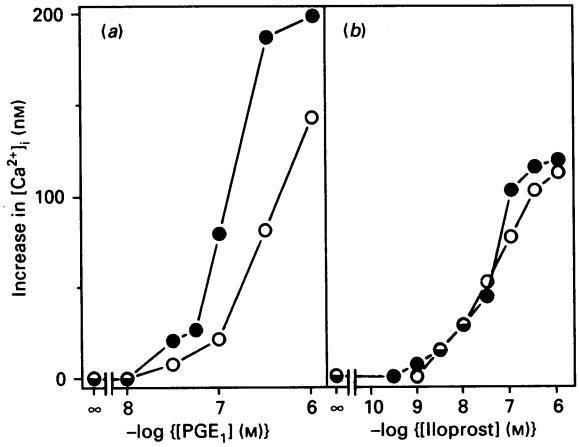

Fig. 3. Concentration–response curves of (*a*) PGE_1- and (*b*) iloprost-induced increases in $[Ca^{2+}]_i$ in HEL cells

HEL cells were treated with pertussis toxin (○, 500 ng/ml) or its vehicle (●, control) for 24 h. Thereafter cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by PGE_1 and iloprost at various concentrations were assessed.

of that of ATP at an equimolar concentration. Adenosine, AMP, adenosine 5′-[β-thio]diphosphate, adenosine 5′-[α,β-methylene]triphosphate, adenosine 5′-[β,γ-methylene]triphosphate, adenosine 5′-[β,γ-imido]triphosphate, guanosine, GDP, guanosine 5′-[β-thio]diphosphate, GTP, guanosine 5′-[γ-thio]-triphosphate, ITP, UMP, uridylyl(3′-5′)uridine, UDP, CMP, CDP and CTP up to 100 μM did not increase $[Ca^{2+}]_i$ (results not shown).

The prostaglandins PGE_1 and PGE_2, the PGE_2 analogue sulprostone and the stable PGI_2 analogues iloprost and cicaprost all increased $[Ca^{2+}]_i$ in a concentration-dependent manner [22–24]. The EC_{50} for PGE_2 was 30 nM, and its effect reached a maximum at 300 nM (Fig. 2). Sulprostone increased $[Ca^{2+}]_i$ with an EC_{50} of 15 nM and a maximum at 1 μM (see Fig. 2). Pertussis toxin abolished the rises in $[Ca^{2+}]_i$ induced by PGE_2 and sulprostone. The EC_{50} value for PGE_1 was 120 nM, and the effect was maximal at 1 μM (Fig. 3). Pertussis toxin partially inhibited the rise in $[Ca^{2+}]_i$ induced by PGE_1 at submaximally and

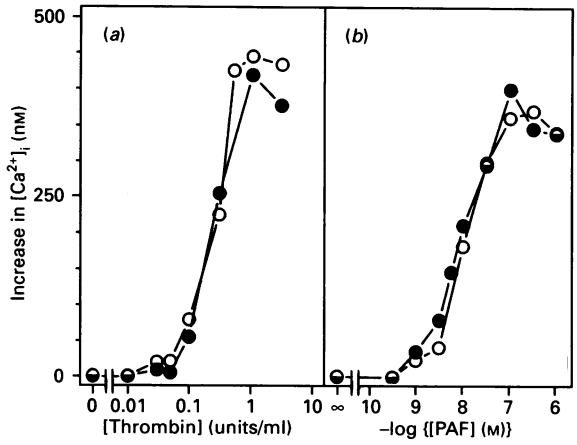

Fig. 4. Concentration–response curves of (*a*) thrombin- and (*b*) PAF-induced increases in $[Ca^{2+}]_i$ in HEL cells

HEL cells were treated with pertussis toxin (○, 500 ng/ml) or its vehicle (●, control) for 24 h. Thereafter cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by thrombin and PAF at various concentrations were assessed.

