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Maitotoxin Effects Are Blocked by SK&F 96365, an Inhibitor of Receptor-Mediated Calcium Entry

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SUMMARY

The dinoflagellate toxin maitotoxin (MTX) elicited a sustained increase of $[Ca^{2+}]_i$ in C6 glioma cells. This response was inhibited by SK&F 96365, a blocker of receptor-mediated calcium entry. In C6 cells, endothelin-1 elicited a rapid but transient increase in $[Ca^{2+}]_i$, followed by a smaller sustained increase. SK&F 96365 inhibited the sustained increase in $[Ca^{2+}]_i$. In both C6 glioma cells and RIN insulinoma cells, MTX elicited a marked influx of $^{45}Ca^{2+}$. SK&F 96365 inhibited MTX-induced $^{45}Ca^{2+}$ influx by 95% at 30 μM. The L-type calcium channel blocker nifedipine, even at 10 μM, inhibited MTX-induced calcium uptake by only 20% in RIN cells and by only 10% in C6 cells. MTX elicited calcium-dependent phosphoinositide breakdown in both C6 and RIN cells. In both cell lines, the MTX-induced phosphoinositide breakdown was inhibited by 90% by SK&F 96365 at 30 μM. Endothelin-1 and carbamylcholine elicited phosphoinositide breakdown in C6 cells and RIN cells, respectively. The stimulations were unaffected by the presence of SK&F 96365 up to 100 μM. In RIN insulinoma cells, MTX elicited calcium-dependent release of insulin. SK&F 96365 at 30 μM inhibited MTX-induced insulin release by 75%, whereas nifedipine, even at 30 μM, inhibited release by only 10%. The blockade of MTX-induced responses by SK&F 96365 indicates that MTX increases intracellular calcium by interacting directly with a calcium-entry system that is similar, in its sensitivity to SK&F 96365, to the calcium-entry system activated by receptors that elicit phosphoinositide breakdown. Activation of phospholipase C and hormone release by MTX also are blocked by SK&F 96365 and, thus, may be secondary to the activation of such a calcium-entry system.

MTX, a marine toxin present in the dinoflagellate *Gambierdiscus toxicus* (1), increases influx of calcium in virtually all cells, evokes release in secretory cells, induces contraction of muscle, and induces phosphoinositide breakdown in all cells studied (2). The actions of MTX are dependent on the presence of extracellular calcium ions. MTX-mediated effects are antagonized in some but not all cells by L-type calcium channel blockers (3, 4). Thus, the effects of MTX on calcium uptake and phosphoinositide breakdown do not appear to be wholly dependent on activation of voltage-dependent L-type calcium channels (2). Indeed, the activation of such voltage-dependent channels is probably indirect, due to a depolarization of membranes subsequent to entry of calcium through an MTX-sensitive channel (5). MTX-induced calcium entry is not an ionophore-like action; unlike ionophores, MTX does not elicit calcium uptake in liposomes (4).

The question of the primary target for the action of MTX remains unanswered. In membranes and permeabilized cells, MTX has no effect on phospholipase C (6). Thus, both an intact cell and extracellular calcium are required for activation of phospholipase C by MTX. It appears likely that the primary target for MTX is not the phospholipase C but a ubiquitous MTX-sensitive calcium channel.

A voltage-insensitive calcium-entry system that represents one such possible membrane target for MTX is a receptor-mediated calcium-entry system present in many if not all cells (7). Activation of receptors that stimulate phosphoinositide breakdown also results, in many cells, in sustained calcium influx (7). As opposed to receptor-mediated stimulation of phospholipase C and resulting phosphoinositide breakdown, receptor-mediated calcium entry is entirely dependent on extracellular calcium (7). Recently, SK&F 96365 was shown to be an inhibitor of such receptor-mediated calcium entry (8). Such channels are not inhibited by dihydropyridines (7). SK&F 96365 does not affect intracellular calcium mobilization in the absence of extracellular calcium (8), indicating that it does not inhibit phospholipase C activation or inositol trisphosphate-elicited release of calcium from internal storage sites.

We report herein that SK&F 96365 also inhibits all MTX-induced actions, namely, intracellular calcium elevation, calcium influx, phosphoinositide breakdown, and hormone release. The potency of SK&F 96365 as an inhibitor of MTX-induced

ABBREVIATIONS: MTX, maitotoxin; SK&F 96365, 1-[3-(4-methoxyphenyl)propoxyl]-1-(4-methoxyphenyl)ethyl-1*H*-imidazole HCl; IP_3, inositol 1,4,5-trisphosphate; IP_1, inositol monophosphate; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

actions is similar to its reported potency (8) as an inhibitor of the receptor-mediated calcium-entry system.

