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Differential Regulation of Thrombin- or ATP-induced Mobilization of Intracellular Ca^{2+} by Prostacyclin Receptor in Mouse Mastocytoma Cells

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Thrombin induced an increase in $[Ca^{2+}]i$ in mouse mastocytoma P-815 cells. This increase was markedly reduced by prior exposure to pertussis toxin (PT) but not by removal of extracellular Ca^{2+}, suggesting that thrombin stimulates phospholipase C via a PT-sensitive GTP-binding protein. ATP also induced an increase in $[Ca^{2+}]i$. This increase was insensitive to PT but completely suppressed on removal of extracellular Ca^{2+}, suggesting that ATP stimulates Ca^{2+} influx in a PT-insensitive manner. Iloprost, a stable prostacyclin analogue, increased the cellular cAMP level and dose-dependently inhibited the thrombin-induced increase in $[Ca^{2+}]i$, whereas the ATP-induced increase in $[Ca^{2+}]i$ was markedly enhanced by iloprost. Cyclic AMP analogues, dibutyryl cAMP and 8-bromo cAMP, also inhibited the increase in $[Ca^{2+}]i$ induced by thrombin and promoted that by ATP, indicating that the inhibitory and stimulatory effects of iloprost are mediated by cAMP. These results suggest that the prostacyclin receptor differentially regulates two distinct Ca^{2+} mobilizing systems via cAMP in mastocytoma cells.

Mast cells have as their main functions, the secretion of histamine and other mediators of allergic reaction in response to stimulation of their IgE receptors (1), and an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]i$) caused by IgE appears to be the primary trigger for initiation of exocytosis. Recently, there has been considerable interest in the secretory responses to stimulation of non-IgE cell surface receptors by such molecules as thrombin, ATP, bradykinin and formylmethionylleucylphenylalanine (2), and these stimulus-secretion mechanisms may also involve intracellular Ca^{2+} mobilization. Among various stimulants, thrombin or ATP is one of the very potent stimulators for histamine release from mast cells (3,4). Whereas thrombin evokes a rapid and transient release within 1 min (3), the secretory response elicited by ATP gradually increases over a 30-min incubation period (4), suggesting that there might be multiple mechanisms for histamine release from mast cells.

In addition to Ca^{2+} mobilization, the adenylate cyclase system is an important signaling pathway in mast cells (5). Increasing cAMP inhibits IgE-triggered histamine

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Abbreviations: PGs, prostaglandins; $[Ca^{2+}]i$, cytosolic free Ca^{2+}; PT, pertussis toxin; G-protein, GTP-binding protein; IP_3, inositol trisphosphate.

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release from mast cells by inhibiting the increase in $[Ca^{2+}]i$ (6). From its inhibitory action, cAMP has been thought to play an important role as a negative feedback regulator for histamine release. However, cross-talk between non-IgE receptor-mediated signal pathways and adenylate cyclase systems remains to be found in mast cells. We previously demonstrated that mouse mastocytoma P-815 cells, neoplastic mast cells, strongly respond to prostacyclin (PGI_2) among various PGs by an elevation in the intracellular cAMP levels (7), and that the cells have a highly specific PGI_2 receptor that is functionally associated with the adenylate cyclase system via a stimulatory GTP-binding protein (G-protein) (8). Thus, we examined the effect of iloprost, a chemically stable PGI_2 analogue, on thrombin- or ATP-induced intracellular Ca^{2+} mobilization. We report here that iloprost inhibits the increase in $[Ca^{2+}]i$ induced by thrombin, whereas it potentiates that induced by ATP.

MATERIALS AND METHODS

Mastocytoma P-815 cells were maintained in the ascitic form in BDF1 mice and harvested from the ascitic fluid of inoculated mice as described previously (8). The cells were washed with a HEPES-buffered saline solution containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES (pH 7.4), and the reactions were started by the addition of the test agent, unless otherwise indicated.

For measurement of $[Ca^{2+}]i$, cells suspended at a density of 10^7 cells/ml were loaded with 3 μM fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37°C. The cells were then washed twice with HEPES-buffered saline containing 0.5% bovine serum albumin (Sigma) and kept at 4°C in the same solution until assayed. After centrifugation, the cells (2×10^6 cells) were resuspended in HEPES-buffered saline without bovine serum albumin, and then the fluorescence intensity was measured at an excitation wavelength of 340 nm or 380 nm and an emission wavelength of 510 nm with a fluorescence spectrophotometer (Jasco, CAF-100), as described previously (9).