maximally effective concentrations, and shifted the concentration–response curve to the right (see Fig. 3). Iloprost increased $[Ca^{2+}]_i$ with an EC_{50} of 40 nM and a maximally effective concentration of 1 μM (see Fig. 3). The concentration–response curve to cicaprost (results not shown) was virtually identical to that of iloprost. Pertussis toxin did not affect the rise in $[Ca^{2+}]_i$ stimulated by iloprost (see Fig. 3) and cicaprost (results not shown). Prostaglandins D_2 and $F_{2\alpha}$ (1 and 10 μM each) did not increase $[Ca^{2+}]_i$ (results not shown).

Thrombin increased $[Ca^{2+}]_i$ with an EC_{50} of 0.3 unit/ml and a maximum at 1 unit/ml (Fig. 4). PAF stimulated an increase in $[Ca^{2+}]_i$ with an EC_{50} of 6 nM and a maximum at 100 nM (Fig. 4). Thrombin- and PAF-induced rises in $[Ca^{2+}]_i$ were not affected by pertussis toxin. The rank order of effectiveness of receptor agonists at maximally effective concentrations in increasing $[Ca^{2+}]_i$ was thrombin = PAF > UTP = ATP > sulprostone > PGE_2 = PGE_1 > iloprost = cicaprost. Collagen (2 μg/ml) did not increase $[Ca^{2+}]_i$ (results not shown).

Fig. 5 shows time courses of the increases in $[Ca^{2+}]_i$ induced by various agonists at maximally effective concentrations in the presence or the absence of extracellular Ca^{2+} ($Ca^{2+}{}_e$). In the presence of $Ca^{2+}{}_e$, ATP caused a rapid increase in $[Ca^{2+}]_i$ to a plateau which was maintained for at least 15 min. In the absence of $Ca^{2+}{}_e$, the rise in $[Ca^{2+}]_i$ was smaller, and $[Ca^{2+}]_i$ declined to the baseline within 2 min. The time courses of the rises in $[Ca^{2+}]_i$ induced by UTP (results not shown) were almost identical to the ones induced by ATP. In the presence of $Ca^{2+}{}_e$, PGE_1 caused a rapid rise in $[Ca^{2+}]_i$ to a peak which declined slowly. In the absence of $Ca^{2+}{}_e$, the magnitude of the rise in $[Ca^{2+}]_i$ induced by PGE_1 was smaller, and $[Ca^{2+}]_i$ returned to the baseline value within 1–2 min. Similar time courses as for PGE_1 were observed when HEL cells were stimulated with the other prostaglandins (Fig. 6). In the presence of $Ca^{2+}{}_e$, thrombin and PAF induced rapid increases in $[Ca^{2+}]_i$ which declined to the baseline within 6 min. In the absence of $Ca^{2+}{}_e$, both agonists induced less prominent increases in $[Ca^{2+}]_i$, and resting values were reached within 3 min.

The homologous and heterologous desensitization patterns of the increases in $[Ca^{2+}]_i$ induced by various prostaglandins (1 μM each) were studied. When HEL cells were stimulated with PGE_1, PGE_2, sulprostone, iloprost or cicaprost, re-addition of the same agonist 3 min later did not result in a second rise in $[Ca^{2+}]_i$