Materials and Methods

MTX was isolated from *G. toxicus* as described (1). MTX is now commercially available from Wako Pure Chemical Industries Ltd. (Richmond, VA). Culture media and sera were obtained from GIBCO (Grand Island, NY). ^{125}I-Insulin, [^{3}H]inositol, and $^{45}CaCl_2$ were from New England Nuclear (Boston, MA). SK&F 96365 was kindly provided by Dr. J. Merritt (Smith Kline & French Research, Ltd., Welwyn, England). Endothelin-1 was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Nifedipine was from Sigma (St. Louis, MO). Fura-2 acetoxymethyl ester was from Molecular Probes, Inc. (Eugene, OR). Guinea pig anti-porcine insulin serum was provided by Dr. Vivian Leclerq (Free University of Brussels, Brussels, Belgium). All other chemicals and reagents were obtained from standard commercial sources.

Cell culture. C6 glioma cells were provided by Dr. P. Fishman (National Institutes of Health, Bethesda, MD). The cells were grown at 37°, in a humidified atmosphere of 95:5 O_2/CO_2, in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. RIN cells were provided by Dr. A. Boyd III (Baylor College, Houston, TX). The cells were grown at 37°, in a humidified atmosphere of 95:5 O_2/CO_2, in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used for 30 passages, and responses were consistent for the duration of the present study.

Intracellular calcium. C6 cells were grown in cell culture medium for 2 days, on glass coverslips arranged on the bottom of a 10-cm Petri dish. The cells were loaded with fura-2 acetoxymethyl ester by aspiration of the cell culture medium and addition of DMEM containing 20 mM HEPES (pH 7.4) and 10 μM fura-2 acetoxymethyl ester. After 30–60 min, the cells were washed twice with buffer containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES (pH 7.4). Coverslips were placed in cuvettes containing 3 ml of buffer composed of 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 0.05% bovine serum albumin, under continuous stirring, at 37°, in a Spex fluorescence spectrophotometer. Agents were added in 5–20 μl, at the indicated final concentrations. Fluorescence was monitored at 340 nm (excitation) and 510 nm (emission). $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz *et al.* (9). Maximal fluorescence was calculated by solubilizing the cells with 0.5% sodium dodecyl sulfate, to release incorporated fura-2. Minimal fluorescence was calculated by quenching fura-2 with 1 mM $MnCl_2$.

$^{45}Ca^{2+}$ uptake. C6 or RIN cells were transferred to 12-well plates and incubated overnight. On the day of the experiment, the cells were preincubated for 10–15 min in buffer containing 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM HEPES (pH 7.4, adjusted with Tris), and, when indicated, channel blockers. The preincubation buffer was then aspirated, and 0.3 ml of influx buffer containing 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM HEPES (pH 7.4, adjusted with Tris), agents and channel blockers, and $^{45}CaCl_2$ (1.0 μCi/ml) was added. After 15 min, the cells were washed with wash buffer containing 150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES (pH 7.4, adjusted with Tris). The cells were solubilized with 0.3 ml of 1% sodium dodecyl sulfate, 0.5 N NaOH, at room temperature for 30–60 min. The NaOH was neutralized with 0.15 ml of 1 N HCl, and the radioactivity was measured with a scintillation counter set to measure ^{14}C. Results were expressed as percentage of control or as nmol of Ca^{2+} influx/well.

Phosphoinositide breakdown. C6 or RIN cells were transferred to 12-well plates and incubated overnight with 10 μCi/mmol [^{3}H] inositol, in 1 ml of cell culture medium. The medium was aspirated and the cells were preincubated for 10–15 min in buffer composed of 108 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSo_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 20 mM HEPES (pH 7.4, adjusted with NaOH), 10 mM LiCl, and indicated channel blockers. Other reagents (MTX, receptor agonists) were then added, and cells were incubated for an additional 15 min at 37°. The incubation buffer was aspirated and 1 ml of 6% trichloroacetic acid was added. Cells were scraped off the bottom of the wells and transferred to 1.5-ml microfuge tubes. The tubes were centrifuged for 2 min (12,000 × *g*), the supernatant was applied to anion exchange columns (Bio-Rad AG 1X8, 100–200 mesh, formate form), and [^{3}H]IP_1 was isolated as described (10). The trichloroacetic acid pellet of each sample was used to measure the incorporation of [^{3}H]inositol into the lipids. Results were expressed as percentage of control or as (cpm of [^{3}H]IP_1/cpm in lipids) × 10,000.

Insulin radioimmunoassay. RIN cells were transferred to 12-well plates and incubated overnight in cell culture medium. Cells were then preincubated for 10–15 min in buffer containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM EDTA, 20 mM HEPES, 5.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and, when indicated, channel blockers (pH 7.4, adjusted with NaOH). After preincubation, agents (MTX, receptor agonists) were added and cells were incubated for an additional 15 min at 37°. The incubation buffer was diluted, and insulin was quantified by radioimmunoassay as described (11, 12), using a dextran-coated charcoal separation with porcine insulin standard, porcine ^{125}I-labeled insulin, and guinea pig anti-porcine insulin serum. Results were expressed as pg of insulin/well or percentage of control.