For measurement of $[^3H]IP_3$ formation, cells suspended at a density of 10^7 cells/ml were labeled with 2 μCi/ml of [^{3}H]inositol (Amersham Corp.) for 2 h at 37°C. The cells were then washed three times with HEPES-buffered saline and preincubated in the buffer containing 10 mM LiCl for 10 min at 37°C. The reaction was started by the addition of the test agents. For termination of the reaction, 10% trichloroacetic acid was added to the cell suspension. Separation of $[^3H]IP_3$ was carried out by Bio-Rad AG-1X8 chromatography, essentially as described by Berridge *et al.* (10).

The cyclic AMP content in the cells was determined as described previously (11).

RESULTS AND DISCUSSION

Thrombin induced a rapid increase in $[Ca^{2+}]i$ from 100 to 700 nM within a few seconds and then the level decreased quickly, the basal level being regained within 1 min (Fig. 1A). Whereas the extent of the maximal increase in $[Ca^{2+}]i$ induced by thrombin was not significantly affected by removal of extracellular Ca^{2+}, it was almost completely abolished by prior exposure to pertussis toxin (PT) at 100 ng/ml for 3 h, suggesting that thrombin stimulates phospholipase C via a PT-sensitive G-protein. Pretreatment for 5 min with 1 μM iloprost and 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) strongly prevented the thrombin-induced increase in $[Ca^{2+}]i$. ATP also induced an increase in $[Ca^{2+}]i$ from 100 to 150 nM, but then the level decreased gradually, the basal level being regained within 3 min (Fig. 1B). The extent of the maximal increase in $[Ca^{2+}]i$ induced by ATP was not significantly affected by prior exposure to PT, but it was almost completely abolished by

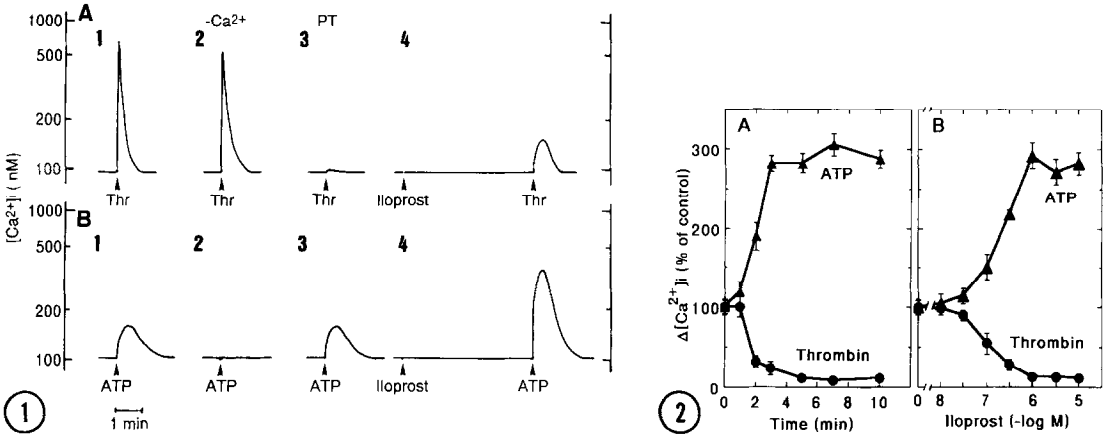

Fig. 1. Effects of iloprost on the thrombin- and ATP-induced increases in $[Ca^{2+}]i$. Fura-2-loaded cells (2 x 10^6 cells) were stimulated with 0.5 U/ml thrombin (A) or 100 μM ATP (B) in buffer (1) or in Ca^{2+}-free buffer containing 0.3 mM EGTA (2). Fura-2-loaded cells exposed to 100 ng/ml PT for 3 h were stimulated with thrombin or ATP (3). After fura-2-loaded cells had been preincubated for 5 min at 37°C with 1 μM iloprost and 0.5 mM IBMX, the cells were stimulated with thrombin or ATP (4). Thrombin or ATP was added at the time indicated by the arrow. The recordings shown are a representative of three independent experiments that yielded similar results.

