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MAITOTOXIN-ELICITED CALCIUM INFLUX IN CULTURED CELLS

EFFECT OF CALCIUM-CHANNEL BLOCKERS

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Abstract—Maitotoxin elicited a marked influx of $^{45}Ca^{2+}$ into NIH 3T3 fibroblast cells. The influx was blocked by imidazoles (econazole, miconazole, SKF 96365, clotrimazole, calmidazolium) with IC_{50} values from 0.56 to 3 μM. Phenylalkylamines (verapamil, methoxyverapamil) and nitrendipine were less potent, and diltiazem was very weak. Among other calcium blockers, the diphenylbutylpiperidines fluspirilene and penfluridol, the diphenylpropylpiperidine loperamide, and the local anesthetic proadifen were quite active with IC_{50} values of 2–4 μM. The pattern of inhibition of maitotoxin-elicited calcium influx did not correspond to the ability of the agents to block elevation of calcium that ensues through calcium-release activated calcium (CRAC) channels after activation of phosphoinositide breakdown by ATP in HL-60 cells. The imidazoles did block CRAC channels, but fluspirilene, penfluridol, loperamide and proadifen were ineffective. Loperamide actually appeared to enhance influx of calcium via the activated CRAC channels. The imidazoles, in particular calmidazolium, caused an apparent influx of calcium and caused a stimulation of phosphoinositide breakdown in HL-60 cells.

Key words: maitotoxin; calcium channels

The structure of maitotoxin, a high molecular weight polycyclic ether isolated from a marine dinoflagellate, was elucidated recently [1]. Maitotoxin has been studied extensively as an activator of calcium influx [2–4]. However, the mechanism(s) whereby this potent toxin elicits calcium influx and phosphoinositide breakdown remains controversial. Clearly, maitotoxin does cause activation of calcium channels in all cells that have been investigated, while having no effect on calcium permeability of lipid bilayers [5, 6]. The initial event now appears likely to be an activation by maitotoxin in a calcium-dependent manner of a voltage-independent nonselective calcium channel [3]. However, it remains possible that maitotoxin also directly activates a so-called "receptor-operated" or CRAC channel‡ [3, 4]. Indeed, maitotoxin-elicited calcium influx is effectively blocked in glioma and insulinoma cells [4] by the imidazole SKF 96365, an inhibitor of CRAC channels, while being partially blocked by SKF 96365 in Mardin-Darby kidney cells [3]. Inhibition of maitotoxin-elicited calcium influx by several imidazoles and a wide structural range of calcium channel blockers has now been examined in NIH 3T3 fibroblast cells and compared with the ability of these blockers to reduce CRAC channel-mediated increases in intracellular calcium levels in HL-60 cells after activation of phosphoinositide breakdown by ATP.

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‡ Abbreviation: CRAC channels, calcium-release activated calcium channels; IP_1, inositol 1-monophosphate; and IP_3, inositol 1,4,5-trisphosphate.

MATERIALS AND METHODS

Materials

Maitotoxin was isolated from *Gambierdiscus toxicus* as described [7] and provided by Dr. T. Yasumoto (Tohoku University, Sendai, Japan). Culture media and sera were obtained from GIBCO (Grand Island, NY). The [^{3}H]inositol (12–24 Ci/mmol) and $^{45}CaCl_2$ (18 Ci/g) were from New England Nuclear (Boston, MA). Fluo-3-AM was from Molecular Probes (Eugene, OR). SKF 96365 was provided by Dr. J. Merritt (Smith Kline & French Research Ltd., Welwyn, England) and Dr. T. J. Torphy (Smith Kline Beecham Pharmaceuticals (King of Prussia, PA), penfluridol by Dr. J. J. Enyeart (Ohio State University, Columbus, OH) and diphenoxylate from Dr. K. Rice (NIH, Bethesda, MD). Other compounds are from standard commercial sources.

Cell culture

NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotics. Cells were grown to confluence and split every 6–9 days. HL-60 cells were grown to suspension in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% calf serum and antibiotics. Cells were differentiated in medium containing 500 μM dibutyryl cyclic AMP for 48 hr before experiments. For phosphoinositide breakdown experiments, [^{3}H]inositol (10 μCi/mL) was present during the final 48 hr.

Influx of $^{45}Ca^{2+}$

NIH 3T3 fibroblast cells were incubated for 1 day in 1 mL of the culture medium containing 0.1 μCi/mL of [^{3}H]leucine in 12-well plates. The cell density after a day reached 1–2 × 10^6 cells/well. The culture medium was removed, and preincubation buffer A, consisting of 150