Results

Intracellular calcium. MTX induced a slow increase in $[Ca^{2+}]_i$ of C6 cells, during an incubation period of 15 min (Fig. 1A). Preincubation of C6 cells with 30 μM SK&F 96365 and subsequent treatment with MTX resulted in a much smaller increase in intracellular calcium.

Endothelin induced a rapid but transient rise in $[Ca^{2+}]_i$, followed by a sustained increase in the resting level of intracellular calcium (Fig. 1B). The rapid initial phase of the transient increase in $[Ca^{2+}]_i$ observed after treatment with endothelin was unaffected, whereas the late phase of the transient increase was reduced by pretreatment with 30 μM SK&F 96365. The subsequent resting level of intracellular calcium was markedly lower than that obtained after treatment with endothelin alone.

Calcium uptake. MTX at 1 ng/ml increased the uptake of $^{45}Ca^{2+}$ in C6 and RIN cells by 38-fold and 21-fold, respectively. This uptake was blocked by SK&F 96365 (Fig. 2). Significant blockade of $^{45}Ca^{2+}$ uptake occurred at 1 μM SK&F 96365 in both cell lines, and maximal blockade of $^{45}Ca^{2+}$ uptake (95%) occurred at 30 μM SK&F 96365 in both C6 and RIN cells. Nifedipine, even at 10 μM, did not significantly inhibit MTX-induced $^{45}Ca^{2+}$ uptake in C6 cells (Fig. 2A) and had only a small effect in RIN cells (Fig. 2B). Neither depolarizing concentrations of potassium ions (40 mM) nor 0.1 μM endothelin-1 evoked a measurable increase in $^{45}Ca^{2+}$ uptake in C6 cells (data not shown). Thapsigargin, an inhibitor of intracellular calcium uptake, at concentrations up to 100 μM did not induce $^{45}Ca^{2+}$ uptake during a 15-min period in RIN cells (data not shown).

Phosphoinositide breakdown. In the presence of extracellular calcium, MTX at 1 ng/ml stimulated phosphoinositide breakdown by 18-fold and 7-fold in C6 and RIN cells, respectively. Pretreatment of C6 or RIN cells with SK&F 96365 caused inhibition of MTX-induced phosphoinositide breakdown in a dose-dependent manner, with significant inhibition at 1 μM SK&F 96365 in C6 cells (Fig. 3A) and 3 μM SK&F 96365 in RIN cells (Fig. 3B). Maximal blockade of MTX-induced phosphoinositide breakdown was about 90% in both cell lines and occurred at 10–30 μM SK&F 96365. Endothelin-1 stimulated phosphoinositide breakdown in C6 cells; at a concentration of 0.1 μM, endothelin-1 stimulated phosphoino-

