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Maitotoxin: Effects on Calcium Channels, Phosphoinositide Breakdown, and Arachidonate Release in Pheochromocytoma PC12 Cells

OKSOON H. CHOI, WILLIAM L. PADGETT, YUKIO NISHIZAWA, FABIAN GUSOVSKY, TAKESHI YASUMOTO, and JOHN W. DALY

Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 (O.H.C., W.L.P., Y.N., F.G., J.W.D.), and Faculty of Agriculture, Tohoku University, Sendai, Japan (T.Y.)

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SUMMARY

Maitotoxin (MTX) increases formation of [^{3}H]inositol phosphates from phosphoinositides and release of [^{3}H]arachidonic acid from phospholipids in pheochromocytoma PC12 cells. Formation of [^{3}H]inositol phosphates is detected within 1 min of incubation even with concentrations as low as 0.3 ng/ml (90 pM) MTX, whereas release of [^{3}H]arachidonic acid is not detected until 20 min even with concentrations as high as 1 ng/ml (300 pM) MTX. Stimulation of arachidonic acid release can be detected at 0.03 ng/ml (9 pM) MTX, whereas 0.1 ng/ml (30 pM) MTX is the threshold for detection of phosphoinositide breakdown. Organic and inorganic calcium channel blockers, except Cd^{2+} and a high concentration of Mn^{2+}, have no effect on MTX-elicited phosphinositide breakdown, whereas inorganic blockers (e.g., Co^{2+}, Mn^{2+}, Cd^{2+}), but not organic blockers (nifedipine, verapamil, diltiazem), inhibit MTX-stimulated arachidonic acid release. All calcium channel blockers, however, inhibited MTX-elicited influx of $^{45}Ca^{2+}$ and the MTX-elicited increase in internal Ca^{2+} measured with fura-2 was markedly reduced by nifedipine. MTX-elicited phosphoinositide breakdown and arachidonic acid release are abolished or reduced, respectively, in the absence of extracellular calcium plus chelating agent. The calcium ionophore A23187 has little or no effect alone but, in combination with MTX, A23187 inhibits MTX-elicited phosphoinositide breakdown and enhances arachidonic acid release, the latter even in the absence of extracellular calcium. The results suggest that different sites and/or mechanisms are involved in stimulation of calcium influx, breakdown of phosphoinositides, and release of arachidonic acid by MTX.

MTX, a marine dinoflagellate toxin, activates voltage-sensitive L-type calcium channels in many cell types (1–3). In addition, MTX stimulates PI breakdown in all cell types as yet tested (4–8), including rat pheochromocytoma PC12 cells (8). The effect of MTX on PI breakdown is not blocked by blockers of voltage-sensitive calcium channels and appears to occur at a lower concentration of MTX than the concentration needed to cause Ca^{2+} influx (5, 8).

Effects of MTX on phospholipases other than the phospholipase C that hydrolyzes PIs to inositol phosphates and diacylglycerides have not been reported. The present study demonstrates that MTX stimulates release of [^{3}H]AA from phospholipids labeled during prior incubation of PC12 cells with [^{3}H]AA. Stimulation of AA release by MTX presumably occurs through a calcium-dependent activation of phospholipase A_2. The effects of MTX on (i) activation of phospholipase A_2, resulting in release of [^{3}H]arachidonic acid, (ii) activation of phospholipase C, resulting in formation of [^{3}H]inositol phosphates, and (iii) activation of calcium channels, resulting in increase of intracellular calcium, are compared in PC12 cells with respect to time course, concentration dependency, and blockade by calcium antagonists and other agents.

Experimental Procedures

Materials. MTX was isolated from *Gambierdiscus toxicus*, as described (9). Its molecular weight as a disodium salt was estimated as 3424.5 ± 0.5. Culture media and sera were from GIBCO (Grand Island, NY). [^{3}H]AA (90–100 Ci/mmol), [^{3}H]inositol (12–17 Ci/mmol), and [^{3}H]inositol mono-1-, bis-1,4-, and tris-1,4,5-phosphate standards for HPLC were from New England Nuclear (Boston, MA). $^{45}CaCl_2$ (18 mCi/mg) was from Amersham (Arlington Heights, IL). Fura-2 (cell-permeant form) was from Molecular Probes (Portland, OR), nifedipine and bovine serum albumin from Sigma (St. Louis, MO), diltiazem from Marion Laboratories (Kansas City, MO), verapamil hydrochloride from A.G. Knoll (West Germany), quinacrine from Aldrich Chemical Co. (Milwaukee, WI), and the calcium ionophore A23187 from Calbiochem (La Jolla, CA). Other chemicals were from standard commercial sources.

ABBREVIATIONS: MTX, maitotoxin; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AA, arachidonic acid; PI, phosphoinositide; HPLC, high pressure liquid chromatography; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Cell culture. PC12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were provided by Dr. G. Guroff (National Institutes of Health, Bethesda, MD). Cells were grown in DMEM with 6% fetal calf serum, 6% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and were kept at 37° in an atmosphere enriched in CO_2.

AA release. Cells in 12-well plates were incubated overnight with [^{3}H]AA (0.5 μCi/ml), except for time course studies where individual plates (60 × 15 mm) were used. The procedure was adopted from Burch *et al.* (10) with slight modifications. The labeled cells were washed three times at 5-min intervals with DMEM containing 20 mM HEPES (pH 7.6). After the final washing, cells were preincubated for 10 min in DMEM containing 20 mM HEPES (pH 7.6) and 0.03% bovine serum albumin. Agents were then added and cells were incubated for 30 min at 37°. At the end of incubation, 200 μl of medium were collected from each well and centrifuged for 1.5 min (12,000 × *g*). A 100-μl aliquot of supernatant was then counted in a liquid scintillation counter. Each set of experiments was done on the same day for consistency of the stage of growth and number of cells. All the experiments involving the calcium ionophore A23187 were done in the absence of bovine serum albumin because A23187 binds to bovine serum albumin (11). "Calcium-free" in AA release assays indicates that no calcium or chelating agent was added in the media. Results are means ± standard errors of three or more experiments performed in triplicate.