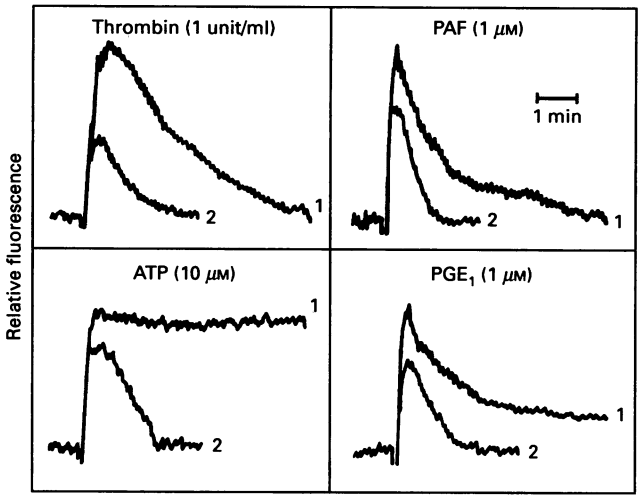

Fig. 5. Time courses of increases in $[Ca^{2+}]_i$ in HEL cells induced by thrombin, PAF, ATP and PGE_1 in the presence and the absence of Ca^{2+}_e

Cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by various agonists at the indicated concentrations were assessed. Trace 1, experiments performed in the presence of Ca^{2+}_e (1 mM); trace 2, experiments performed in the presence of EGTA (1 mM). Superimposed original tracings are shown.

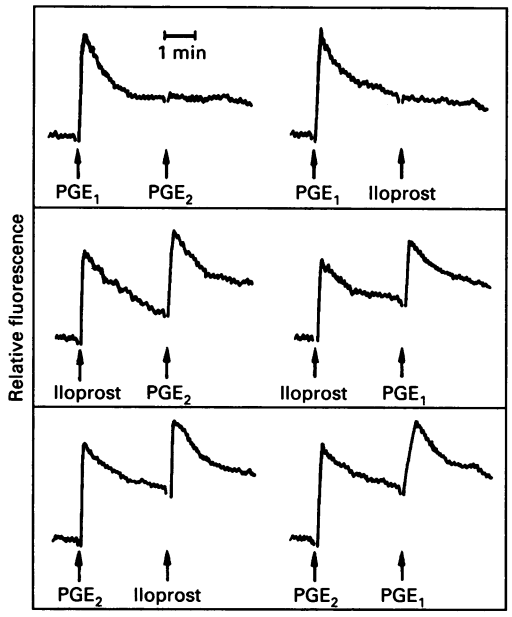

Fig. 6. Heterologous desensitization of prostaglandin-induced increases in $[Ca^{2+}]_i$ in HEL cells

Cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by prostaglandins were assessed. Arrows indicate the addition of prostaglandins (1 μM each). The second stimulus was added 3 min after the first stimulus. Original tracings are shown.

(results not shown). PGE_1 desensitized the response to PGE_2 and iloprost (Fig. 6). In contrast, neither iloprost nor PGE_2 desensitized the response to PGE_1. PGE_2 did not desensitize the increases in $[Ca^{2+}]_i$ induced by iloprost and *vice versa*. The desensitization patterns of sulprostone and cicaprost (results not shown) were identical to those of PGE_2 and iloprost respectively.

In human platelets, adrenaline enhances the increases in $[Ca^{2+}]_i$ induced by agonists such as the thromboxane A_2 analogue

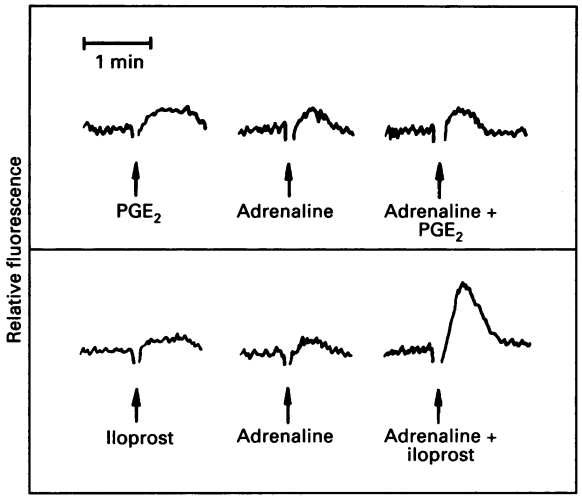

Fig. 7. Time courses of increases in $[Ca^{2+}]_i$ induced in HEL cells by adrenaline, PGE_2, iloprost and adrenaline plus PGE_2 or iloprost

Cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by the agonists were assessed. Arrows indicate the addition of stimuli (10 mM each). Original tracings are shown.