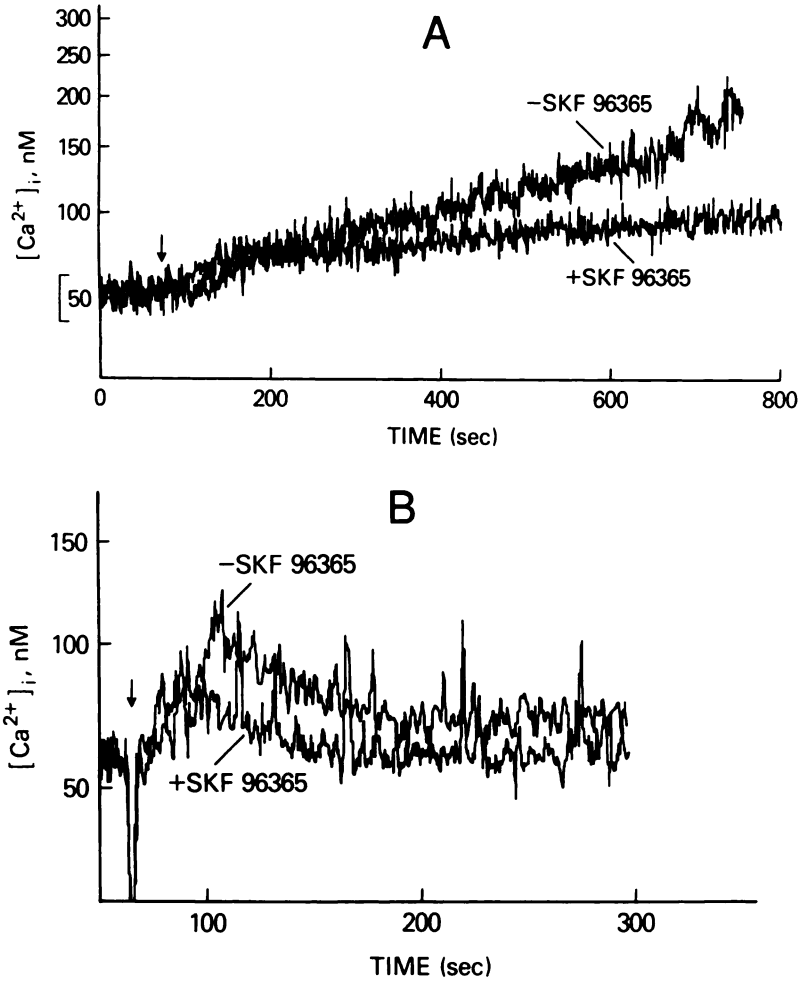

Fig. 1. Increases in intracellular calcium in C6 cells treated with MTX (A) or endothelin-1 (B). C6 cells were grown on glass coverslips and loaded with fura-2 acetoxymethyl ester, as described in Materials and Methods. SK&F 96365 (10 μM) was added, where indicated, 10 min before either 1 ng/ml MTX or 0.1 μM endothelin-1. *Arrow*, time at which either MTX or endothelin-1 was added. The tracings are from representative experiments that were repeated at least three times with similar results.

sitide breakdown by 10-fold. In contrast to the inhibitory effect of SK&F 96365 on MTX-induced phosphoinositide breakdown, the stimulation of phosphoinositide breakdown by endothelin was not inhibited by SK&F 96365 (Table 1). In RIN cells, the muscarinic agonist carbamylcholine stimulated phosphoinositide breakdown in a dose-dependent manner (data not shown), with maximal stimulation of 3-fold at a concentration of 100 μM carbamylcholine (Table 1). In contrast to the inhibitory effect of SK&F 96365 on MTX-induced phosphoinositide breakdown, SK&F 96365 did not inhibit carbamylcholine-induced phosphoinositide breakdown in RIN cells. The L-type calcium channel blocker nifedipine, at 10 μM, had no effect on MTX-induced phosphoinositide breakdown in C6 or RIN cells, on endothelin-induced phosphoinositide breakdown in C6 cells, or on carbamylcholine-induced phosphoinositide breakdown in RIN cells (data not shown).

Insulin release. MTX at 1 ng/ml stimulated the release of insulin in RIN cells by 13-fold (data not shown). Pretreatment of RIN cells with SK&F 96365 inhibited MTX-elicited insulin release in a dose-dependent fashion (Fig. 4). Nifedipine at 1–10 μM had no inhibitory effect on MTX-elicited insulin release (Fig. 4).

Discussion

In PC12 cells and HIT cells, MTX-induced changes in intracellular calcium or calcium uptake can be partially blocked with L-type calcium channel blockers (3, 13, 14). In the same cells, however, MTX-induced phosphoinositide breakdown is unaffected by calcium channel blockers. Therefore, MTX-mediated actions on phosphoinositide breakdown are not dependent on activation of voltage-dependent calcium channels. Indeed, in BC3H1 cells (15), C6 glioma cells (this paper and Ref. 15), aortic myocytes (16), and synaptosomes (17), MTX-elicited calcium influx is not antagonized by nifedipine, suggesting that L-type voltage-dependent calcium channels are a nonessential component of MTX-induced calcium uptake. However, unlike stimulation of phosphoinositide breakdown by receptor agonists, MTX-elicited stimulation of phosphoinositide breakdown is dependent on extracellular calcium, suggesting that activation of phospholipase C by MTX involves the influx of calcium through plasma membranes (6). Furthermore, in membranes or in permeabilized cells, where there is no calcium gradient, MTX has no effect on activation of phospholipase C (6). Thus, an attractive hypothesis is that MTX activates calcium influx via a ubiquitous calcium-entry system, whose function is tightly linked to activation of phospholipase C.

Receptor-mediated calcium entry is observed in many cell types and results in prolonged and sustained elevations in calcium (7). Such receptor-mediated calcium influx has been proposed to be triggered by the emptying of intracellular calcium stores (18). However, in a neuroblastoma cell line, emptying of calcium stores by thapsigargin did not trigger calcium influx (19). Similarly, in the present study with RIN cells, thapsigargin failed to induce any detectable uptake of $^{45}Ca^{2+}$