Fig. 2. Time courses and dose dependencies of the effects of iloprost on the thrombin- and ATP-induced increase in $[Ca^{2+}]i$.
A, Fura-2-loaded cells (2 x 10^6 cells) were incubated for the indicated times at 37°C with 1 μM iloprost and 0.5 mM IBMX. B, Fura-2-loaded cells were incubated for 5 min at 37°C with the indicated concentrations of iloprost and 0.5 mM IBMX. The cells stimulated by 0.5 U/ml thrombin (●) and 100 μM ATP (▲), and the peak $[Ca^{2+}]i$ levels induced by these stimulants were determined as described under "Materials and Methods". The values shown represent percentages of the respective controls and are means ± S. E. for triplicate experiments. The values of the increase in $[Ca^{2+}]i$ in controls were 631 ± 29 nM for thrombin and 41.6 ± 4.1 nM ATP respectively.

removal of extracellular Ca^{2+}, suggesting that the ATP-induced increase in $[Ca^{2+}]i$ is ascribable to the entry of extracellular Ca^{2+}. The P_2 purinergic receptor agonist, 2-methylthio-ATP, also induced an increase in $[Ca^{2+}]i$ (data not shown), suggesting that ATP-induced Ca^{2+} mobilization is mediated by a P_2 purinergic receptor. In sharp contrast, iloprost in the presence of IBMX markedly potentiated the ATP-induced increase in $[Ca^{2+}]i$. Pretreatment with IBMX alone had no influence on the increase in $[Ca^{2+}]i$ induced by either thrombin or ATP (data not shown). Fig. 2 shows the time courses and dose dependencies of the effects of iloprost on the thrombin- and ATP-induced Ca^{2+} mobilization. Inhibition of the thrombin-induced increase in $[Ca^{2+}]i$ was observed within 2 min and was complete by 5 min after the addition of 1 μM iloprost (Fig. 2A). Potentiation of the ATP-induced increase in $[Ca^{2+}]i$ by 1 μM iloprost showed the almost same time course as the case of thrombin. The thrombin-induced increase in $[Ca^{2+}]i$ was prevented by pretreatment for 5 min with iloprost in a dose-dependent manner, the half-maximal concentration for the inhibition being 100 nM (Fig. 2B). Dose-dependency of the potentiation of ATP-induced increase in $[Ca^{2+}]i$ by iloprost is similar to that of the inhibition of thrombin action.

Table I
Effects of various PGs, cAMP analogues and TPA on the thrombin- and ATP-induced increase in $[Ca^{2+}]i$ and the cellular cAMP level

Addition	Δ$[Ca^{2+}]i$		cAMP
	Thrombin	ATP	
	% of control		pmol/10^6 cells
1 μM Iloprost	12.9 ± 4.5	292 ± 16	61.5 ± 2.8
1 μM PGE_1	30.3 ± 6.7	220 ± 16	34.5 ± 3.1
1 μM PGE_2	60.0 ± 6.3	187 ± 21	23.5 ± 5.6
1 μM $PGF_{2\alpha}$	83.1 ± 8.2	125 ± 13	4.5 ± 2.1
1 μM PGD_2	95.0 ± 3.7	112 ± 10	3.8 ± 1.8
1 mM DbcAMP	32.9 ± 8.2	194 ± 19	
1 mM 8Br-cAMP	38.0 ± 10	205 ± 17	

After fura-2-loaded cells (2 x 10^6 cells) had been preincubated for 5 min at 37°C with or without the indicated agents in the presence of 0.5 mM IBMX, the cells were stimulated with 0.5 U/ml thrombin or 100 μM ATP. The peak $[Ca^{2+}]i$ levels induced by these stimulants were determined as described under "Materials and Methods". The values shown represent percentages of the respective controls and are means ± S. E. for triplicate experiments. For measurement of cAMP, cells (1 x 10^6 cells) were incubated for 5 min at 37°C with various PGs in the presence of 0.5 mM IBMX, and then the cAMP levels were determined as described under "Materials and Methods". The values of the increase in $[Ca^{2+}]i$ in controls were 623 ± 61 nM for thrombin and 42.5 ± 3.5 nM for ATP respectively.

We next compared the effects of various PGs on both thrombin- and ATP-induced Ca^{2+} mobilization. As shown in Table I, iloprost was the most efficient in inhibiting the thrombin-induced increase in $[Ca^{2+}]i$ and in potentiating the ATP-induced increase in $[Ca^{2+}]i$. PGE_1 and PGE_2 showed weaker effects, in that order, but $PGF_{2\alpha}$ and PGD_2 were much less potent. These effects were well correlated with the ability of each PG to produce an increase in the cAMP level. Furthermore, the cAMP analogues, dibutyryl cAMP and 8-bromo cAMP, also inhibited the thrombin-induced increase in $[Ca^{2+}]i$ and potentiated the ATP-induced increase in $[Ca^{2+}]i$. These results taken together demonstrate that the inhibitory and stimulatory effects of iloprost were mediated by cAMP.