PI breakdown. Cells in 12-well plates were incubated overnight with [^{3}H]inositol (1 μCi/ml) in inositol-free medium. The labeled cells were washed once with buffer A (118 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). The cells were then preincubated for 10 min in buffer A containing 10 mM LiCl (osmolarity was maintained by reducing NaCl to 108 mM). Agents were then added and cells were incubated for 30 min at 37°. At the end of the incubation, cells were scraped from the plates and transferred to 1.5-ml microfuge tubes. After centrifugation for 1 min (12,000 × *g*), the supernatants were discarded and 1 ml of 6% trichloroacetic acid was added. The tubes were sonicated with a probe sonicator, vortexed, and centrifuged for 1.5 min (12,000 × *g*). The supernatant was applied to anion exchange columns (Bio-Rad AG 1 × 8, 100–200 mesh, formate form). The separation procedure by anion exchange chromatography was as described by Berridge *et al.* (12). For some experiments, HPLC was used for separation of inositol phosphates. For HPLC determination, 400 μl of 5% perchloric acid and 10 μl of a mixture of phytate hydrolysate (0.9 mg/ml P_i) (13), adenosine, and adenosine phosphates (2 mM of adenosine, AMP, ADP, and ATP) were added, instead of 1 ml of 6% trichloroacetic acid. Adenosine and adenosine phosphates were added to provide for monitoring HPLC by UV adsorption. The tubes were sonicated with a probe sonicator, vortexed, and centrifuged for 1.5 min (12,000 × *g*). The supernatant of each tube was transferred to another set of tubes and 350 μl of a mixture (1:1) of 1,1,2-trichloro-trifluoroethane (Freon) and tri-*n*-octylamine and 5 μl of phenol red were added to each tube. The tubes were shaken in a microfuge shaker for 5 min and centrifuged for 1.5 min (12,000 × *g*). Aliquots (300 μl) of the upper layer were transferred to autosampler vials, neutralized with 10 μl of 0.2 N NaOH, and then applied to the HPLC column. The separation procedure was as described (14). A Zorbax column (5-μm particle size) was utilized. The mobile phases were 0.01 M (solvent A) and 1.2 M (solvent B) ammonium phosphate. The elution solvent was a gradient with the following sequence: 100% solvent A (0–5 min), solvent B from 0 to 65% (5–50 min), solvent B from 65 to 0% (50–55 min), and 100% solvent A (55–75 min). Retention time for standards were as follows: [^{3}H]inositol mono-1-phosphate, 10.6 min; [^{3}H]inositol bis-1,4-phosphate, 19.7 min; [^{3}H]inositol tris-1,4,5-phosphate, 29.4 min. The [^{3}H]inositol mono-1-phosphate peak was followed by another [^{3}H]inositol monophosphate (12.0 min) assumed to be the [^{3}H]inositol mono-4-phosphate. In most experiments, the relative amounts of presumed [^{3}H]inositol mono-4-phosphate were about 60% of the amounts of [^{3}H]inositol mono-1-phosphate. The perchloric acid precipitate was used for the measurement of incorporation of [^{3}H]inositol into lipids. The pellet was suspended in 0.5 ml of a 1:1 mixture of 1 M KCl and 10 mM inositol in methanol. Chloroform (0.5 ml) was added to the suspension and lipids were extracted by shaking for 10 min. An aliquot of the chloroform layer (0.2 ml) was transferred to a scintillation vial and evaporated to dryness. Radioactivity in the lipid fraction was measured after adding 6 ml of Betafluor (National Diagnostics, Manville, NJ). The results were calculated as cpm of inositol phosphate/10,000 cpm of lipids (15). [^{3}H]Inositol phosphates in time course experiments were measured using anion exchange columns as described (15). "Calcium-free" in th PI breakdown assay indicates a final calcium concentration of 10 μM. Because buffer A contains 0.5 mM EDTA, calcium and magnesium concentrations had to be adjusted for "calcium-free" condition. All the data are means ± standard errors of three or more experiments performed in triplicate.

Uptake of $^{45}Ca^{2+}$. Cells labeled with [^{3}H]leucine for 2 days were plated in 6-well plates overnight. The labeled cells were preincubated for 10 min in 0.5 ml of a preincubation buffer (10 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM HEPES, pH 7.4 adjusted with Tris at 22°, 240 mM sucrose, and indicated channel blockers and channel activators). After the preincubation buffer was aspirated, 0.5 ml of an influx buffer, containing 150 NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, 50 mM HEPES (pH 7.4 adjusted with Tris at 22°), agents, and $^{45}CaCl_2$ (0.5 μCi/well), was added. After incubation for 2 min at 22°, cells were washed three times with a wash buffer (150 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES, pH 7.4 adjusted with Tris at 22°). The cells were solubilized with 0.5 ml of 1% sodium dodecyl sulfate/0.5 N NaOH at room temperature for 30 to 60 min. After the NaOH was neutralized with 0.25 ml of 1 N HCl, the radioactivity (^{3}H and ^{45}Ca) was determined using a scintillation counter set to measure ^{3}H/^{14}C. Results are means ± standard errors of three or more experiments performed in triplicate. The amount of [^{3}H]leucine incorporation allowed normalization for cell density in different wells.

Determination of $[Ca^{2+}]_i$. PC12 cells were suspended in buffer A (same as PI breakdown buffer without lithium) containing 10 μM fura-2 (cell-permeant form). After a 30-min incubation at 37°, cells were centrifuged and the supernatant was discarded. Cells were washed twice before fluorometric determinations were performed. Aliquots containing 1–2 × 10^6 cells were added to temperature-controlled cuvettes (37°). Fluorescence was determined in a Perkin Elmer LS-5 spectrofluorometer at an excitation wavelength of 354 nm and emission wavelength of 510 nm. Calibration was performed by obtaining maximum fluorescence after Triton X-100 lysis of cells and then after a subsequent addition of 10 mM EGTA. Results are means ± standard errors of seven experiments performed in triplicate.

Results

MTX caused both AA release and PI breakdown in a concentration-dependent manner in PC12 cells (Fig. 1). Stimulation of AA release can be detected at 0.03 ng/ml (9 pM) MTX (data not shown) and reaches a maximum at 0.5 ng/ml (150 pM). PI breakdown can be detected at 0.1 ng/ml (30 pM), reaches a maximum at about 1 ng/ml (300 pM), and returns to control levels at higher concentrations. Time courses of AA release (Fig. 2) and PI breakdown (Fig. 3) were studied. In the absence of MTX, release of [^{3}H]AA slowly increased from 261 ± 34 cpm at 0 min to 926 ± 58 cpm at 60 min; cpm in [^{3}H]inositol phosphates after 45 min were not significantly different from cpm in [^{3}H]inositol phosphates at 0 min (data not shown). PI breakdown was detected as early as 1 min of incubation with 0.2 ng/ml (60 pM), 0.5 ng/ml (150 pM), or 1 ng/ml (300 pM) MTX, whereas stimulation of AA release was not detected until after 20 min of incubation with the same concentrations of MTX. Formation of inositol monophosphate slowly increased with 0.5 ng/ml MTX, reaching a plateau by 30 min,

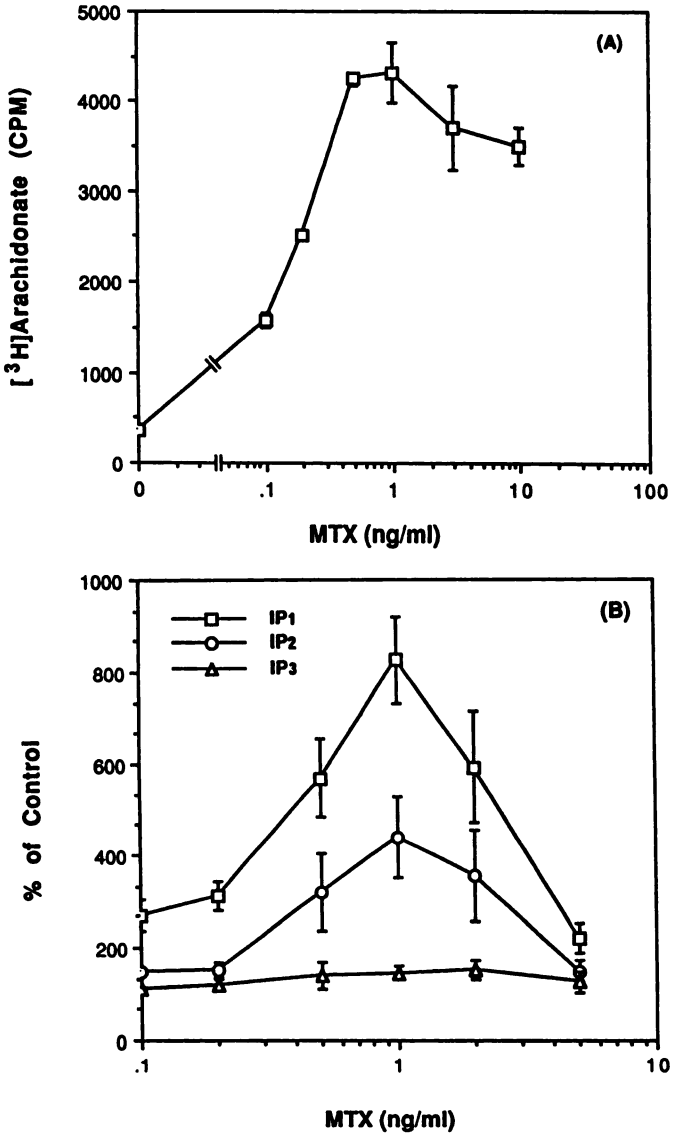

Fig. 1. Concentration response of MTX for [^{3}H]AA release (A) and formation of [^{3}H]inositol phosphate (B) in PC12 cells. Cells prelabeled with either [^{3}H]AA (A) or [^{3}H]inositol (B) were incubated with different concentrations of MTX for 30 min. [^{3}H]Inositol monophosphate was measured by anion exchange chromatography (see Experimental Procedures). Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*. IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; IP_1, inositol monophosphate.