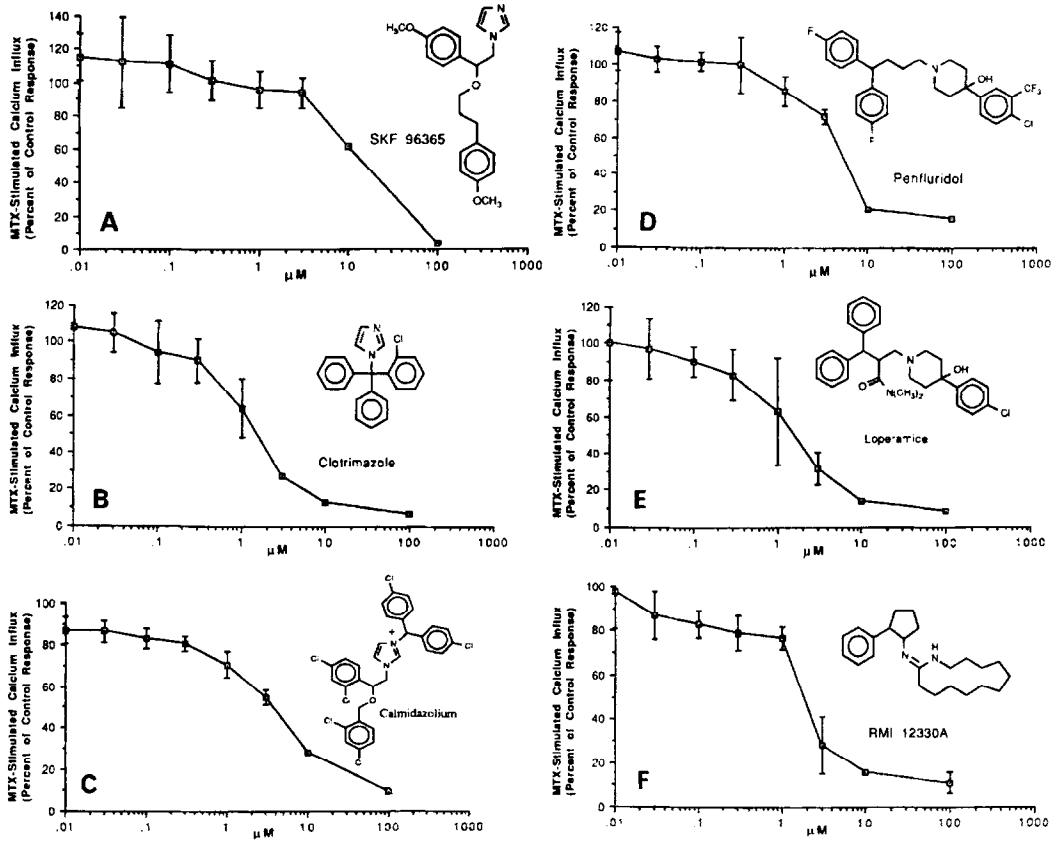

Fig. 1. Inhibition of maitotoxin-elicited influx of $^{45}Ca^{2+}$ by calcium channel blockers in NIH 3T3 cells. Cells were incubated with maitotoxin (2 ng/mL) and various concentrations of (A) SKF 96365, (B) clotrimazole, (C) calmidazolium, (D) penfluridol, (E) loperamide, and (F) RMI 12330A. Uptake of $^{45}Ca^{2+}$ was determined as described in Materials and Methods. Values are means ± SEM (N = 3). The basal value for uptake of $^{45}Ca^{2+}$ was 200 cpm/5 × 10^5 cells. Maitotoxin-stimulated values were about 8000 cpm/5 × 10^5 cells.

mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES (pH 7.4 adjusted by Tris), was added. After 12 min of preincubation, buffer A was replaced with an influx buffer consisting of buffer A containing $^{45}CaCl_2$ (1.5 μCi/mL). Maitotoxin was added to the influx buffer in 1–3 μL/well of DMSO. The other agents were dissolved in the influx buffer. Incubation time was 15 min at 22°. After removal of the influx buffer by aspiration, the cells were washed three times with buffer A. Then the cells were solubilized with 0.25 mL of 1% sodium dodecyl sulfate in 0.5 N NaOH. The solution was transferred to a scintillation vial, followed by neutralization with 0.5 N HCl, and mixing with 8 mL Hydrofluor. Radioactivity was counted with $^3H/^{14}C$ windows. The amount of incorporated [^{3}H]leucine was used to normalize cell numbers in each well.

Intracellular calcium levels

The cells were suspended in a 1:1 mixture of RPMI medium and Krebs-Ringer buffer at about 10^6 cells/mL. Fluo 3-AM (10 μg/10 μL DMSO) was added, and cells were left in the dark for 40 min. The cells were centrifuged and resuspended in Krebs-Ringer buffer. For each experiment, 2 mL of cell suspension was added to cuvettes with magnetic stir bar, and fluorescence was followed with a Spex fluorescence spectrophotometer. Maitotoxin or other agents were added in 1–20 μL. Fluorescence was monitored at 506 nm (excitation) and 526 nm (emission) as described [8].

Phosphoinositide breakdown

Differentiated [^{3}H]inositol-labeled HL-60 cells were washed with buffer B (108 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSo_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose and 20 mM HEPES, pH 7.4) containing 10 mM LiCl. After resuspending the cells in buffer B at a density of 2–3 × 10^6 cells/mL, aliquots of cells (1 × 10^6 cells/tube) were incubated at 37° for 10 min, and then agents were added. Incubations were carried for 5 min and were stopped by adding 12% trichloroacetic acid. After centrifugation at 12,000 *g* for 5 min, the upper phase was transferred to anion exchange columns (Bio-Rad AG 1X8, 100–200 mesh formate form). Separation and elution of [^{3}H]inositol phosphates were performed as described [9].

Data analysis

The IC_{50} values were obtained from concentration-response curves by computer analysis using the GraphPAD computer program (Graph PAD Software Inc., San

Diego, CA). Statistical evaluations were performed with Student's *t*-test.

RESULTS

The influx of $^{45}Ca^{2+}$ elicited by maitotoxin was inhibited in a concentration-dependent manner by a wide range of calcium channel blockers. Representative inhibition curves are shown in Fig. 1. The IC_{50} values were estimated from such curves, using computer analysis. It should be noted that in many cases the curves did not appear monophasic. The IC_{50} values are tabulated in Table 1. None of the agents caused significant influx in $^{45}Ca^{2+}$ in 3T3 cells when tested alone.

The maitotoxin-elicited increase in intracellular calcium, as assessed from the fluo-3 signal in HL-60 cells, and the effects of several structural classes of blockers are shown in Figs. 2 and 3. In the absence of external calcium (EGTA present), maitotoxin did not increase intracellular levels of calcium in HL-60 cells (data not shown). The imidazoles econazole, miconazole, and calmidazolium at 30 μM all caused a marked increase in intracellular calcium alone (Fig. 2A, B and E), but also largely prevented the maitotoxin-elicited increases in intracellular calcium. SKF 96365 and clotrimazole, in contrast, had minimal effects on intracellular calcium, but did block the maitotoxin response (Fig. 2C and D). Methoxyverapamil (D-600) at 30 μM partially inhibited the maitotoxin-elicited increase in intracellular calcium (Fig. 3A). Nitrendipine at 30 μM caused a slight inhibi-

Table 1. Effects of calcium channel blockers on maitotoxin (MTX)-elicited calcium influx and on MTX- and CRAC channel-elicited increases in intracellular calcium