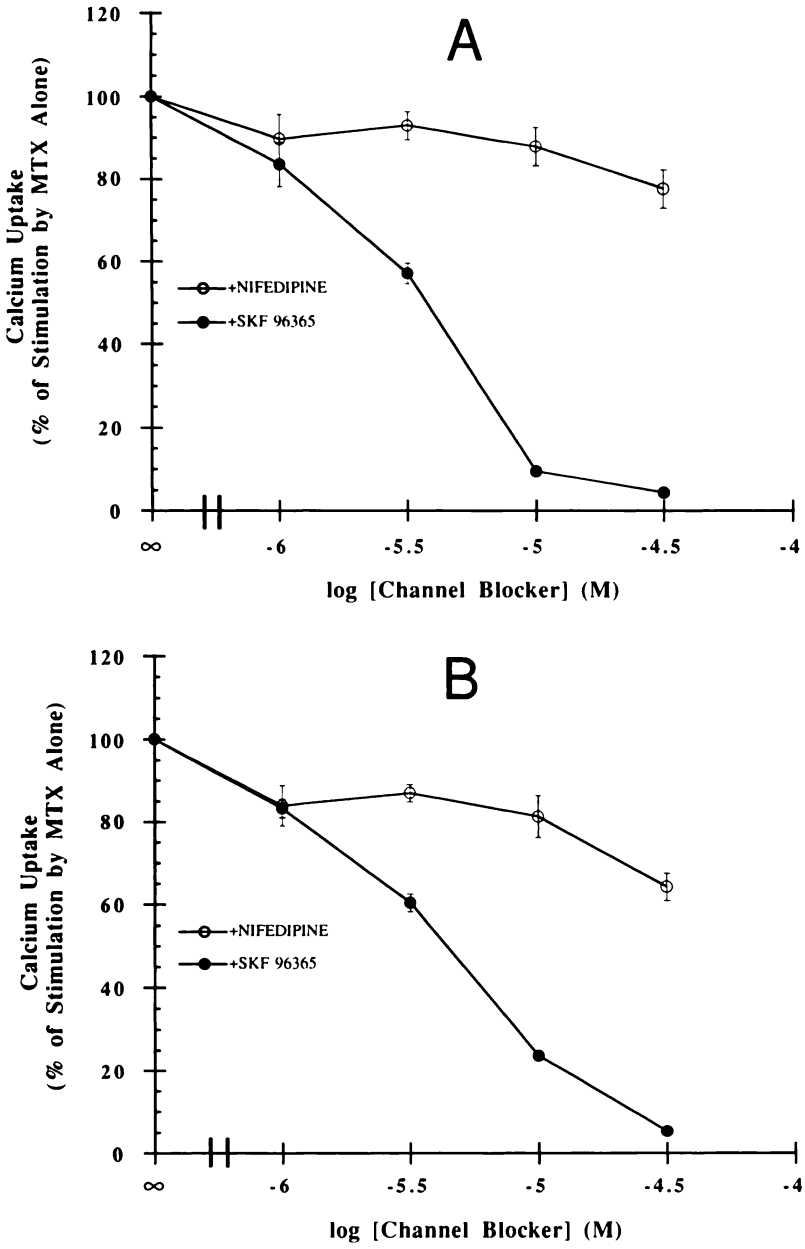

Fig. 2. Inhibition of MTX-induced $^{45}Ca^{2+}$ uptake by SK&F 96365 and nifedipine in C6 cells (A) and in RIN cells (B). Cells were preincubated in the presence of SK&F 96365 or nifedipine for 10–15 min before addition of 1 ng/ml MTX and $^{45}Ca^{2+}$-containing solution. After 15 min, cells were washed and calcium content was determined as described in Materials and Methods. Basal uptake was 0.80 ± 0.11 nmol of calcium in C6 cells and 0.98 ± 0.10 nmol of calcium in RIN cells; MTX elicited an uptake of 30.74 ± 2.44 nmol of calcium and 20.89 ± 6.97 nmol of calcium in C6 and RIN cells, respectively. Values correspond to means ± standard errors of three independent experiments, each performed in duplicate. Error bars smaller than the size of the symbol are not shown.

(see Results). Regardless of the mechanism underlying receptor-mediated calcium influx, activation of the same receptors results in a rapid increase of IP_3 formation and a transient and rapid increase in $[Ca^{2+}]_i$ due to IP_3-elicited release of intracellular stores of calcium, followed by a sustained elevation in $[Ca^{2+}]_i$ due to receptor-mediated calcium entry. Such a pattern of calcium mobilization was observed in C6 glioma cells after treatment with endothelin-1 (Fig. 1B), which has been shown to stimulate phosphoinositide breakdown in these cells (20). The "transient peak" due to endothelin in C6 cells was relatively broad and thus may reflect both release from internal stores and initiation of receptor-mediated calcium entry, particularly in the later phase of the transient peak. MTX, on the other hand, induced a slow increase in intracellular calcium (Fig. 1A) and a marked stimulation of phosphoinositide breakdown (Fig. 3, legend).

SK&F 96365 is an inhibitor of receptor-mediated calcium entry, as shown in human platelets (8), neutrophils (8), and PC12 cells (19). In addition, it can inhibit L-type voltage-dependent calcium channels (8). SK&F 96365 has no effects on ATP-elicited calcium influx (8), nor does a close structural analog have any effects on *N*-methyl-D-aspartate-activated channels (21). Therefore, such compounds do not block all membrane channels in a nonspecific fashion. SK&F 96365 inhibited endothelin-1-induced calcium entry in C6 cells, as evidenced by the reduction of the late sustained elevation of $[Ca^{2+}]_i$ observed in the absence of SK&F 96365 (Fig. 1B). SK&F 96365 did not affect endothelin-1-mediated inositol phosphate generation in C6 glioma cells and had no effect on the initial phase of the transient elevation in $[Ca^{2+}]_i$. In platelets and neutrophils, the release of internal calcium via IP_3 after receptor stimulation was not affected by SK&F 96365 (8).