As the stimulation of phosphoinositide metabolism has been shown to be followed by Ca^{2+} mobilization from the endoplasmic reticulum via IP_3, we examined the effect of iloprost on phosphoinositide metabolism enhanced by various stimulants. As shown in Table II, thrombin and ATP stimulated the accumulation of $[^3H]IP_3$. Whereas the accumulation of $[^3H]IP_3$ induced by thrombin was not affected by removal of extracellular Ca^{2+}, that by ATP was absolutely dependent on extracellular Ca^{2+} (data not shown). Pretreatment for 5 min with iloprost and IBMX markedly prevented the accumulation of $[^3H]IP_3$ induced by thrombin, but enhanced that induced by ATP. NaF plus $AlCl_3$ has been demonstrated to induce inositol phosphate formation through a direct activation of the G-protein for its coupling to phospholipase C (12). Furthermore, it has been demonstrated

Table II
Effect of iloprost on the thrombin, ATP, NaF + $AlCl_3$ and ionomycin-induced accumulation of IP_3

Addition	$[^3H]IP_3$ (% of control)	
	None	+Iloprost and IBMX
0.5 U/ml Thrombin	237 ± 8.5	131 ± 25
100 μM ATP	205 ± 30	289 ± 25
20 mM NaF + 10 μM $AlCl_3$	220 ± 4.1	221 ± 5.9
1 μM Ionomycin	235 ± 7.8	233 ± 14

After $[^3H]$inositol-labeled cells (2 x 10^6 cells) had been preincubated for 5 min at 37°C with or without 1 μM iloprost and 0.5 mM IBMX, the cells were further incubated with 0.5 U/ml thrombin or 100 μM ATP for 30 sec, or with 20 mM NaF plus $AlCl_3$ or 1 μM ionomycin for 5 min. $[^3H]IP_3$ formed was determined as described under "Materials and Methods". The values shown represent percentages of the control and are means ± S. E. for triplicate experiments. The radioactivity of the control (n = 3) was 73.3 ± 2.9 dpm/10^6 cells for IP_3.

that an increase in $[Ca^{2+}]i$ could directly activate phospholipase C (13). In mastocytoma cells, the addition of 20 mM NaF plus $AlCl_3$ or the Ca^{2+} ionophore, ionomycin (1 μM), was also found to induce the formation of $[^3H]IP_3$. However, the formation induced by NaF plus $AlCl_3$ and by ionomycin was not affected by pretreatment with iloprost, indicating that iloprost did not affect the phospholipase C activity and the ability of the G-protein to activate phospholipase C. Judging from the thrombin-induced accumulation of IP_3 and PT-sensitive intracellular Ca^{2+} mobilization, thrombin may stimulate phospholipase C via activation of a PT-sensitive G-protein, leading to the accumulation of IP_3 and subsequent mobilization of Ca^{2+} from intracellular stores. Because phosphoinositide metabolism stimulated by ionomycin and NaF plus $AlCl_3$ was not affected by iloprost (Table II), the inhibitory effect of iloprost is toward a component(s) specific to the thrombin-induced phosphoinositide metabolism, and not toward phospholipase C itself or coupling of the activated G-protein to phospholipase C. It is known that cAMP reduces the agonist-induced production of IP_3 and other responses in a variety of cells (14,15). In platelets, cAMP inhibits the sequence of events leading to activation of platelets promoted by thrombin, and this inhibition is thought to be due to phosphorylation by cAMP-dependent protein kinase of specific proteins which participate in thrombin-induced activation of phospholipase C. Several substrates for cAMP-dependent protein kinase have been reported in human platelets (16) and human erythroleukemia cells (17), but the exact mechanism underlying this inhibition is not well understood.

In contrast, ATP may trigger Ca^{2+} entry through a receptor-gated ion channel and in turn activate Ca^{2+}-sensitive phospholipase C, considering that the ATP-induced increase in $[Ca^{2+}]i$ and accumulation of IP_3 are absolutely dependent on extracellular Ca^{2+}, and that ionomycin induces the accumulation of IP_3. The site of action of iloprost appears to be a

component(s) specific for the ATP-induced Ca^{2+} influx system, but the mechanism by which activation of the prostacyclin receptor results in sensitization of ATP-induced Ca^{2+} mobilization remains unclear.

Thrombin and ATP are thought to induce histamine release through different Ca^{2+} mobilization systems. Since the inhibitory and stimulatory effects of iloprost are specific for the receptor-mediated Ca^{2+} mobilization, the regulation of receptor-mediated Ca^{2+}-mobilizing signals by the prostacyclin receptor is entirely dependent on which receptor or Ca^{2+} mobilizing system is being stimulated.

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