Agent	MTX-elicited $^{45}Ca^{2+}$ influx in 3T3 Cells* IC_{50} (μM)	Fluo-3 fluorescence in HL-60 cells: Effect alone†	MTX-elicited signal‡ (Tested at 30 μM)	CRAC-channel signal§
Imidazoles				
Econazole	2.7 ± 0.2	↑↑	Blocks	Inhibits
Miconazole	2.2 ± 0.3	↑	Blocks	Inhibits
SKF 96365	1.8 ± 0.2	None	Blocks	Inhibits
Clotrimazole	0.56 ± 0.12	↑	Blocks	Inhibits
Calmidazolium	2.3 ± 0.4	↑↑↑	Blocks	Indeterminate
Phenylalkylamines				
R-Verapamil	21 ± 3	ND‖	ND	ND
S-Verapamil	25 ± 7	ND	ND	ND
Methoxyverapamil (D-600)	16 ± 5	None	Inhibits	No effect
Dihydropyridines				
Nitrendipine	21 ± 5	None	Inhibits	No effect
Benzothiazepines				
Diltiazem	105 ± 23	None	No effect	No effect
Diphenylbutylpiperidines				
Fluspirilene	3.9 ± 0.1	↑	Blocks	No effect
Pimozide	16 ± 4	None	Blocks	No effect
Penfluridol	3.2 ± 0.7	↑↑↑	Blocks	No effect
Diphenylpropylpiperidines				
Loperamide	1.6 ± 0.2	None	Blocks	↑↑
Diphenoxylate	2.9 ± 0.9	None	Inhibits	No effect
Piperazines				
Cinnarizine	13 ± 1	None	Inhbits	No effect
Flunarizine	12 ± 2	ND	ND	ND
Other blockers				
WB4101	22 ± 5	None	Inhibits	Inhibits
W-7	7.2 ± 0.6	None	ND	No effect
Bepridil	6.7 ± 0.6	↑	Blocks	Inhibits
Proadifen (SKF 525a)	3.9 ± 0.3	None	Blocks	No effect
RMI 12330A	1.8 ± 0.2	None	Blocks	No effect
Benzamil	7.4 ± 0.5	None	Inhibits	No effect
Quinidine	12 ± 3	None	Inhibits	No effect
Trifluoperazine	4.6 ± 0.4	None	Blocks	No effect

* Inhibition of maitotoxin-elicited $^{45}Ca^{2+}$ influx was measured in NIH 3T3 cells as described in Materials and Methods. The IC_{50} values are means ± SEM (N = 3).

† Effect of agent at 30 μM on fluo-3 fluorescence was determined in HL-60 cells as described in Materials and Methods. Arrows indicate an increase, with the magnitude of the increase as follows ↑↑↑ > ↑↑ > ↑.

‡ Effect of agent at 30 μM on maitotoxin-elicited increase in fluo-3 fluorescence was determined in HL-60 cells as described in Materials and Methods.

§ Effect of agent at 30 μM on CRAC channel-dependent increase in fluo-3 fluorescence after ATP stimulation in HL-60 cells was determined as described in Materials and Methods. Arrows indicate an increase in the CRAC-channel-dependent increase in fluo-3 fluorescence.

‖ ND = not determined.

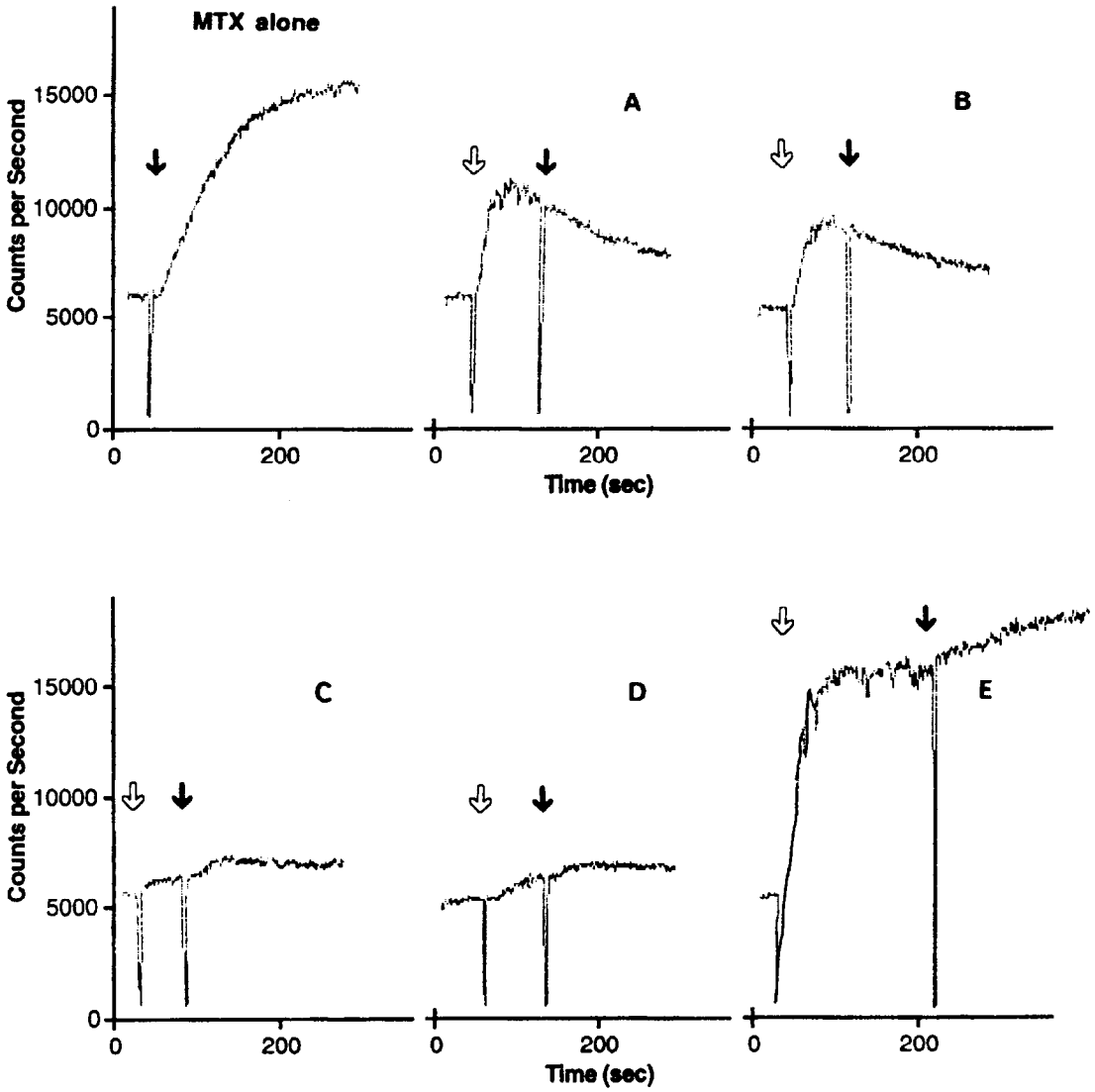

Fig. 2. Inhibition of the maitotoxin-elicited increase in levels of intracellular calcium by imidazoles in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods with maitotoxin (2 ng/mL) either alone or in the presence (A–E) of an imidazole at a 30 μM final concentration. The solid arrow indicates the addition of maitotoxin. The open arrow indicates the addition of (A) econazole, (B) miconazole, (C) SKF 96365, (D) clotrimazole and (E) calmidazolium. EGTA (0.5 mM) prevented the maitotoxin response (data not shown). The graphs are from typical experiments.