whereas maximal stimulation of inositol monophosphate formation with 1 ng/ml MTX was reached in 5 min. Formation of the various inositol phosphate isomers was measured by HPLC after 1-min incubations with 0.3 ng/ml and 1 ng/ml MTX (Table 1). Inositol mono-4-phosphate increased to the greatest extent, suggesting that inositol tris-1,4,5-phosphate had been mainly metabolized to inositol mono-4-phosphate within 1 min. It should be noted that, although results with MTX within a set of experiments were highly reproducible, results with batches of cells at different stages of growth or with cells at different passages showed more variability.

Stimulation of AA release, PI breakdown, and calcium influx induced by MTX were differentially inhibited by organic and inorganic calcium channel blockers. Organic calcium channel

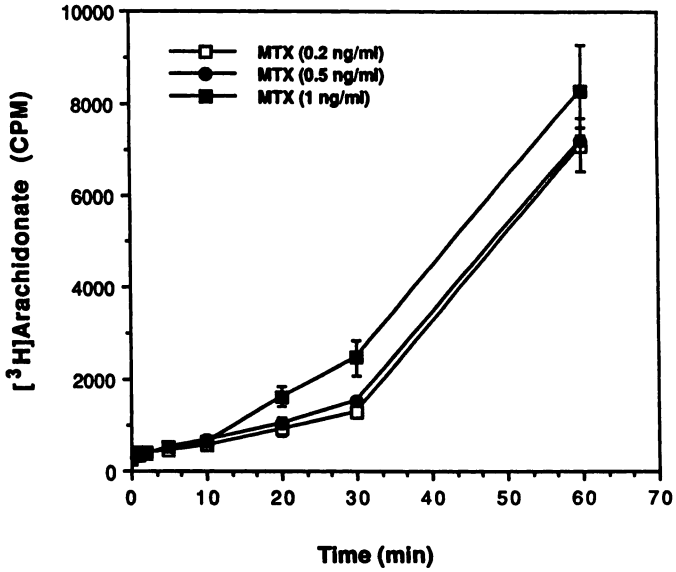

Fig. 2. Time course for [^{3}H]AA release in the presence of MTX in PC12 cells. Cells prelabeled with [^{3}H]AA were incubated with 0.2, 0.5, and 1 ng/ml MTX for the indicated period of time. Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

blockers have no effect on stimulation of either AA release or PI breakdown by MTX, while partially blocking MTX-induced calcium influx (Table 2). Nifedipine (1 μM) had no effect on AA release elicited by MTX over a range of concentrations from 0.1 to 3 ng/ml (data not shown). All the inorganic calcium channel blockers tested (Cd^{2+}, Mn^{2+}, Co^{2+}) inhibited AA release and calcium influx, whereas only Cd^{2+} and a high concentration (5.0 mM) of Mn^{2+} inhibited PI breakdown (Table 2).[1] Cd^{2+} inhibited both AA release, PI breakdown, and calcium influx in a concentration-dependent manner (Fig. 4). The shallow concentration-response curves of Cd^{2+} for inhibition of MTX-elicited AA release and calcium influx are in contrast to the concentration-response curve for inhibition of MTX-elicited PI breakdown. MTX-stimulated AA release, PI breakdown, and calcium influx were measured in the absence and presence of Cd^{2+} (Fig. 5). Blockade of MTX-stimulated AA release and PI breakdown by Cd^{2+} was noncompetitive, whereas blockade of MTX-stimulated calcium influx was overcome by increasing the concentration of MTX, suggesting a pseudocompetitive effect. Nifedipine (1 μM) inhibited the increase in concentration of intracellular calcium measured with fura-2 from $\Delta[Ca^{2+}]_i$ of 192 ± 23 nM (mean ± standard error, seven experiments) with 1 ng/ml MTX alone to $\Delta[Ca^{2+}]_i$ of 47 ± 12 nM (mean ± standard error, seven experiments) in the presence of nifedipine. The effects of nifedipine (1 μM) on MTX-elicited calcium influx, intracellular calcium concentration, PI breakdown, and AA release are summarized in Fig. 6. The inhibitory effects of nifedipine on the MTX-stimulated increase in calcium uptake and intracellular calcium are in marked contrast to the lack of effect of nifedipine on MTX-stimulated AA release and PI breakdown. Clearly, the magnitude of calcium influx elicited by MTX does not correlate with the magnitude of effects of MTX on PI breakdown or AA release, i.e., with nifedipine (Fig. 6) and with inorganic calcium blockers such as cadmium (Fig.

[1] $CdCl_2$ (1 mM) was not effective in inhibiting MTX-induced PI breakdown in NCB-20 cells (14).

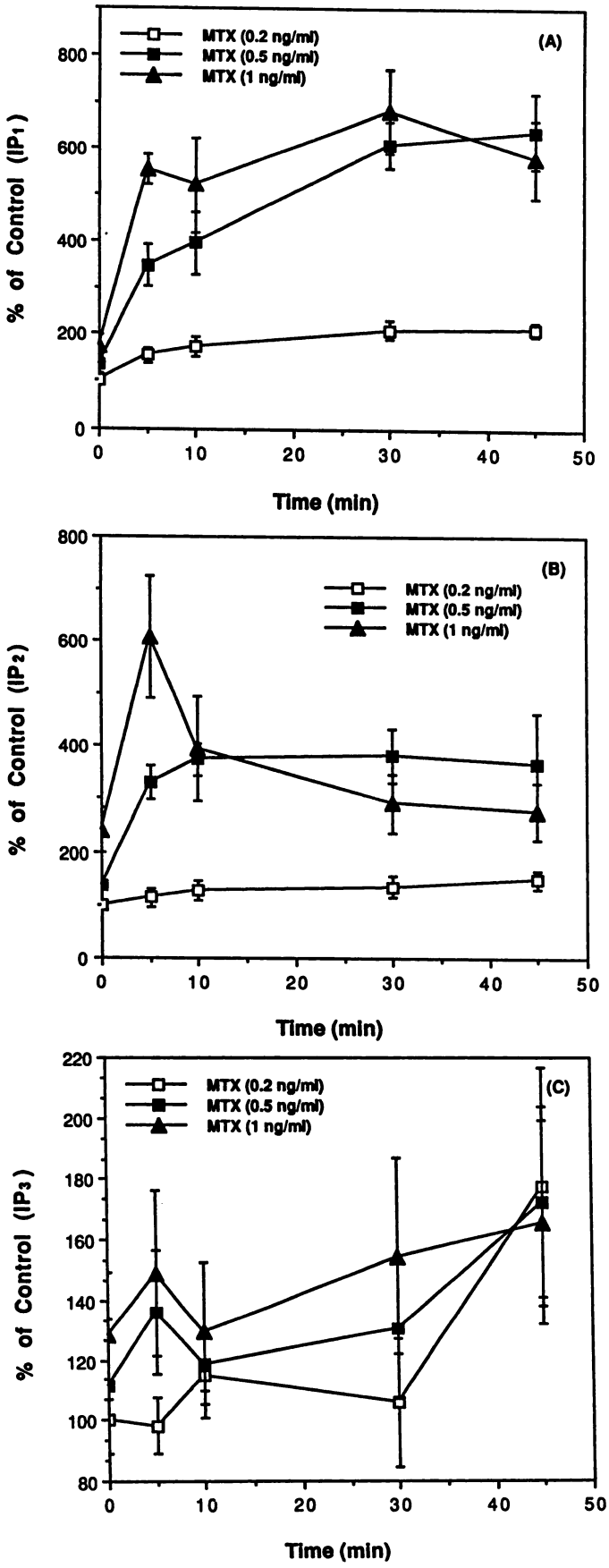

Fig. 3. Time course of [^{3}H]inositol phosphate formation in the presence of MTX in PC12 cells. Cells prelabeled with [^{3}H]inositol were incubated with 0.2, 0.5, and 1 ng/ml MTX for the indicated period of time. [^{3}H] Inositol phosphates were measured by anion exchange chromatography (see Experimental Procedures). A, [^{3}H]Inositol monophosphate; B, [^{3}H] inositol bisphosphate; C, [^{3}H]inositol trisphosphate. Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