MTX-induced increases in $[Ca^{2+}]_i$ in NCB20 (22), PC12 (22), HL60 (6), and C6 cells (23) (Fig. 1A) are calcium dependent and are typically slower than those observed with receptor agonists that induce IP_3 generation and release of internal

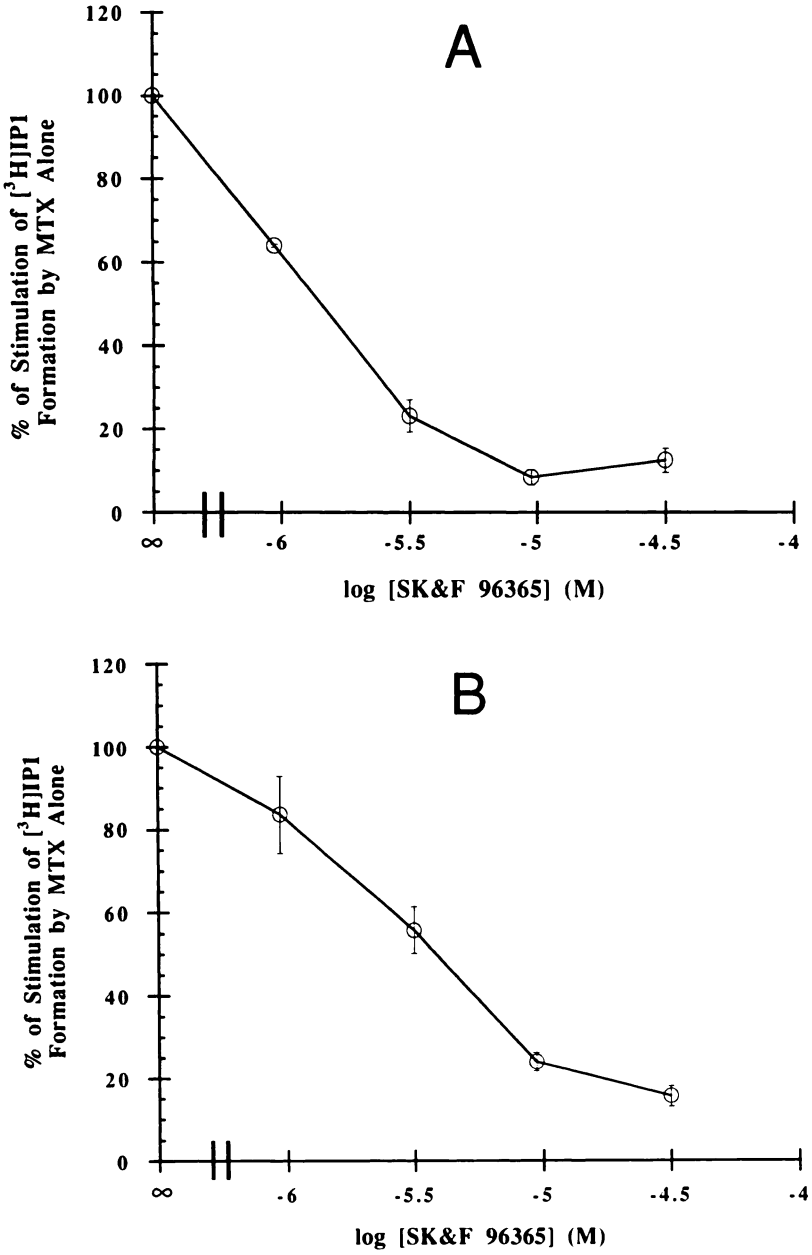

Fig. 3. Inhibition of MTX-induced phosphoinositide breakdown by SK&F 96365 in C6 cells (A) and in RIN cells (B). Cells were preincubated in the presence of SK&F 96365 for 10–15 min before addition of 1 ng/ml MTX. After 15 min, cells were washed and $[^3H]IP_1$ content was determined as described in Materials and Methods. Basal accumulation of $[^3H]IP_1$ was 156 ± 56 cpm and 168 ± 13 cpm in C6 and RIN cells, respectively; MTX elicited an accumulation of 2889 ± 381 cpm and 1216 ± 327 cpm of $[^3H]IP_1$ in C6 and RIN cells, respectively. Values correspond to means ± standard errors of three independent experiments, each performed in duplicate.