tion (data not shown). Diltiazem at 30 μM had no effect (data not shown). The diphenylbutylpiperidines fluspirilene and pimozide at 30 μM blocked the maitotoxin response (data not shown). The diphenylbutylpiperidine penfluridol at 30 μM itself caused a slow increase in intracellular calcium, but did appear to block the maitotoxin response (Fig. 3B). The diphenylpropylpiperidines loperamide and diphenoxylate at 30 μM blocked the maitotoxin response (Fig. 3C and data not shown). Cinnarizine at 30 μM inhibited the maitotoxin response (Fig. 3D). Bepridil, proadifen, and RMI 12330A at 30 μM blocked the maitotoxin response (data not shown and Fig. 3E). At 10 μM, fluspirilene, pimozide, penfluridol and loperamide still caused a complete blockade of the maitotoxin response; bepridil, proadifen and RMI 12330A now caused only a marked inhibition and methoxyverapamil, diphenoxylate and cinnarizine caused only a minimal inhibition (data not shown). The effects of the various agents, tested at 30 μM, on maitotoxin-elicited increases in intracellular calcium in HL-60 cells are summarized in Table 1. Quantitative analysis was not attempted, but it is clear that all compounds that inhibited maitotoxin-elicited $^{45}Ca^{2+}$ influx in 3T3 cells with IC_{50} values of 2–22 μM (Table 1) did at 30 μM either block or inhibit maitotoxin-elicited increases in intracellular calcium in HL-60 cells.

The effects of the various blockers on the CRAC channels, which are responsible for a sustained increase in intracellular calcium that occurs in HL-60 cells after treatment with ATP, were determined. ATP and UTP through P_{2u}-receptors elicited a similar concentration-dependent phosphoinositide breakdown in HL-60 cells

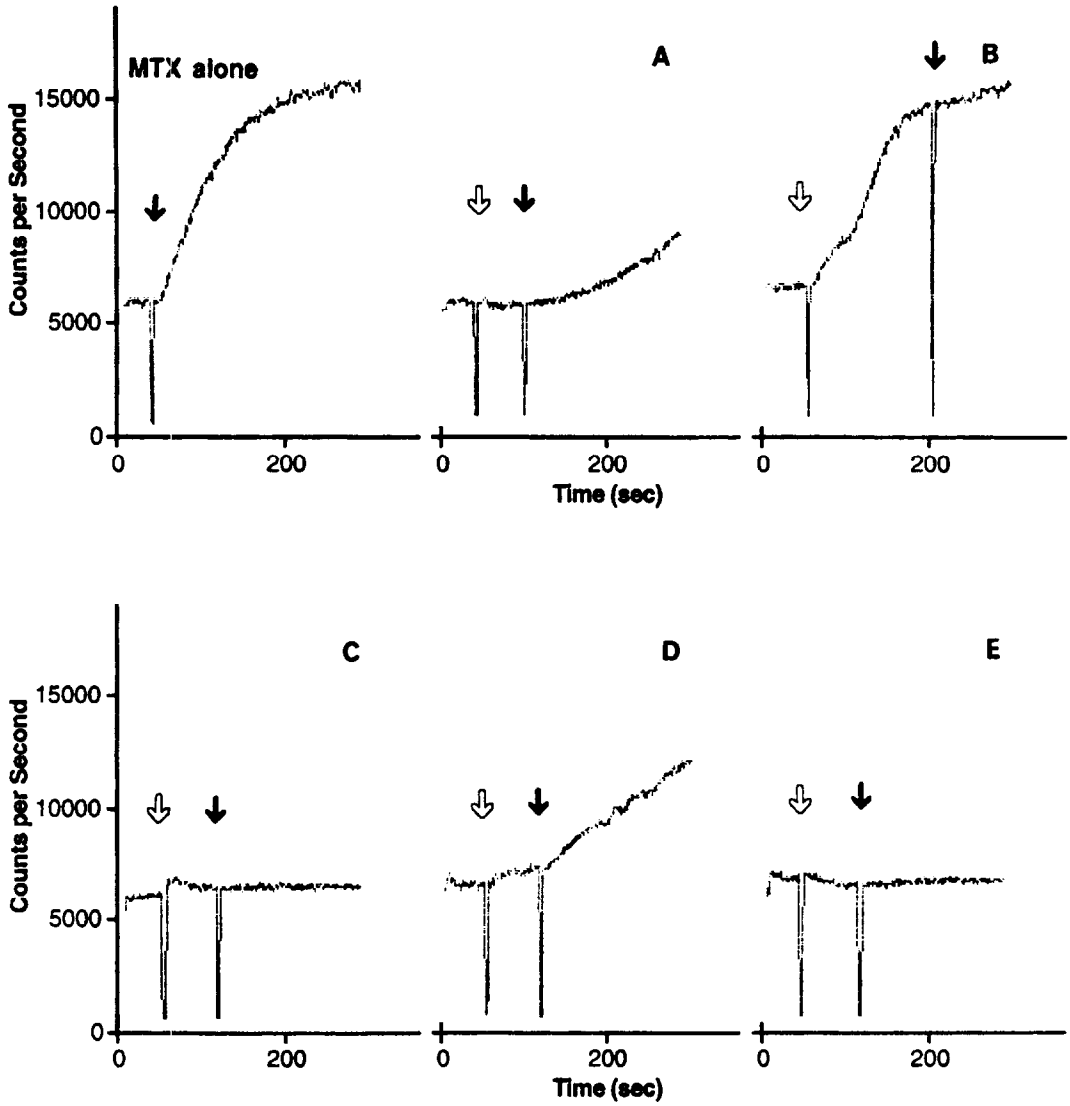

Fig. 3. Inhibition of maitotoxin-elicited increase in levels of intracellular calcium by calcium channel blockers in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods with maitotoxin (2 ng/mL) either alone or in the presence of a calcium channel blocker (A–E) at a 30 μM final concentration. The solid arrow indicates the addition of maitotoxin. The open arrow indicates the addition of (A) methoxyverapamil, (B) penfluridol, (C) loperamide, (D) cinnarizine, and (E) RMI 12330A. The graphs are from typical experiments.