4) the MTX-elicited calcium influx can be markedly reduced with no effect on MTX-elicited PI breakdown.

The calcium dependency for MTX-induced AA release and PI breakdown was determined (Fig. 7). In calcium-free media, the potency of MTX for stimulation of AA release was markedly reduced. A further reduction in potency occurred in the presence of EDTA but, at a concentration of 900 pM, MTX still elicited AA release (Fig. 7A). This is in contrast to the lack of effect of MTX on PI breakdown in the presence of EDTA (Fig. 7B). The potency of MTX for stimulation of AA release was also reduced by the absence of sodium in the medium (Fig. 7A), whereas the potency of MTX for stimulation of PI breakdown was not changed by the absence of sodium in the medium (Fig. 7B). Stimulation of AA release by MTX was maximal at a concentration range of 2–5 mM calcium and returned towards control levels at higher concentrations of calcium (Fig. 8).

Effects of the calcium ionophore A23187 on basal and MTX-stimulated AA release and PI breakdown were studied in regular and calcium-free medium. A23187 (10 μM) markedly potentiated MTX-stimulated AA release in regular medium (Fig. 9A), whereas only a slight potentiation was observed in the calcium-free medium (Fig. 9B). MTX-induced PI breakdown was almost completely inhibited by coincubation with 10 μM A23187 in regular medium (Fig. 9C), whereas in calcium-free medium there was a slight potentiation with the combination of MTX and A23187 (Fig. 9D).

Quinacrine, a putative phospholipase A_2 inhibitor, inhibited with similar potency MTX-induced AA release, PI breakdown, and calcium influx (Fig. 10).

Discussion

MTX activates voltage-dependent calcium channels, as shown by calcium influx (1–3). Such activation appears to be responsible for stimulation by MTX of neurotransmitter release, contraction of smooth muscle, and stimulation of cardiac function, because such effects of MTX are blocked by organic blockers of voltage-dependent calcium channels. MTX also stimulates PI breakdown in many, if not all, cell types (4–8). The stimulatory effects of MTX on PI breakdown occur at concentrations of MTX as low as 0.1 ng/ml (30 pM) and are not blocked by organic calcium channel blockers. However, effects of MTX on the phospholipases catalyzing hydrolysis of PIs to inositol phosphates and diacylglycerides are dependent on extracellular calcium, suggesting that MTX at concentrations of 100–1500 pM may merely elicit an influx of calcium and that the calcium then stimulates phospholipase C. As yet, MTX has not been shown to have a direct effect on phospholipase C in membrane preparations (6, 16). MTX has no ionophore activity (17) and the high potency suggests interaction with a specific calcium transport system. Lack of blockade by classical channel blockers suggests that stimulation by MTX of voltage-dependent calcium channels is not involved. In view of the fact that other phospholipases in cell membranes, such as phospholipase A_2, are also calcium dependent, the effects of MTX on both hydrolysis of PIs by phospholipase C to inositol

TABLE 1

MTX-elicited formation of [^{3}H]inositol phosphate isomers in PC12 cells

Cells prelabeled with [^{3}H]inositol were preincubated, as described in Experimental Procedures, in buffer A containing LiCl for 10 min and then MTX was added. After 1 min of incubation, [^{3}H]inositol phosphate isomers were measured by HPLC (see Experimental Procedures). Results are mean ± standard error (three experiments). Statistically significant differences from control values were determined by Student's t-test.

MTX	Inositol mono-1-phosphate	Inositol mono-4-phosphate	Inositol bisphosphate	Inositol tris-1,3,4-phosphate	Inositol tris-1,4,5-phosphate
ng/ml			*% of control*		
0.3	154 ± 14[a]	196 ± 30[b]	152 ± 25[b]	135 ± 21	133 ± 43
1	267 ± 61[b]	402 ± 85[b]	279 ± 42[a]	145 ± 38	212 ± 22[a]

[a] $p \leq 0.01$.
[b] $p \leq 0.05$.

TABLE 2

Effects of calcium channel blockers on MTX-induced AA release, PI breakdown, and calcium uptake in PC12 cells

Calcium channel blockers	AA release[a] (MTX, 0.3 ng/ml)	PI breakdown[b] (MTX, 0.5 ng/ml)	$^{45}Ca^{2+}$ uptake[c] (MTX, 0.5 ng/ml)
	% of the response of MTX alone		
Nifedipine (1 μM)	106 ± 9	85 ± 4	60 ± 6
Nifedipine (10 μM)	97 ± 1	123 ± 2	49 ± 6
Verapamil (10 μM)	69 ± 4	140 ± 30	34 ± 5
Diltiazem (10 μM)	71 ± 3	114 ± 10	56 ± 10
$CoCl_2$ (1 mM)	22 ± 4	121 ± 41	54 ± 10
$MnCl_2$ (1 mM)	30 ± 8	70 ± 15	38 ± 10
$MnCl_2$ (5 mM)	4 ± 2	41 ± 2	−3 ± 6
$CdCl_2$ (1 mM)	24 ± 3	2 ± 2	−3 ± 4

[a] Cells prelabeled with [^{3}H]AA were preincubated as described in Experimental Procedures for 10 min and then MTX and calcium channel blockers were added. After 30 min of incubation at 37°, an aliquot of the incubation medium was collected and counted for radioactivity. Results are means ± standard error (three experiments or more).

[b] Cells prelabeled with [^{3}H]inositol were preincubated, as decscribed in Experimental Procedures, in buffer A containing LiCl for 10 min and then MTX and calcium channel blockers were added. After 30 min of incubation, [^{3}H]inositol mono-1-phosphate was measured by HPLC (see Experimental Procedures). Results are mean ± standard error (three experiments).

[c] Cells were preincubated in preincubation mixture with agents for 10 min at 22° and then $^{45}Ca^{2+}$ was added for 2 min as described in Experimental Procedures. After incubations were stopped, cells were washed three times and solubilized with 0.5 ml of 1% sodium dodecyl sulfate/0.5 N NaOH added for 30 to 60 min. Values are means ± standard error (three experiments).