calcium. The effect of SK&F 96365 on MTX-induced increase in $[Ca^{2+}]_i$ was examined. The MTX-mediated increase in $[Ca^{2+}]_i$ in C6 glioma cells was inhibited by SK&F 96365 (Fig. 1A). MTX-induced $^{45}Ca^{2+}$ uptake in C6 glioma cells and in RIN cells was almost completely inhibited by SK&F 96365, in a dose-dependent manner (Fig. 2). Because SK&F 96365 also inhibits L-type voltage-dependent calcium channels (8), the possible contribution of such channels to the MTX-elicited responses was defined using the selective L-type channel-blocker nifedipine. In C6 glioma cells and RIN cells, MTX-induced $^{45}Ca^{2+}$ uptake was not significantly or only slightly inhibited by 10 μM nifedipine (Fig. 2). These results indicate that MTX induces calcium entry primarily by activating a SK&F 96365-sensitive, nifedipine-insensitive calcium-entry system. Whether the SK&F 96365-sensitive calcium-entry system activated by MTX is identical to the receptor-mediated calcium-entry system is unknown. The potency of SK&F 96365 versus MTX in the present study was similar to its reported potency versus receptor-mediated calcium entry (8). However, SK&F 96365 has not been investigated with respect to other

TABLE 1

Lack of effect of SK&F 96365 on stimulation of [^{3}H]IP$_1$ formation by endothelin-1 in C6 glioma cells and by carbamylcholine in RIN insulinoma cells

Cells were labeled with [^{3}H]inositol overnight and preincubated for 10–15 min with buffer or buffer containing 10 μM SK&F 96365. Cells were then incubated with 1.0 ng/ml MTX, 0.1 μM endothelin-1, or 100 μM carbamylcholine for 15 min, and [^{3}H] IP$_1$ was extracted and analyzed as described in Materials and Methods. Values represent means ± standard errors of three independent experiments, each performed in duplicate.

Treatment	[^{3}H]IP$_1$
	cpm
C6 cells	
Control	124.2 ± 6.2
Endothelin-1 (0.1 μM)	860.5 ± 39.2
+SK&F 96365 (10 μM)	1095.5 ± 167.0
MTX (1.0 ng/ml)	2803.8 ± 208.0
+SK&F 96365 (10 μM)	221.3 ± 8.5
RIN cells	
Control	95.7 ± 7.2
Carbamylcholine (100 μM)	302.2 ± 32.1
+SK&F 96365 (10 μM)	354.3 ± 22.1
MTX (1.0 ng/ml)	1280.8 ± 250.4
+SK&F 96365 (10 μM)	297.6 ± 59.8

calcium channels such as the calcium-activated nonselective cation channels or N-type voltage-dependent calcium channels or P-channels. Until further studies on the specificity of SK&F 96365 are completed, the present results remain consistent with an identity of MTX-activated channels and receptor-activated channels but certainly do not prove such an identity.

Certain other aspects of a comparison of MTX-activated calcium entry and receptor-mediated calcium entry need to be considered. First, the influx of calcium mediated by MTX is much greater than the influx mediated by receptor activation. Indeed, endothelin does not elicit a measurable influx of $^{45}Ca^{2+}$ into C6 glioma cells. Two possible explanations suggest themselves. The first is that the MTX-sensitive calcium-entry system is unrelated to the receptor-mediated calcium-entry system, with the two systems sharing only a similar sensitivity to blockade by SK&F 96365. However, another possibility is that MTX fully activates the receptor-mediated calcium-entry system, whereas receptor agonists and/or depletion of calcium stores cause only a partial activation of the calcium-entry system. Related to this aspect of the comparison is the MTX-evoked activation of phosphoinositide breakdown, a phenomenon dependent on extracellular calcium and presumably linked to an initial activation by MTX of a calcium-entry system. Receptor agonists, unlike MTX, often cause only initial transient activation of phosphoinositide breakdown. In many cells, the sesquiterpene thapsigargin, through inhibition of reuptake of calcium into intracellular organelles, can elicit calcium entry from extracellular media, presumably by the same calcium-entry system activated by receptor agonists (24, 25). Thapsigargin does not stimulate phosphoinositide breakdown (2). It could be proposed that the sustained influx of calcium elicited by receptor agonists or thapsigargin is below a threshold required to elicit activation of phospholipase C, whereas the influx of calcium elicited by MTX is above such a threshold. Alternatively, the calcium-entry system activated by MTX may be unique in its coupling to activity of phospholipase C and thus in this respect would not be identical to the receptor-mediated calcium-entry system. Such questions require further research, and SK&F 96365 provides a key agent for further study on the relationship between the two SK&F 96365-sensitive calcium-entry systems.