with EC_{50} values of about 10 μM (data not shown), leading to the expected IP_3-dependent transient release of intracellular calcium, followed by a sustained elevation in intracellular calcium, which was dependent on influx of calcium from the medium. Inclusion of EGTA had little effect on the transient increase in intracellular calcium, but eliminated the sustained influx (data not shown). Imidazoles at 30 μM inhibited CRAC channel-dependent increases in intracellular calcium (Figs. 4 and 5A–C). But, in addition, calmidazolium (Fig. 5D–F) and to a lesser extent econazole (Fig. 4A) and miconazole (Fig. 4B) caused an increase in intracellular calcium, as was the case when these imidazoles were tested alone (Fig. 2A, B and E). SKF 96365 (Fig. 4C) and clotrimazole (Fig. 5A–C) showed no or only minimal stimulatory effects on intracellular calcium levels. The calmidazolium-elicited increase in intracellular levels of calcium prevented detection of any effects on CRAC channels (Fig. 5D–F). Of the fourteen non-imidazole compounds, all of which blocked maitotoxin-elicited $^{45}Ca^{2+}$ influx in 3T3 cells, and either blocked or inhibited maitotoxin-elicited increases in intracellular calcium in HL-60 cells, only WB4101 and bepridil (Fig. 6D and E) caused any inhibition of the CRAC channel-mediated elevation of intracellular calcium in HL-60 cells. The lack of effect of the potent maitotoxin blockers, fluspirilene, pimozide, and proadifen are shown in panels A, B and F of Fig. 6. Penfluridol caused a further slow increase in calcium (Fig. 6C) as was the case when this agent was tested alone (Fig. 3B). Loperamide was remarkable, being among the most potent blockers of maitotoxin responses, but instead of blocking the CRAC

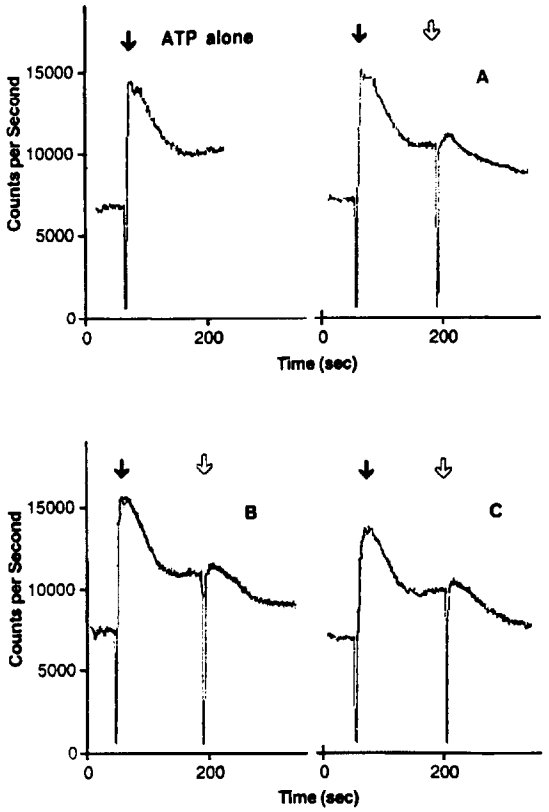

Fig. 4. Effect of imidazoles on the sustained elevation of intracellular calcium after an ATP-elicited release of intracellular calcium and a resultant activation of CRAC channels in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods. ATP in a final concentration of 10 μM was added as indicated by the solid arrow either alone, or followed by addition of an imidazole (A–C) at a 30 μM final concentration. The open arrow indicates the addition of (A) econazole, (B) miconazole, and (C) SKF 96365. Graphs are from typical experiments.

channel-mediated sustained elevation in intracellular calcium, loperamide caused a further increase in intracellular levels of calcium (Fig. 7). In the absence of activation of CRAC channels, loperamide had *no* effect on intracellular levels of calcium (Fig. 7E and F, and data not shown). The threshold for the enhancing effect of loperamide on CRAC channel-mediated elevation of intracellular calcium was about 3 μM (Fig. 7B).

The various blockers were tested for effects on phosphoinositide breakdown. The imidazoles econazole, miconazole and calmidazolium, which elevated intracellular calcium in HL-60 cells, also increased phosphoinositide breakdown (Table 2). Clotrimazole, which has no effect on intracellular calcium, also slightly increased phosphoinositide breakdown. SKF 96365, which has only a minimal effect on intracellular calcium, had no effect on phosphoinositide breakdown. The stimulation of phosphoinositide breakdown elicited by ATP and by the imidazoles appeared additive (Table 2). Most of the other agents had minimal effects on phosphoinositide breakdown alone or when tested with ATP (data not shown). However, fluspirilene and pimozide at 10 μM slightly increased phosphoinositide breakdown. Penfluridol and loperamide had no effect on phosphoinositide breakdown (data not shown).

DISCUSSION

The nature of the so-called "receptor-operated" calcium channels that are responsible for the calcium influx that ensues after depletion of IP_3-sensitive stores of intracellular calcium has remained elusive [10]. Such channels function regardless of whether depletion of internal calcium stores has been elicited through receptor-mediated activation of IP_3 formation or whether the depletion occurs after treatment with thapsigargin, an inhibitor of a Ca^{2+}-ATPase that is responsible for reuptake of intracellular calcium into IP_3-sensitive storage sites. Thus, a more appropriate term for these channels is that proposed recently [10], namely calcium-release activated calcium channel or CRAC channel. In spite of intensive studies, the mechanism whereby the seemingly ubiquitous CRAC channels are activated through depletion of intracellular calcium has not been defined. A diffusible small messenger has been proposed [11, 12], as has an involvement of inositol 1,3,4,5-tetrakisphosphate [13], tyrosine kinase [14], phosphoprotein phosphatases [12, 15], and cytochrome P450 [16]. The CRAC channels are not readily blocked by classical inhibitors (nifedipine, verapamil, diltiazem) of voltage-dependent calcium channels, and the discovery in 1990 that an imidazole derivative, SKF 96365, inhibits CRAC channels [17] provided a useful tool for the study of such channels. A number of such imidazoles are now known to block CRAC channels [16, 18, 19]. Such imidazoles, originally developed as antifungal agents, are not specific blockers of CRAC channels, but also inhibit voltage-dependent calcium channels [17, 18], potassium channels [20, 21], cytochrome P450 [22, 23], and Ca^{2+}-ATPases [18, 24]. The demonstration that the imidazole SKF 96365, unlike classical calcium channel blockers, effectively blocks maitotoxin-elicited calcium influx in cultured cells [4] suggested that maitotoxin might be activating CRAC channels. The present study was designed to explore this possibility by comparing the abilities of a number of imidazoles and a variety of other calcium channel blockers to antagonize maitotoxin-elicited influx of $^{45}Ca^{2+}$ in 3T3 cells and maitotoxin-elicited elevation of intracellular calcium in HL-60 cells with the abilities of these compounds to antagonize CRAC channels that activate after stimulation of IP_3 by ATP in HL-60 cells (cf. Ref. 25). The results indicate that while the various imidazoles did antagonize both maitotoxin responses and the CRAC channel-mediated increase in intracellular calcium, there were many other agents that effectively blocked maitotoxin responses in both 3T3 and HL-60 cells, but had no effect on the CRAC channel-mediated elevation in intracellular calcium (Table 1). Thus, the maitotoxin-activated channel appears clearly different from the CRAC channels.