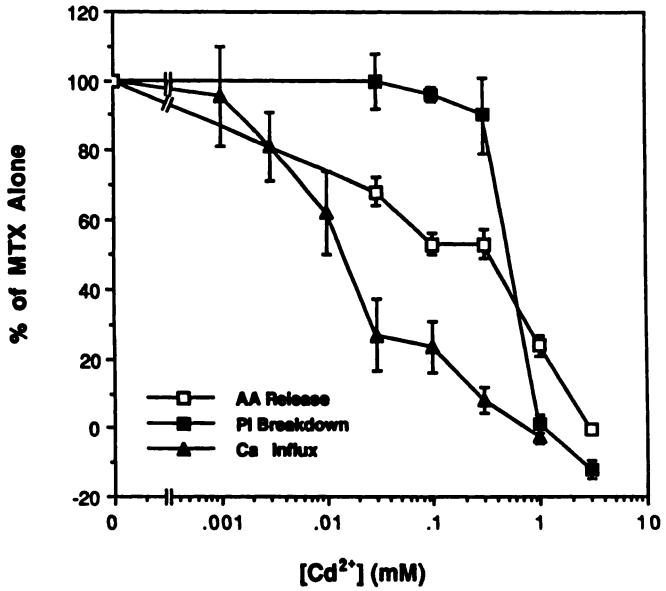

Fig. 4. Blockade of MTX-induced [^{3}H]AA release (□), PI breakdown (■), and calcium influx (▲) by $CdCl_2$ in PC12 cells. Cells prelabeled with [^{3}H]AA for AA release, [^{3}H]inositol for PI breakdown, or [^{3}H]leucine for calcium influx experiments were incubated for 30 min with 0.3 ng/ml (for AA release) or 0.5 ng/ml (for PI breakdown) MTX or for 2 min with 0.5 ng/ml (for calcium influx) in the presence of different concentrations of $CdCl_2$. [^{3}H]Inositol mono-1-phosphate was measured by HPLC. For calcium influx experiments, cells were incubated with agents and $^{45}Ca^{2+}$ for 2 min. Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

phosphates and on hydrolysis of phospholipids by phospholipase A_2 to AA have been compared in pheochromocytoma PC12 cells. In addition, the effects of MTX on $^{45}Ca^{2+}$ influx and on internal levels of calcium were determined.

MTX caused marked stimulation of AA release in PC12 cells. Stimulation of AA release occurred at lower concentration of MTX than the concentration required for PI breakdown; MTX-induced AA release reached maximum at 0.5 ng/ml MTX, whereas MTX-induced PI breakdown reached a maximum at 1 ng/ml (Fig. 1). However, PI breakdown was detected earlier (1 min of incubation with MTX) (Table 1) than stimulation of AA release (20 min of incubation with MTX) (Fig. 2). MTX-induced AA release reached maximum at calcium concentrations of 2 to 5 mM (Fig. 8). In the case of PI breakdown in PC12 cells, stimulation by MTX has been shown to be maximal at 1.5 mM calcium for [^{3}H]inositol monophosphate formation, 2.5 mM for [^{3}H]inositol bisphosphate formation, and 0.5 mM for [^{3}H]inositol trisphosphate formation and to return to control level at higher concentrations of calcium (13). The MTX concentration-response curve for stimulation of AA release was shifted to the right in the absence of extracellular calcium and was further shifted to the right with EDTA (500 μM). However, at high concentrations, MTX still stimulated AA release even in the presence of EDTA (Fig. 7A). Furthermore, the concentration of Cd^{2+} at which calcium influx was completely blocked did not completely block AA release (Fig. 5). Calcium is essential for the activation of mammalian membrane phospholipase A_2 (18), suggesting that EDTA or Cd^{2+} cannot completely remove calcium essential for MTX-elicited activation of this enzyme.

In contrast to the stimulatory effects of MTX on AA release in the absence of any extracellular calcium, MTX at 150 pM did not stimulate PI breakdown in the presence of EDTA, suggesting either that calcium influx is required or that calcium, which is essential for the action of MTX on PI breakdown, is removed by EDTA. Concentrations of Cd^{2+} that completely blocked MTX-elicited influx of $^{45}Ca^{2+}$ also blocked the stimulatory effects of MTX on PI breakdown (Fig. 5). Thus, the effects of MTX on PI breakdown are more dependent on extracellular calcium than are the effects of MTX on AA release. Whether MTX can, in high concentrations, mobilize membranal or intracellular calcium, resulting in AA release but not PI breakdown, is unknown.

MTX-induced calcium influx in PC12 cells was not completely inhibited by nifedipine (Table 2), whereas 50 mM KCl-

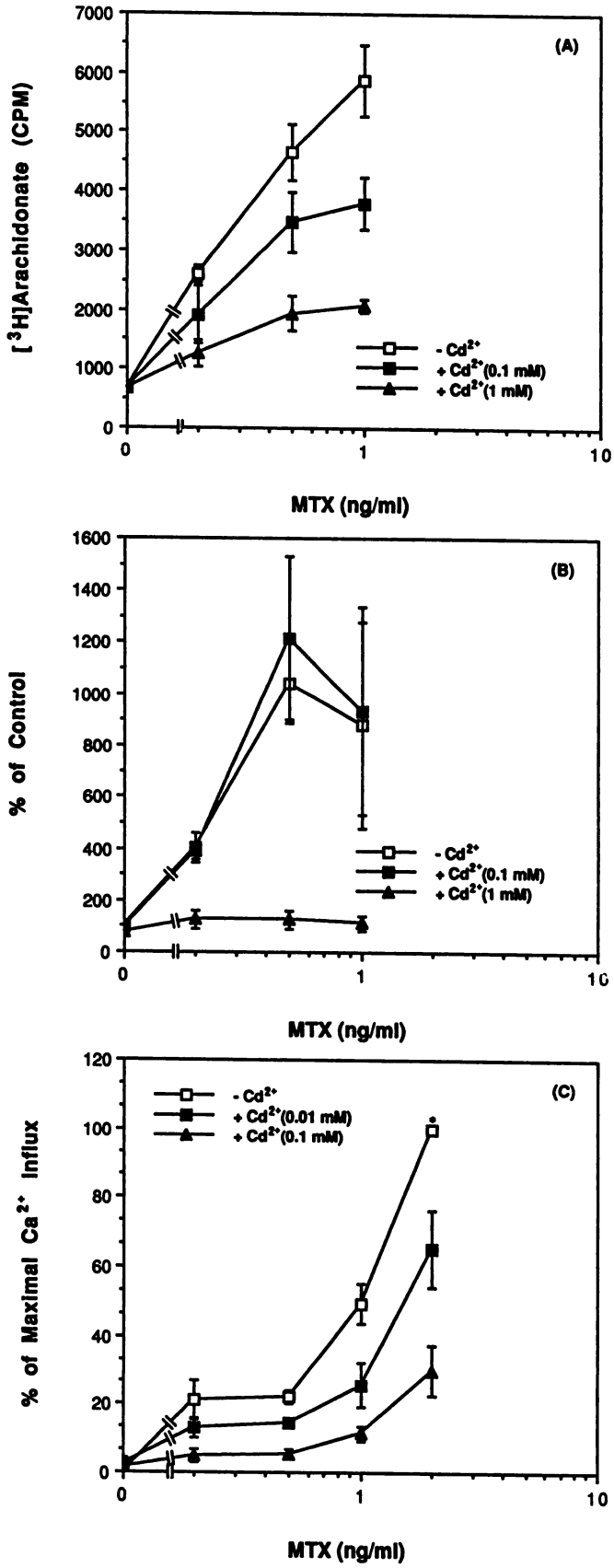

Fig. 5. Concentration response of MTX for AA release (A), PI breakdown (B), and calcium influx (C) in the presence of various concentrations of Cd^{2+} in PC12 cells. Cells prelabeled with either [^{3}H]AA, [^{3}H]inositol, or [^{3}H]leucine were incubated with MTX for 30 min for AA release and PI breakdown or 2 min for calcium influx. B, [^{3}H]Inositol mono-1-phosphate was measured by HPLC. C, The *asterisk* indicates the point assigned a value of 100%. Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