U46619, thrombin and ADP [25]. In HEL cells, we examined the effect of adrenaline at a threshold concentration (10 nM) on the increase in $[Ca^{2+}]_i$ induced by various agonists at threshold concentrations. Simultaneous addition of adrenaline and iloprost (10 nM) resulted in a markedly higher increase in $[Ca^{2+}]_i$ than that induced by either agonist alone (Fig. 7). Adrenaline did not potentiate the increase in $[Ca^{2+}]_i$ induced by PGE_2 (10 nM) (Fig. 7). When added together with PGE_1 (30 nM), PAF (5 nM), thrombin (0.01 unit/ml), the thromboxane A_2 analogue U46619 (10 nM), ATP, ADP or UTP (100 nM each), adrenaline failed to enhance the increase in $[Ca^{2+}]_i$ induced by these agonists (results not shown).

The effects of an activator of protein kinase C, PMA, on increases in $[Ca^{2+}]_i$ induced by agonists at maximally effective concentrations were studied. PMA (100 nM) completely inhibited the responses to ATP and PGE_1 (Fig. 8), and diminished the increases in $[Ca^{2+}]_i$ induced by thrombin and PAF. The increases in $[Ca^{2+}]_i$ induced by UTP, PGE_2, sulprostone and iloprost were also completely inhibited by PMA (results not shown). The differential effects of PMA suggest that protein kinase C inhibits receptor-mediated rises in $[Ca^{2+}]_i$ by interfering with early steps of the signal transduction cascade, e.g. at the level of plasma membrane receptors.

The cell-permeant analogue of cyclic AMP, dibutyryl cyclic AMP (1 mM), did not in itself increase $[Ca^{2+}]_i$, and did not affect the rises in $[Ca^{2+}]_i$ induced by ATP, UTP, PGE_2, sulprostone, PGE_1, iloprost, PAF or thrombin at submaximally and maximally stimulatory concentrations (results not shown).

SK&F 96365 was recently shown to inhibit receptor-mediated Ca^{2+} influx in human platelets and neutrophils [26]. In HEL cells, SK&F 96365 (10, 30 and 100 μM) had no effect on the rise in $[Ca^{2+}]_i$ induced by thrombin (1 unit/ml) (results not shown). The effect of membrane depolarization induced by gramicidins S and D on rises in $[Ca^{2+}]_i$ was examined. Neither gramicidin S (100 nM) nor gramicidin D (1 μM) had any effect on resting $[Ca^{2+}]_i$ or on the increase in $[Ca^{2+}]_i$ stimulated by thrombin (1 unit/ml) (result not shown). Additionally, the organic blockers of voltage-gated Ca^{2+} channels, diltiazem (10 μM) and isradipine (1 μM), did not affect the thrombin-induced rise in $[Ca^{2+}]_i$ (results not shown). The effects of the inorganic blockers of cation channels, La^{2+} and Ni^{2+}, on agonist-induced rises in $[Ca^{2+}]_i$ were