The imidazoles when tested alone had no significant effect on influx of $^{45}Ca^{2+}$ in 3T3 cells, but did cause a significant increase in intracellular levels of calcium in HL-60 cells. Calmidazolium (a quaternary derivative of miconazole) was the most effective in this regard, while clotrimazole and SKF 96365 were the least effective, having no or nearly no effect. Calmidazolium is generally considered to be a potent calmodulin antagonist

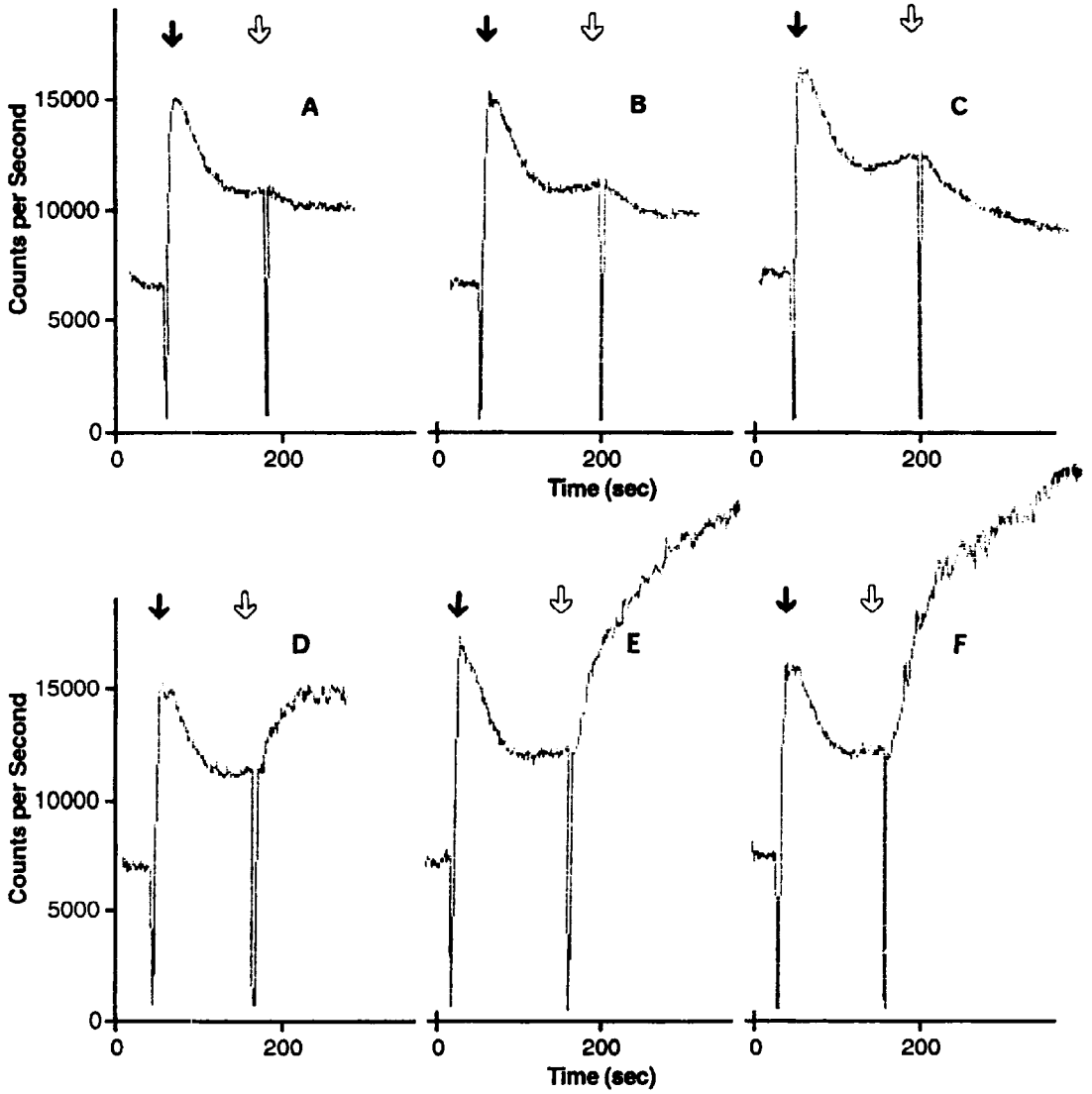

Fig. 5. Effect of the imidazoles clotrimazole and calmidazolium on the sustained elevation of intracellular calcium after an ATP-elicited release of intracellular calcium and a resultant activation of CRAC channels in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods. ATP in a final concentration of 10 μM was added as indicated by the solid arrow, followed by addition of an imidazole (A–F). The open arrow indicates the addition of clotrimazole (A, 3 μM; B, 10 μM; C, 30 μM) or calmidazolium (D, 3 μM; E, 10 μM; F, 30 μM). Graphs are from typical experiments.

[26], but it was shown recently to block calcium channels in pituitary cells [27]. SKF 96365 has been reported to activate a nonselective cation channel [21].

Two diphenylpropylpiperidines, namely the antidiarrheal loperamide and the analgetic diphenoxylate, were potent versus the maitotoxin responses, but neither inhibited the CRAC channel-mediated increase in intracellular calcium. Instead, loperamide, but not diphenoxylate, caused a remarkable further increase in intracellular levels of calcium when CRAC channels had been activated through receptor-mediated IP_3 formation and calcium depletion, using ATP (Fig. 7) or fMLP (data not shown), or through thapsigargin-mediated inhibition of calcium uptake into storage sites (data not shown). Loperamide had no effect alone on intracellular levels of calcium and very effectively blocked maitotoxin-elicited increases in intracellular calcium.