Stimulation of phosphoinositide breakdown by MTX has been observed in all cells tested (23). The stimulation of phosphoinositide breakdown by MTX is dependent on the presence of extracellular calcium (23). Moreover, MTX-mediated stimulation of phosphoinositide breakdown does not require accumulation of cytosolic calcium, because an intracellular calcium chelator, BAPTA, did not prevent the stimulation of phosphoinositide breakdown by MTX in HL60 cells (6), nor did a calcium ionophore mimic the effects of MTX on phosphoinositide breakdown (6). SK&F 96365 inhibited MTX-elicited stimulation of phosphoinositide breakdown in both C6 glioma and RIN insulinoma cells (Fig. 3), with potency similar to that observed for inhibition of $^{45}Ca^{2+}$ uptake in these cells (Fig. 2). Nifedipine, as reported for other cells (14), did not affect MTX-elicited stimulation of phosphoinositide breakdown. It appears likely that calcium uptake and phosphoinositide breakdown

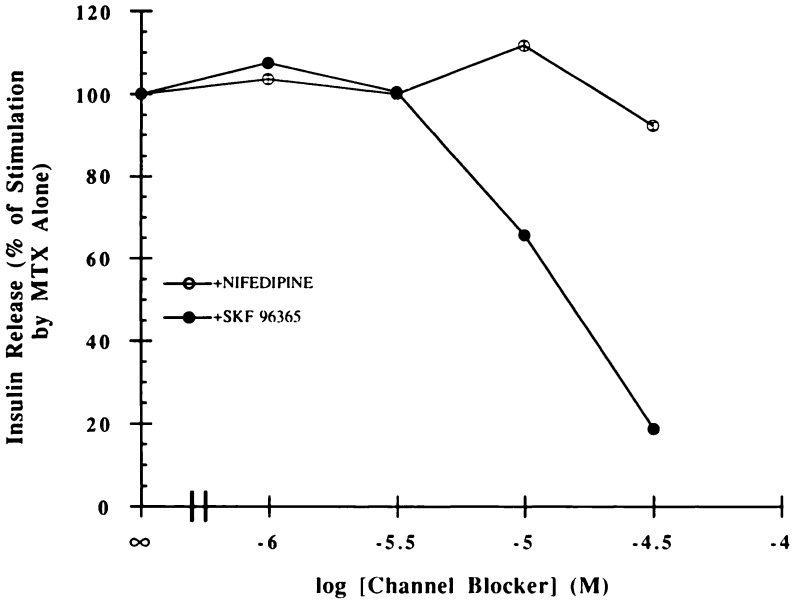

Fig. 4. Inhibition of MTX-induced insulin release by SK&F 96365 in RIN cells. Cells were preincubated in the presence of SK&F 96365 or nifedipine for 10–15 min before addition of 1 ng/ml MTX. After 15 min, cells were washed and insulin content in the medium was determined as described in Materials and Methods. Basal release was 13.3 ± 4.0 ng of insulin/well; MTX alone elicited release of 176.1 ± 47.1 ng of insulin/well. Values correspond to means ± standard errors of three independent experiments, each performed in duplicate. Error bars smaller than the size of the symbols are not shown.

induced by MTX are both mediated by the same SK&F 96365-sensitive pathway and that the initial event is a direct activation of a calcium-entry system by MTX.

In RIN insulinoma cells, MTX elicited the release of insulin (Fig. 4). This action was dependent on the presence of extracellular calcium (data not shown). Stimulatory effects of MTX on insulin release also occur in another insulin-secreting cell line, the HIT cells (14). In HIT cells, MTX-induced release of insulin is dependent on calcium but is only partially inhibited by the calcium channel blocker nifedipine, suggesting that mechanisms other than activation of L-type calcium channels can sustain MTX-induced release of insulin in these cells (14). MTX-induced phosphoinositide breakdown in HIT cells is unaffected by nifedipine (14), and it was proposed that the release of insulin and the phosphoinositide breakdown induced by MTX are interrelated (14). In RIN cells, SK&F 96365 (30 μM) inhibited MTX-induced insulin release by 75% (Fig. 4). At that concentration, SK&F 96365 also inhibited MTX-induced phosphoinositide breakdown (Fig. 3B) and calcium uptake (Fig. 2B) in RIN cells, suggesting that all of the actions of MTX share a SK&F 96365-sensitive initial step. MTX at higher concentrations (3 ng/ml) overcame the inhibition of insulin release by SK&F 96365, suggesting an apparent competitive blockade (data not shown).

In summary, we present evidence for an inhibitory action of SK&F 96365, a blocker of receptor-mediated calcium entry, on MTX-induced calcium-dependent actions. Such inhibition does not involve the action of SK&F 96365 on L-type calcium channels, because nifedipine has minimal effects in paradigms where SK&F 96365 can block MTX-induced responses almost completely. Because the stimulations of calcium uptake, phosphoinositide breakdown, and insulin release induced by MTX are inhibited by SK&F 96365 with similar potency, it is suggested that first MTX interacts with an SK&F 96365-sensitive calcium-entry system and then calcium, through this system, is responsible for all the biological actions of MTX, namely phospholipid turnover, release of hormones and neurotransmitters, and perhaps muscle contraction. Activation of voltage-dependent calcium channels by MTX in some cells may occur because of an initial depolarization due to influx of calcium through the MTX-sensitive receptor-mediated calcium-entry system. If the site of primary action for MTX is the receptor-mediated calcium-entry system, MTX may represent a direct activator of such a "channel."

Acknowledgments

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