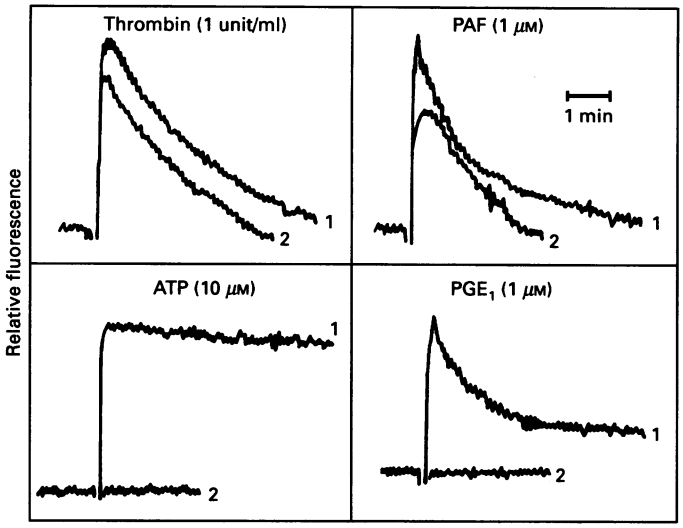

Fig. 8. Time courses of increases in $[Ca^{2+}]_i$ induced in HEL cells by thrombin, PAF, ATP and PGE1: effect of PMA

Cells were harvested and loaded with fura-2, and the increases in $[Ca^{2+}]_i$ induced by various agonists at the indicated concentrations were assessed. Trace 1, experiments performed in the absence of PMA; trace 2, experiments performed in the presence of PMA (100 nM). PMA was added to cells 3 min prior to stimuli. Superimposed original tracings are shown.

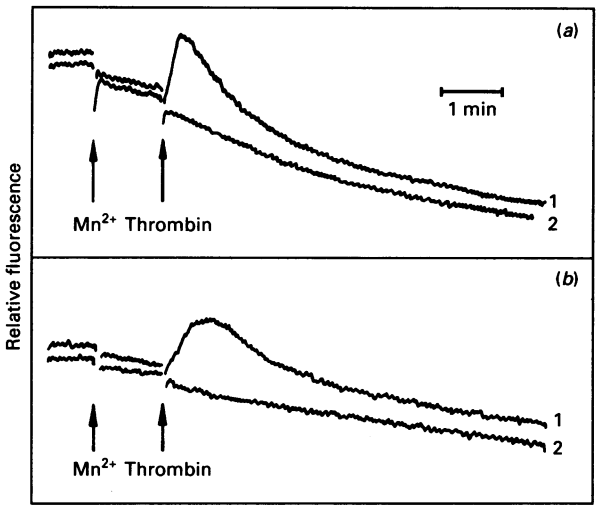

Fig. 9. Effect of Mn^{2+} on fura-2 fluorescence of thrombin-stimulated HEL cells

Cells were harvested and loaded with fura-2, and fura-2 fluorescence was monitored at an excitation wavelength of 340 nm (trace 1) or 360 nm (trace 2). The emission wavelength was 500 nm. Experiments were performed in the absence of Ca^{2+}_e. The arrows indicate the addition of $MnCl_2$ (100 μM) and thrombin (1 unit/ml). (*a*) Experiments in the absence of $NiCl_2$, (*b*) experiments in the presence of $NiCl_2$ (5 mM). $NiCl_2$ was added to cells 2 min prior to $MnCl_2$. Superimposed original tracings are shown.

examined. $LaCl_2$ (1 mM) did not affect resting or thrombin-induced increases in $[Ca^{2+}]_i$ (results not shown). By contrast, $NiCl_2$ (5 mM) decreased the effect of thrombin (1 unit/ml) to that seen in the absence of Ca^{2+}_e (see Fig. 5) (results not shown).

In order to answer the question of whether thrombin stimulated influx of bivalent cations, quenching of fura-2 fluorescence by Mn^{2+} was studied. At an excitation wavelength of 340 nm, fluorescence is increased by Ca^{2+} and decreased by Mn^{2+}. At an excitation wavelength of 360 nm, fluorescence is again quenched by Mn^{2+}, but is insensitive to Ca^{2+} [20]. At both excitation wavelengths, Mn^{2+} induced a slow decrease in fluorescence, indicating basal Mn^{2+} influx (Fig. 9). At an excitation wavelength of 340 nm, thrombin (1 unit/ml) transiently increased fluorescence, reflecting release of Ca^{2+} from internal stores. At an excitation wavelength of 360 nm, thrombin substantially increased fluorescence quenching. Ni^{2+} blocked the latter process, but not the transient increase in fluorescence at 340 nm (Fig. 9).