A wide range of other agents that can affect calcium channels were tested (Table 1). Many were effective versus maitotoxin responses, but none were effective versus the CRAC channels. These agents included flusperilene, a potent blocker of N-type calcium channels [28], penfluridol, a selective blocker of T-type calcium channels [29], cinnarizine, an agent previously reported to block maitotoxin responses in insulinoma cells [30], proadifen, a local anesthetic that is well known as an inhibitor of cytochrome P450 [31], and the adenylate cyclase inhibitor RMI 12330A [32].

Maitotoxin-elicited influx of calcium appears to very effectively stimulate breakdown of phosphoinositides in

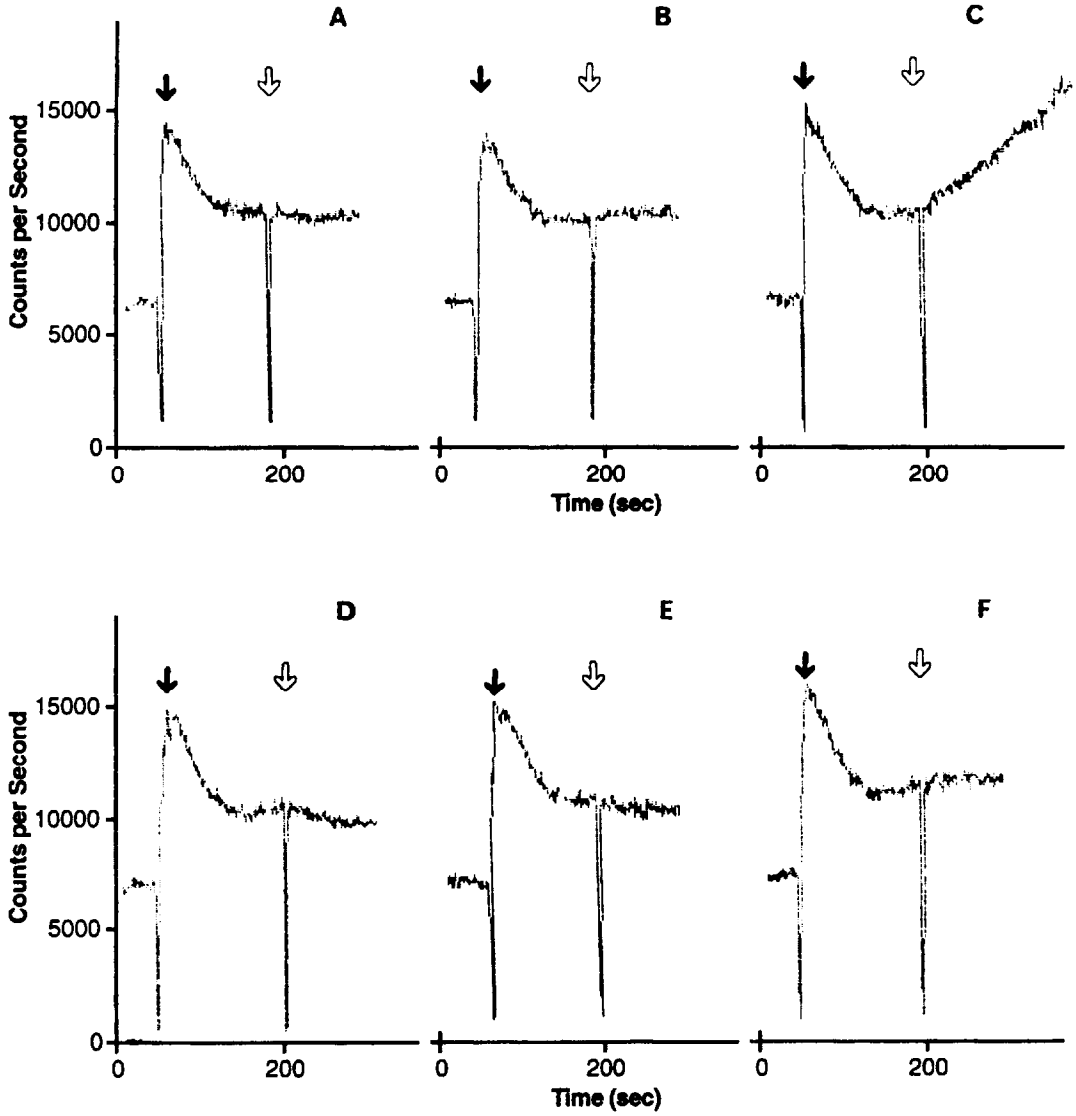

Fig. 6. Effect of calcium channel blockers on the sustained elevation of intracellular calcium after an ATP-elicited release of intracellular calcium and a resultant activation of CRAC channels in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods. ATP in a final concentration of 10 μm was added as indicated by the solid arrow, followed by addition of a calcium channel blocker (A–F) at a 30 μM final concentration. The open arrow indicates the addition of (A) fluspirilene, (B) pimozide, (C) penfluridol, (D) WB4101, (E) bepridil, and (F) proadifen.

a variety of cell types [2]. In differentiated HL-60 cells, the stimulation of calcium influx elicited by maitotoxin paralleled an increase in formation of IP_3 [33]. The IP_3 should then cause release of intracellular calcium and presumably an activation of CRAC channels. It would appear, however, that continued massive influx of calcium via maitotoxin-activated channels may prevent any detection of any CRAC channels that are activated after maitotoxin.

ATP and UTP through a P_{2u} receptor elicit phosphoinositide breakdown in HL-60 cells (cf. Ref. 25), which results in release of intracellular calcium and activation of CRAC channels. None of the current calcium antagonists prevented ATP-elicited phosphoinositide breakdown (data not shown), but the imidazoles miconazole, econazole, clotrimazole and calmidazolium did increase phosphoinositide breakdown when tested alone. It appears likely that this stimulation is due to the increase in calcium levels elicited by the imidazoles. A calcium ionophore, ionomycin, does increase calcium levels and phosphoinositide breakdown in differentiated HL-60 cells [33].