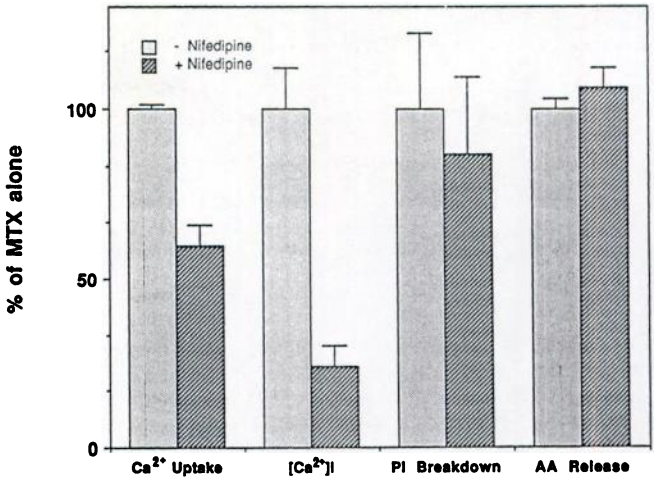

Fig. 6. MTX-induced $^{45}Ca^{2+}$ uptake, increase in concentration of intracellular Ca^{2+}, PI breakdown, and [^{3}H]AA release in the absence and presence of nifedipine (1 μM) in PC12 cells. Either 0.3 ng/ml (AA release), 0.5 ng/ml (PI breakdown, $^{45}Ca^{2+}$ influx), or 1 ng/ml ($[Ca^{2+}]_i$) MTX was used. For assays see Experimental Procedures.

induced calcium influx was completely inhibited by nifedipine (data not shown). Thus, in PC12 cells, MTX elicits an influx of calcium both through activation of L-type calcium channels that are sensitive to blockade by nifedipine and through activation of other channels or mechanisms. High potassium apparently activates only nifedipine-sensitive L-type calcium channels and has only slight effects on PI breakdown (data not shown). MTX-induced AA release and PI breakdown were not inhibited at all by nifedipine or by two other organic calcium channel blockers, namely verapamil and diltiazem (Table 2). Clearly, MTX-induced AA release and PI breakdown are not dependent on calcium influx through L-type calcium channels. Quinacrine is often purported to be a selective phospholipase A_2 inhibitor but, in the present study, it inhibited equally effectively both MTX-elicited AA release, PI breakdown, and calcium influx (Fig. 10). Thus, quinacrine should be used cautiously, if at all, as a selective phospholipase A_2 inhibitor.

The data do not provide clear insight into the mechanisms of stimulation of calcium channels, phospholipase C, and phospholipase A_2 by MTX. One hypothesis would be that MTX interacts with a high affinity site associated with calcium channels and/or calcium transporters and that transport of calcium to phospholipases results in activation of such enzymes. As a corollary of this hypothesis, the transporters associated with activation of phospholipase C would be entirely dependent on availability of extracellular calcium, whereas transporters associated with phospholipase A_2 could to some degree utilize membranal or intracellular calcium for the activation of the enzyme. It is possible that calcium would act at an intramembrane site rather than an intracellular site to activate phospholipases. Consonant with this proposal, it was reported that calcium-dependent PI breakdown with MTX occurs in frog oocytes even after microinjection of the oocytes with EGTA (19). Furthermore, MTX elicits PI breakdown in HL-60 cells

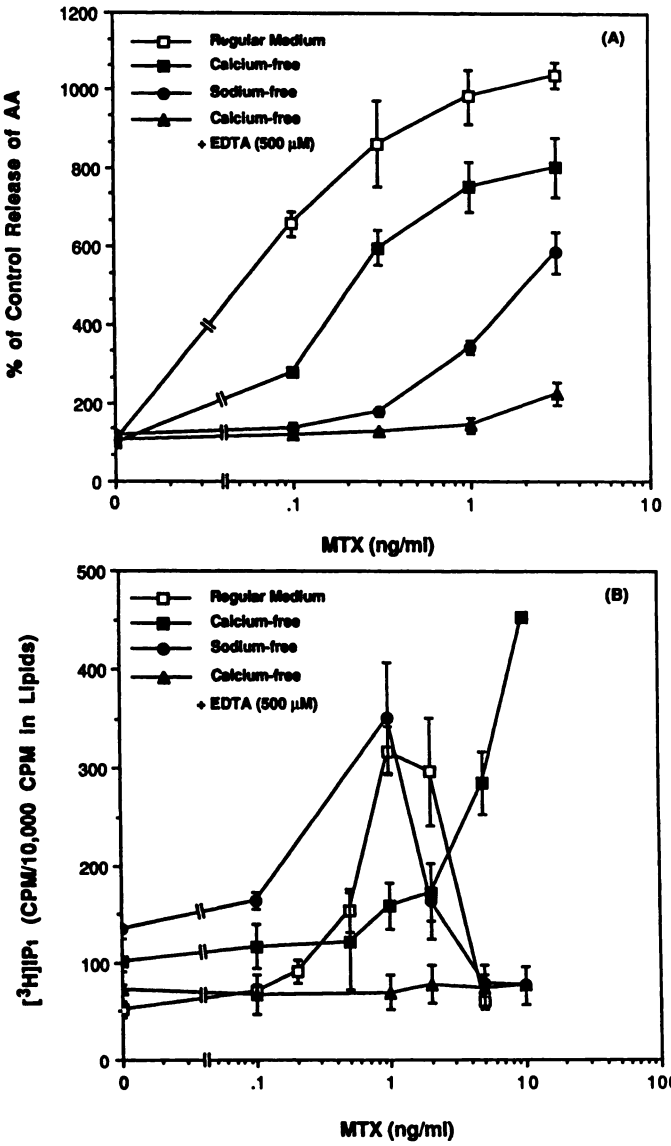

Fig. 7. Concentration response of MTX for [^{3}H]AA release (A) and for PI breakdown (B) in regular (DMEM containing 20 mM HEPES), calcium-free sodium-free, or calcium-free plus EDTA medium in PC12 cells. Cells prelabeled with [^{3}H]AA were incubated with different concentrations of MTX for 30 min in the indicated medium. In sodium-free medium, osmolarity was adjusted with choline chloride (110 mM) and sucrose (130 mM) for AA release and or choline chloride (118 mM) for PI breakdown. [^{3}H]Inositol mono-1-phosphate was measured by HPLC (see Experimental Procedures). Results are means ± standard errors (three experiments except for B, last point in calcium-free). No *error bars* indicate standard error less than the *size* of *symbols*.

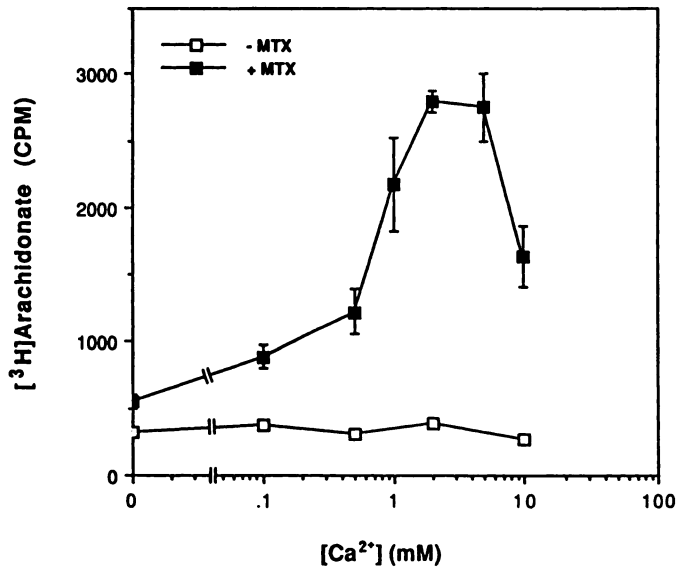

Fig. 8. Effects of calcium concentration on [^{3}H]AA release in the presence (■) or absence (□) of MTX in PC12 cells. Cells prelabeled with [^{3}H]AA were incubated for 30 min with or without 0.3 ng/ml MTX in the presence of different concentrations of calcium. The indicated concentrations of calcium were based on addition of calcium to Ca^{2+}-free medium. Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

even under conditions where intracellular calcium is chelated by BAPTA (16).