In order to assess the functional role of agonist-induced rises in $[Ca^{2+}]_i$, β-glucuronidase release, superoxide formation and cell aggregation were measured. The total activity of β-glucuronidase in HEL cells amounted to 0.07 ± 0.01 nmol·min^{-1}·10^6 cells^{-1} ($n = 3$), a value comparable with that of human neutrophils [21]. Thrombin, ATP, UTP and PGE_1, employed at concentrations that were maximally or supramaximally effective in increasing $[Ca^{2+}]_i$, did not activate any of the above-mentioned functions. Additionally, PMA (100 nM) did not stimulate superoxide formation or cell aggregation in HEL cells (results not shown).

DISCUSSION

We studied the effects of various nucleotides on $[Ca^{2+}]_i$ in HEL cells. The order of effectiveness of adenine nucleotides, at maximally effective concentrations, in increasing $[Ca^{2+}]_i$ (ATP > adenosine 5′-[γ-thio]triphosphate > ADP) suggests that their effects are mediated via $P_{2\gamma}$-like purinoceptors. This assumption is supported by the finding that adenosine, AMP, adenosine 5′-[α,β-methylene]triphosphate and adenosine 5′-[β,γ-methylene]-triphosphate did not increase $[Ca^{2+}]_i$ in these cells [27,28]. UTP was found to be equipotent and as effective as ATP in increasing $[Ca^{2+}]_i$ in HEL cells (Fig. 1). In the pluripotent promyelocytic human cell line HL-60, the relative effectiveness of nucleotides in increasing $[Ca^{2+}]_i$ is similar to that in HEL cells [29]. In undifferentiated and differentiated HL-60 cells, pertussis toxin partially inhibits the increase in $[Ca^{2+}]_i$ induced by ATP, adenosine 5′-[γ-thio]triphosphate and UTP [19,30]. These findings suggest the involvement of both pertussis toxin-sensitive and -insensitive G-proteins in signal transduction pathways activated by nucleotides in HL-60 cells. In HEL cells, pertussis toxin had no effect on either ATP- or UTP-induced rises in $[Ca^{2+}]_i$ (Fig. 1). Similar to its effect in HL-60 cells, PMA completely inhibited nucleotide-induced rises in $[Ca^{2+}]_i$ in HEL cells (See Fig. 8) [30]. In HL-60 cells, stimulatory effects of UTP were suggested to be mediated through pyrimidinoceptors [31]. The fact that ADP increased $[Ca^{2+}]_i$ in HEL cells, whereas UDP did not, suggests that in these cells UTP also acts through pyrimidinoceptors. The order of effectiveness of nucleotides in activating HEL cells is dissimilar to that in platelets. In the latter cells, ATP and UTP are competitive antagonists for ADP, and ADP is the most effective nucleotide for platelet activation [27]. Also dissimilar to platelets, adrenaline did not potentiate the increase in $[Ca^{2+}]_i$ induced by ADP in HEL cells [25]. All these data suggest that HEL cells possess phagocyte-like nucleotide receptors coupled to pertussis toxin-insensitive G-proteins.

It is well known that receptors for PGI_2 mediate activation of adenylate cyclase, with a subsequent increase in cyclic AMP, in various cell types including platelets, vascular smooth muscle cells, mastocytoma cells and HEL cells [8,24,32–34]. Most unexpectedly, we found that iloprost and cicaprost increased $[Ca^{2+}]_i$, as did PGE_1, PGE_2 and sulprostone (Figs. 2 and 3). However, iloprost does not only act as agonist at PGI_2 receptors, but also at PGE_2 receptors [23,24]. Our data indicate that the E_2-