In summary, the wide range of potencies for calcium channel blockers versus maitotoxin-elicited calcium flux argues for the presence of a calcium ion channel with selectivity as to interactions with various agents. The profile of potency for the various agents as blockers is not commensurate with any known voltage-dependent calcium channel, nor as shown in the present study is it commensurate with CRAC channels. Recently, another

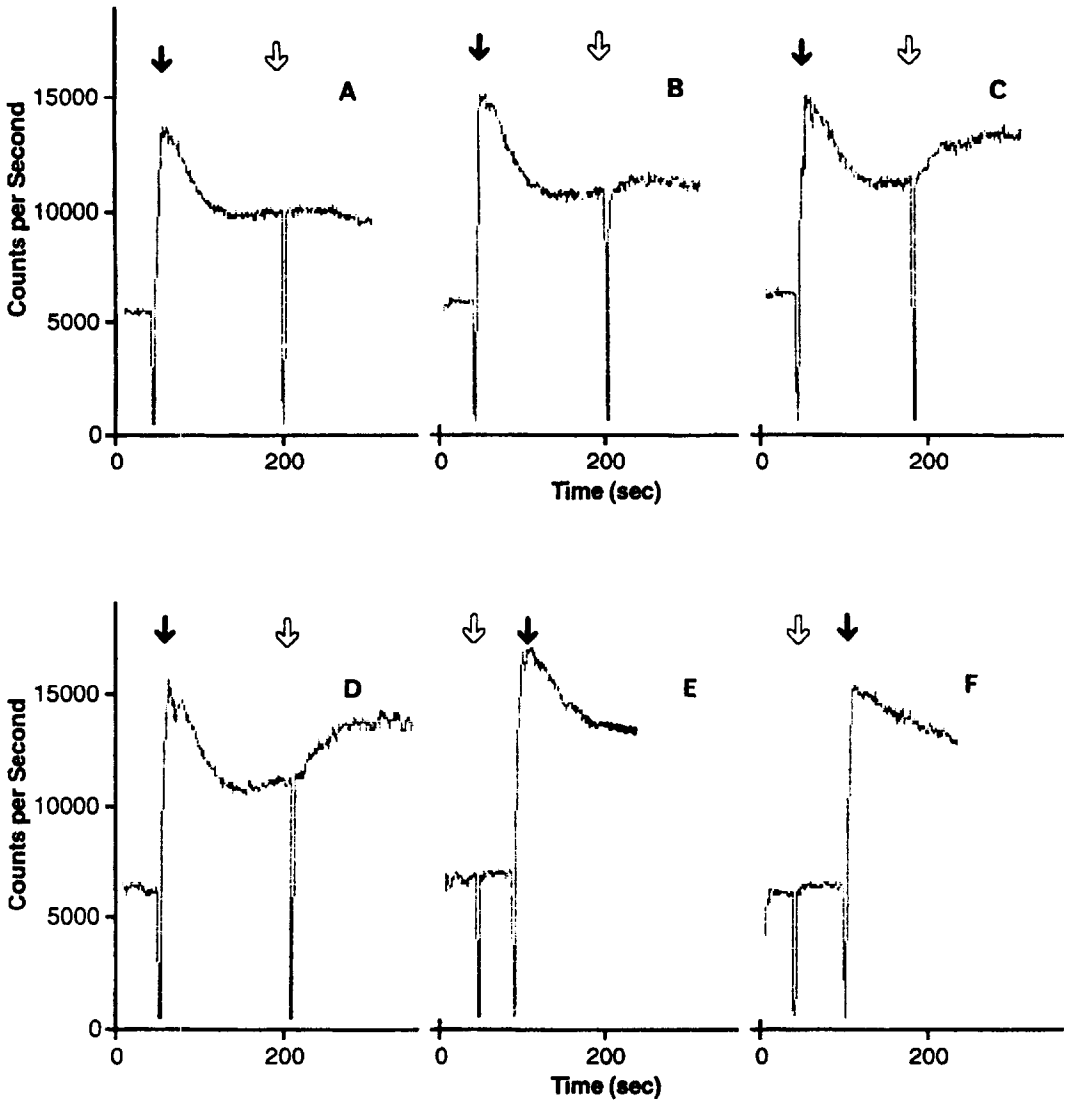

Fig. 7. Effect of loperamide on levels of intracellular calcium in HL-60 cells. Cells were labeled with fluo-3-AM and incubated as described in Materials and Methods. ATP in a final concentration of 10 μM was added as indicated by the solid arrow followed by loperamide (A–D) or preceded by loperamide (E and F) as indicated by the open arrow. Key: ATP followed by loperamide (A, 1 μM; B, 3 μM; C, 10 μM; D, 30 μM) or loperamide (E, 10 μM; F, 30 μM) followed by ATP.

laboratory also reported that maitotoxin- and CRAC channel-mediated increases in calcium levels in cells showed different sensitivities to blockade [34]. In that study, CRAC channel-mediated responses were blocked by gadolinium ions, whereas maitotoxin responses were not. It is, of course, possible that maitotoxin-activated channels differ in their properties from the native channel. Many of the inhibition curves versus maitotoxin-elicited $^{45}Ca^{2+}$ influx in 3T3 fibroblasts appeared biphasic with a small portion of the maitotoxin response being more sensitive to blockade. Thus, the maitotoxin response may represent effects at more than one component or channel. This has been proposed recently, based on electrophysiological evidence [3].

The most potent blockers of maitotoxin-elicited calcium flux were clotrimazole, loperamide and RMI 12330A. SKF 96365, fluspirilene, penfluridol, and proadifen also were very potent. The imidazole blockers of CRAC channels were confirmed to be effective in blocking maitotoxin channels, but also were found to cause elevations in levels of intracellular calcium, at least in part probably due to activation of calcium channels. Calmidazolium was much more effective than the nonquarternary imidazoles with respect to causing increases in intracellular levels of calcium. The diphenylpropylpiperidine loperamide had the unique property of further increasing levels of intracellular calcium, but only when levels were already elevated due to activation of CRAC channels. The nature of the channel that is activated by maitotoxin remains in question, but the potent and structurally unrelated blockers, defined in the present study, provide a series of

Table 2. Effect of imidazole on phosphoinositide breakdown in HL-60 cells

Imidazole	$[^3H]IP_1$ generation (% of control)	
	−ATP	+ATP
None	100	182 ± 8*
Econazole	131 ± 3*†	249 ± 14†
Miconazole	128 ± 8‡	231 ± 33‡
SKF 96365	103 ± 5	220 ± 13
Clotrimazole	121 ± 9‡	260 ± 14†
Calmidazolium	180 ± 11†	258 ± 4†

Imidazoles at 10 μM were incubated with HL-60 cells for 15 min, and $[^3H]IP_1$ generation from [^{3}H]inositol-labeled phosphoinositides was determined in the presence or absence of 10 μM ATP as described in Materials and Methods. Values are means ± SEM (N = 3). Control value for $[^3H]IP_1$ generation was 300 ± 32 cpm/10,000 cpm membrane lipid.

* $P < 0.01$ relative to absence of ATP.

† $P < 0.01$ relative to absence of imidazole.

‡ $P < 0.05$ relative to absence of imidazole.

valuable tools for investigation of maitotoxin-elicited responses.

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