MTX has been suggested to be a pore-forming agent (7, 20). However, other studies even with very high concentrations of MTX demonstrated no pore-forming or ionophore activity for MTX in liposomes or mitochondria (17). Because of its size and polarity, it appears unlikely that MTX will even penetrate across plasma membranes of cells. The present study provides further data suggesting that MTX does not act as an ionophore in stimulating AA release or PI breakdown. Thus, the calcium channel ionophore A23187 had no effect on the basal AA release and PI breakdown (Fig. 9), demonstrating that MTX must act in a different manner than this ionophore to stimulate phospholipid metabolism in PC12 cells. Furthermore, the ionophore A23187 significantly potentiated MTX-stimulated AA release in both regular and calcium-free medium, whereas MTX-stimulated PI breakdown was markedly inhibited by A23187. A mechanism to account for the differential effects of A23187 on MTX-stimulated AA release and PI breakdown in PC12 cells is not clear.

Interestingly, absence of extracellular sodium markedly reduced the potency of MTX with respect to AA release (Fig. 7A), while not reducing the potency of MTX to stimulate PI breakdown (Fig. 7B). The data indicate that extracellular sodium is necessary for AA release, but not for PI breakdown. It has been noted that MTX-induced calcium influx into NG108-15 cells was decreased in sodium-free medium (3). However, MTX was found to have no effect on sodium flux in these cells and the involvement of sodium in MTX-elicited opening of voltage-sensitive calcium channels (3) and in MTX-stimulated AA release remains unexplained.

Possible interrelationships between MTX-elicited channel activation, PI breakdown by phospholipase C, and AA release by phospholipase A_2 in intact cells clearly remain poorly defined. One consideration is whether the early activation of PI breakdown by MTX could lead to activation of phospholipase A_2. Receptor control of phospholipase A_2 and phospholipase C have been shown to be independent in thyroid cells (21). However, in the case of MTX, activation of protein kinase C by the PI breakdown product diacylglycerol (see Ref. 14) might lead to enhancement of AA release, in analogy to the stimulatory effects on AA release of exogenous activators (phorbol esters) of protein kinase C (22, 23). And it has been proposed that diacylglycerol itself, independent of activation of protein kinase C, induces AA release in 3T3 fibroblasts (24). Although not conclusive, the present data do not support a relationship between the rapid effects of MTX on PI breakdown and the

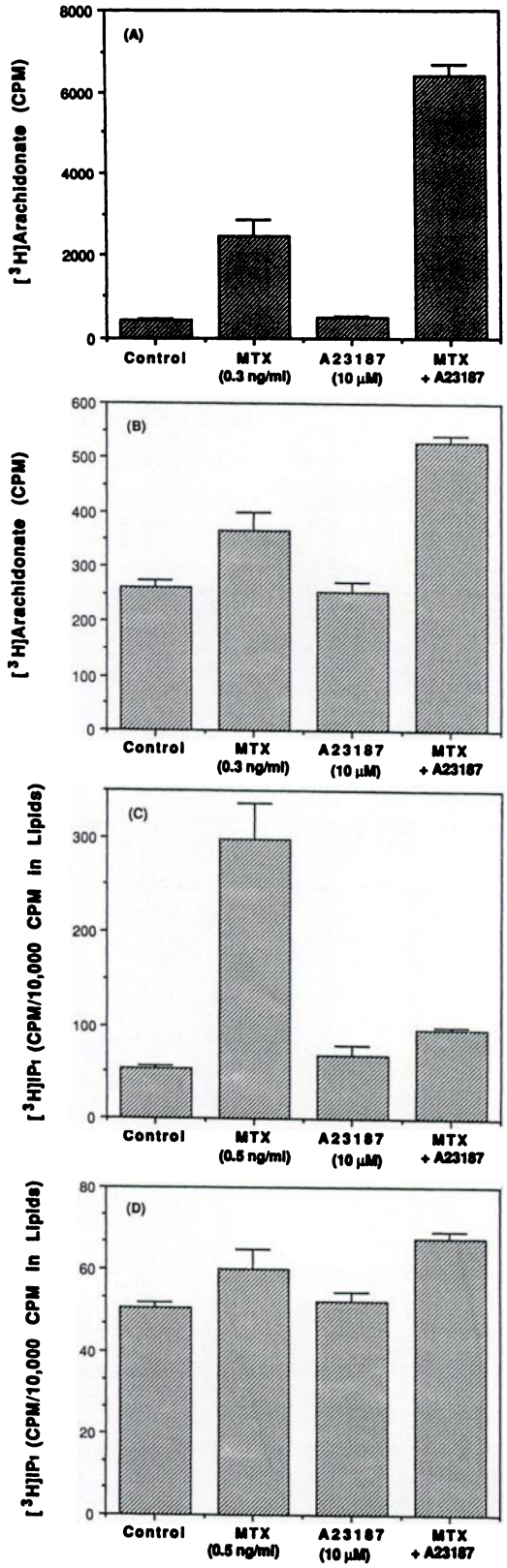

Fig. 9. Effects of calcium ionophore A23187 on MTX-induced [^{3}H]AA release (A and B) and [^{3}H]inositol mono-1-phosphate formation (C and D) in PC12 cells. Experiments were performed in regular (A and C) or calcium-free medium (B and D). Cells prelabeled with either [^{3}H]AA (A and B) or [^{3}H]inositol (C and D) were incubated with agents for 30 min. For A and B, bovine serum albumin was not included in the incubation medium. [^{3}H]Inositol mono-1-phosphate was measured by HPLC (see Experimental Procedures). Results are means ± standard errors (three experiments).

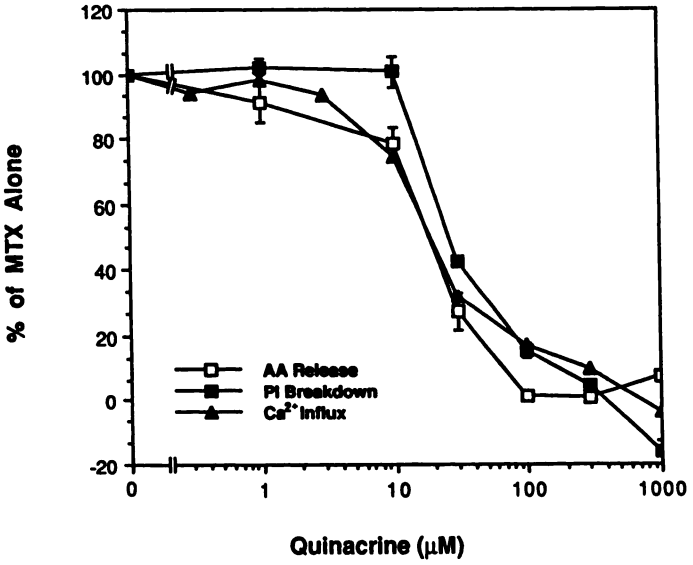

Fig. 10. Concentration-dependent blockade by quinacrine of MTX-induced [^{3}H]AA release (□), PI breakdown (■), and calcium influx (▲) in PC12 cells. Cells prelabeled with either [^{3}H]AA, [^{3}H]inositol, or [^{3}H]leucine were incubated with MTX (0.3 ng/ml for AA release and 0.5 ng/ml for PI breakdown and calcium influx) for 30 min (for AA release and PI breakdown) or 2 min (for calcium influx) with $^{45}Ca^{2+}$ (see Experimental Procedures). [^{3}H]Inositol mono-1-phosphate was measured by HPLC (see Experimental Procedures). Results are means ± standard errors (three experiments). No *error bars* indicate standard error less than the *size* of *symbols*.

slower effects on AA release. Thus, MTX stimulates AA release at lower concentrations than PI breakdown. Furthermore, at high concentrations, MTX can still stimulate AA release, while having minimal effects on PI. Finally, EDTA completely blocks MTX-elicited PI breakdown, whereas EDTA only reduces potency of MTX with respect to AA release. If an increase in the intracellular calcium level due to inositol trisphosphate formation was involved in MTX-induced AA release, then a calcium ionophore alone also should stimulate AA release. The ionophore A23187 alone did not stimulate AA release (Fig. 9A). Finally, if both calcium and diacylglycerol, products of MTX-elicited PI breakdown, are involved in AA release, then incubation of cells with a combination of calcium ionophore and phorbol ester should stimulate AA release; this treatment had no effect on AA release (data not shown).