type prostaglandins on the one hand and the PGI_2 analogues on the other increased $[Ca^{2+}]_i$ through different receptors. The increases in $[Ca^{2+}]_i$ induced by PGE_2 and sulprostone were completely inhibited by pertussis toxin, whereas the toxin did not affect the increases in $[Ca^{2+}]_i$ induced by iloprost and cicaprost. The former finding suggests involvement of G_i-proteins, and the latter finding suggests that the PGI_2 receptor couples to a G-protein that is different from G_i. Additionally, the E_2-type prostaglandins did not desensitize the response to iloprost or cicaprost, and *vice versa* (see Fig. 6). Moreover, adrenaline enhanced the increases in $[Ca^{2+}]_i$ stimulated by iloprost but not by PGE_2 (Fig. 7). Furthermore, cicaprost is devoid of agonist activity at PGE_2 receptors [23,24]. To our knowledge, stimulatory effects of PGI_2 analogues on $[Ca^{2+}]_i$ have not been observed. Whether the effects of iloprost and cicaprost in HEL cells are attributable to the presence of a PGI_2 receptor subtype or to tumour-cell-associated aberrations in the interactions of receptors with G-proteins and/or effector systems remains to be determined. It will also be of interest to study the effects of PGI_2 analogues on $[Ca^{2+}]_i$ in normal haemopoietic progenitor cells.

In contrast to PGE_2 and the PGI_2 analogues, which apparently increased $[Ca^{2+}]_i$ through different receptors, PGE_1 mimicked the effects of both of these agonists. This suggests that PGE_1 activates both of the above receptors. This notion is supported by the following findings. The increase in $[Ca^{2+}]_i$ induced by PGE_1 was partially inhibited by pertussis toxin (Fig. 3). This suggests involvement of different G-proteins, one of them being G_i. Additionally, PGE_1 desensitized the response to PGE_2 and iloprost or cicaprost, but there was no reciprocal effect. By analogy, PGE_1-induced vasodilation also involves stimulation of PGE_2 and PGI_2 receptors [35].

In human platelets, various prostaglandins such as PGE_1 and PGI_2 cause a G_s-protein-mediated stimulation of adenylate cyclase [32]. This process is assumed to play a role in inhibition of platelet activation, including receptor-mediated rises in $[Ca^{2+}]_i$ [32]. In agreement with the data obtained for platelets, iloprost inhibits thrombin- and PAF-induced inositol phosphate generation [14]. Apparently, the stimulatory effects of prostaglandins on $[Ca^{2+}]_i$ in HEL cells are not mediated through an increase in cyclic AMP, as dibutyryl cyclic AMP failed to mimic their effects. Additionally, the prostaglandin-mediated increases in $[Ca^{2+}]_i$ were not affected by a rise in cyclic AMP. Thus stimulatory effects of prostaglandins in HEL cells on cyclic AMP on the one hand and on $[Ca^{2+}]_i$ on the other may be independently regulated. Similar to the situation in HEL cells, PGE_2 was reported to increase $[Ca^{2+}]_i$ in HL-60 cells, and this effect was not mimicked by a cell-permeant analogue of cyclic AMP [36]. Unlike in HEL cells, the effect of PGE_2 in HL-60 cells was pertussis toxin-insensitive, and the effects of PGI_2 analogues were not investigated [36].

Among all the agonists studied, thrombin and PAF were the most effective. In contrast to inositol phosphate generation, the thrombin-induced increase in $[Ca^{2+}]_i$ was pertussis toxin-insensitive (Fig. 4) [17]. Intriguingly, pertussis toxin completely blocked the thrombin-induced inhibition of adenylate cyclase and greatly decreased but did not abolish thrombin-stimulated inositol phosphate generation [17]. Thus it is possible that the residual inositol phosphate generation was sufficient to increase $[Ca^{2+}]_i$.