At present, three effects of MTX observed in intact cells are (i) activation of calcium channels, leading to calcium influx, (ii) activation of phospholipase C, leading to PI breakdown, and (iii) activation of phospholipase A_2, leading to AA release. A comparison of dose-response relationships, time courses, dependence on extracellular calcium, effects of inorganic and organic calcium channel blockers, and interactions with calcium ionophores suggests that different mechanisms and/or sites are involved in the three effects of MTX. However, it is possible that a single high affinity site for MTX involved with calcium translocation subserves all effects of MTX. The delineation of molecular mechanisms involved in activation of calcium channels in most if not all cells, PI breakdown in all cells

Experimental Procedures). Results are means ± standard errors (three experiments).

as yet tested, and AA release undoubtedly will require development of cell-free systems and a radiolabeled MTX. As yet cell-free systems in which MTX has effects on phospholipid metabolism have not been developed (6, 16).

References

1. Takahashi, M. Y., Ohizumi, and T. Yasumoto. Maitotoxin, a Ca^{2+} channel activator candidate. *J. Biol. Chem.* **257**:7287–7289 (1982).
2. Ohizumi, Y., and T. Yasumoto. Contraction and increase in tissue calcium content induced by maitotoxin, the most potent known marine toxin, in intestinal smooth muscle. *Br. J. Pharmacol.* **79**:3–5 (1983).
3. Freedman, S. B., R. J. Miller, D. M. Miller, and D. R. Tindall. Interactions of maitotoxin with voltage-sensitive calcium channels in cultured neuronal cells. *Proc. Natl. Acad. Sci. USA* **81**:4582–4585 (1984).
4. Gusovsky, F., T. Yasumoto, and J. W. Daly. Maitotoxin, a potent general activator of phosphoinositide breakdown. *FEBS Lett.* **243**:307–312 (1989).
5. Berta, P., F. Sladeczek, J. Derancourt, M. Durand, P. Travo, and J. Haiech. Maitotoxin stimulates the formation of inositol phosphates in rat aortic myocytes. *FEBS Lett.* **197**:349–352 (1986).
6. Gusovsky, F., T. Yasumoto, and J. W. Daly. Maitotoxin stimulates phosphoinositide breakdown in neuroblastoma hybrid NCB-20 cells. *Cell. Mol. Neurobiol.* **7**:317–322 (1987).
7. Sladeczek, F., B. H. Schmid, R. Alonso, L. Vian, A. Tep, T. Yasumoto, R. N. Cory, and J. Bockaert. New insights into maitotoxin action. *Eur. J. Biochem.* **174**:663–670 (1988).
8. Gusovsky, F., J. W. Daly, T. Yasumoto, and E. Rojas. Differential effects of maitotoxin on ATP secretion and on phosphoinositide breakdown in rat pheochromocytoma cells. *FEBS Lett.* **233**:139–142 (1988).
9. Yokoyama, A., M. Murata, Y. Oshima, T. Iwashita, and T. Yasumoto. Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. *J. Biochem.* **104**:184–187 (1988).
10. Burch, R. M., A. Luini, D. E. Mais, D. Corda, J. Y. Vanderhoek, L. D. Kohn, and J. Axelrod. α_1-Adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. *J. Biol. Chem.* **261**:11236–11241 (1986).
11. Hoffman, T., E. F. Lizzio, J. Suissa, D. Rotrosen, J. A. Sullivan, G. L. Mandell, and E. Bonvini. Dual stimulation of phospholipase activity in human monocytes: role of calcium-dependent and calcium-independent pathways in arachidonic acid release and eicosanoid formation. *J. Immunol.* **140**:3912–3918 (1988).
12. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**:473–482 (1983).
13. Wreggett, K. A., L. R. Howe, J. P. Moore, and P. F. Irvine. Extraction and recovery of inositol phosphates from tissues. *Biochem. J.* **245**:933–934 (1987).
14. Gusovsky, F., T. Yasumoto, and J. W. Daly. Calcium-dependent effects of maitotoxin on phosphoinositide breakdown and on cyclic AMP accumulation in PC12 and NCB-20 cells. *Mol. Pharmacol.* **36**:44–53 (1989).
15. Gusovsky, F., and J. W. Daly. Formation of inositol phosphates in synaptoneurosomes of guinea pig brain: stimulatory effects of receptor agonists, sodium channel agents and sodium and calcium ionophores. *Neuropharmacology* **27**:95–105 (1988).
16. Gusovsky, F., J. A. Bitran, T. Yasumoto, and J. W. Daly. Mechanism of maitotoxin-stimulated phosphoinositide breakdown in HL-60 cells. *J. Pharmacol. Exp. Ther.*, in press.
17. Takahashi, M., M. Tatsumi, Y. Ohizumi, and T. Yasumoto. Ca^{2+} channel activating function of maitotoxin, the most potent marine toxin known, in clonal rat pheochromocytoma cells. *J. Biol. Chem.* **258**:10944–10949 (1983).
18. Bormann, B. J., C.-K. Huang, W. M. Mackin, and E. L. Becker. Receptor-mediated activation of a phospholipase A_2 in rabbit neutrophil plasma membrane. *Proc. Natl. Acad. Sci. USA* **81**:767–770 (1984).
19. Bernard, V., A. Laurent, J. Derancourt, M. Clement-Durand, A. Picard, C.L. Peuch, P. Berta, and M. Doree. Maitotoxin triggers the cortical reaction and phosphatidylinositol-4,5-bisphosphate breakdown in amphibian oocytes. *Eur. J. Biochem.* **174**:655–672 (1988).
20. Yoshii, M., A. Tsunoo, Y. Kuroda, C. H. Wu, and T. Narahashi. Maitotoxin-induced membrane current in neuroblastoma cells. *Brain Res.* **424**:119–125 (1987).
21. Burch, R. M., A. Luini, and J. Axelrod. Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. USA* **83**:7201–7205 (1986).
22. Burch, R. M., and D. A. Kniss. Modulation of receptor-mediated signal transduction by diacylglycerol mimetics in astrocytes. *Cell. Mol. Neurobiol.* **8**:251–257 (1988).
23. Ho, A. K., and D. C. Klein. Activation of α_1-adrenoceptors, protein kinase C, or treatment with intracellular free Ca^{2+} elevating agonists increases pineal phospholipase A_2 activity. *J. Biol. Chem.* **262**:11764–11770 (1987).
24. Burch, R. M. Diacylglycerol stimulates phospholipase A_2 from Swiss 3T3 fibroblasts. *FEBS Lett.* **234**:283–286 (1988).

Send reprint requests to: Oksoon H. Choi, LBC, NIDDK, NIH, Bldg. 8A, Rm. 1A19, Bethesda, MD 20892.