In HEL cells, thrombin, PAF, ATP, UTP and the prostaglandins increased $[Ca^{2+}]_i$ through both mobilization from internal stores and sustained Ca^{2+} influx (Fig. 5). We studied the effects of various drugs on the agonist-induced increases in $[Ca^{2+}]_i$ to evaluate the properties of the channel which mediated the Ca^{2+} influx. The lack of effect of diltiazem and isradipine on rises in $[Ca^{2+}]_i$ argues against the presence of voltage-gated Ca^{2+} channels in HEL cells, and this finding is in agreement with earlier published results on platelets and neutrophils [37,38]. Additionally, gramicidin S and D did not affect the agonist-induced rise in $[Ca^{2+}]_i$ in HEL cells. In platelets, gramicidin D inhibits thrombin-induced Ca^{2+} influx, whereas in neutrophils gramicidin D does not affect a formyl-peptide-induced increase in $[Ca^{2+}]_i$ [38,39]. The finding that SK&F 96365 did not affect the increase in $[Ca^{2+}]_i$ in HEL cells does not argue against the presence of receptor-stimulated cation channels in these cells. SK&F 96365 may discriminate between different types of receptor-stimulated cation channels. In platelets and neutrophils, SK&F 96365 blocks Ca^{2+} influx, but it is ineffective in blocking ATP-linked Ca^{2+} channels in rabbit artery smooth muscle and in the human neurosecretory cell line PC12 [26,40]. Similar to neutrophils, HL-60 cells and platelets, there is receptor-mediated Mn^{2+} influx in HEL cells which is blocked by Ni^{2+} (Fig. 8) [20,36,38,41–43]. These data show that HEL cells possess receptor-stimulated cation channels with properties similar but not identical to those in other myelocytic cells.

An increase in $[Ca^{2+}]_i$ is known to play a role in the regulation of numerous cell functions [32,44]. In HEL cells, we found a dissociation between agonist-induced rises in $[Ca^{2+}]_i$ on the one hand and β-glucuronidase release, superoxide formation and cell aggregation on the other hand. Interestingly, the specific β-glucuronidase activity in HEL cells was found to be similar to that in human neutrophils [21], but in contrast to the situation with these latter cells, β-glucuronidase could not be released by agonists. In undifferentiated and differentiated HL-60 cells, ATP and UTP increase $[Ca^{2+}]_i$, but only in the differentiated cells do they induce β-glucuronidase release [19]. HEL cells did not generate superoxide upon stimulation, similar to the situation with undifferentiated HL-60 cells [19]. In platelets and neutrophils, increases in $[Ca^{2+}]_i$ may be prerequisites for receptor-mediated aggregation [32,45]. In HEL cells, thromboxane A_2 induced a shape change but no aggregation [11]. The dissociations between rises in $[Ca^{2+}]_i$ and lack of functional activation in HEL cells suggest that proximal signal transduction components are present in these cells, whereas components of the distal signal transduction pathway and/or effector proteins are missing. It is conceivable that these distal components are expressed during the later stages of megakaryocytic and myelocytic differentiation. Possibly the PGE_2- and ATP-induced increases in $[Ca^{2+}]_i$ are early signals for the differentiation of HEL cells, as in HL-60 cells these agonists promote myeloid differentiation [46,47]. Regardless of the functional role, the differences in the time courses and amplitude of rises in $[Ca^{2+}]_i$ induced by various agonists in HEL cells indicate that they may be non-equivalent.

In conclusion, HEL cells possess a number of receptors which mediate increases in $[Ca^{2+}]_i$ through pertussis toxin-sensitive and -insensitive pathways. Receptor agonists increase $[Ca^{2+}]_i$ by mobilization from internal stores and influx from the extracellular space through receptor-stimulated cation channels with properties similar to those of phagocytes and platelets. Thus HEL cells may be a valuable system for investigating thrombocytic and phagocytic aspects of signal transduction and differentiation.

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After submission of this paper, Wu *et al.* [48] reported that PGE_1 and PGE_2 activated phospholipase D in HEL cells in a pertussis-toxin-sensitive manner.

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