0026-895X/89/010044-10$02.00/0

MOLECULAR PHARMACOLOGY, 36:44-53

Calcium-Dependent Effects of Maitotoxin on Phosphoinositide Breakdown and on Cyclic AMP Accumulation in PC12 and NCB-20 Cells

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Received December 14, 1988; Accepted March 31, 1989

SUMMARY

The marine dinoflagellate toxin maitotoxin (MTX) stimulates phosphoinositide breakdown in pheochromocytoma PC12 cells and in neuroblastoma hybrid NCB-20 cells. In both cell lines, the stimulation of phosphoinositide breakdown by MTX is dependent on extracellular calcium, but it is not reduced by organic or inorganic calcium channel blockers. In PC12 cells, the maximal stimulation of phosphoinositide breakdown occurs at 1.5 mM $[Ca^{2+}]_o$, whereas in NCB-20 cells the maximal stimulation is observed at 2.5–4.5 mM $[Ca^{2+}]_o$. Phosphoinositide breakdown is known to lead to formation of both inositol phosphates and diacylglycerols. The latter, through stimulation of protein kinase C, would, like phorbol esters, be expected to augment cyclic AMP accumulation in PC12 cells and to inhibit receptor-mediated cyclic AMP accumulation in NCB-20 cells. MTX does potentiate forskolin-induced accumulation of cyclic AMP in PC12 cells and does inhibit prostaglandin E_2-induced accumulation of cyclic AMP in NCB-20 cells. The effects of MTX on accumulation of cyclic AMP are calcium dependent and the concentrations of calcium required for maximal responses are the same as the ones required for maximal stimulation of phosphoinositide breakdown. MTX increases intracellular calcium in both cell lines, as measured by calcium-quin2 fluorescence. But the effects of MTX on forskolin- and prostaglandin E_2-mediated cyclic AMP accumulation are not mimicked by a calcium ionophore and are not blocked by nifedipine, a calcium channel blocker. Translocation of protein kinase C occurs after treatment with MTX in both cell lines; the protein kinase C activity and content are reduced in the cytosol and increased in membranes after exposure to either MTX or a phorbol ester. The results confirm previous studies on the heterogeneous input of protein kinase C to cyclic AMP-generating systems performed with phorbol esters and demonstrate the utility of MTX as a unique tool for studies of systems that involve second messengers generated through stimulation of phosphoinositide breakdown.

MTX, a potent marine toxin present in the dinoflagellate *Gambierdiscus toxicus*, induces activation of calcium fluxes and release of neurotransmitters (1). In addition, MTX stimulates calcium-dependent breakdown of phosphoinositides in aortic myocytes (2), neuroblastoma hybrid NCB-20 cells (3), PC12 cells (4), and a large variety of other cell types (5, 6). Thus, MTX represents a potentially useful tool for the investigation of the effect of second messengers generated through phosphoinositide breakdown on physiological and biochemical functions in various cell types. One such function involves the purported effects of phosphoinositide breakdown on cyclic AMP-generating systems; receptor-mediated stimulation of phosphoinositide breakdown by phospholipase C has been proposed to enhance the responsiveness of cyclic AMP-generating systems in brain slices (7), pinealocytes (8), and smooth muscle (9). Receptor-mediated activation of phospholipase A_2 also has been proposed to be involved in enhancing responsiveness of cyclic AMP-generating systems (10). It would appear likely that activation of protein kinase C by the diacylglycerols that are generated during phosphoinositide breakdown is responsible for enhanced responses of cyclic AMP-generating systems. In support of this hypothesis, phorbol esters, which mimic the stimulatory effects of diacylglycerols on protein kinase C, also augment accumulations of cyclic AMP in brain slices (11), pinealocytes (12), and smooth muscle (9). Phorbol esters also augment cyclic AMP accumulations in pheochromocytoma PC12 cells (13), in primary cultures of myoblasts (14) and in S49 lymphoma cells (15), but in these systems effects of phosphoinositide breakdown on cyclic AMP accumulation have not been demonstrated. On the other hand, phorbol esters reduce cyclic AMP accumulations in thyroid cells (16), C6 glioma cells (17), pituitary GH3 cells (18), turkey erythrocytes (19), hepatocytes (20), Leydig cells (21), fibroblasts (22), and NCB-20 cells (23). It is not known whether stimulation of phosphoino-

ABBREVIATIONS: MTX, maitotoxin; HEPES, 4-(hydroxymethyl)-1-piperazine ethanesulfonic acid; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethylbenzoate; HPLC, high pressure liquid chromatography; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

sitide breakdown in such systems will, like phorbol esters, have inhibitory effects on cyclic AMP accumulation. In several cultured cells, receptor-mediated stimulation of phosphoinositide breakdown can be marginal and the receptors coupled to phospholipase C can also be inhibitory directly to cyclic AMP-generating systems (see Refs. 13 and 23). Through the use of MTX, we now demonstrate that, in pheochromocytoma PC12 cells, stimulation of phosphoinositide breakdown enhances responsiveness of cyclic AMP-generating systems, as had phorbol esters (13). On the other hand, in neuroblastoma hybrid NCB-20 cells, stimulation of phosphoinositide breakdown by MTX reduces responsiveness of cyclic AMP-generating systems, again in parallel with the inhibitory effect of phorbol esters (23). MTX-elicited increases in intracellular calcium do not appear to be responsible for the effects on cyclic AMP generation, but rather the effects appear to be linked to MTX-elicited translocation of protein kinase C from cytosol to membranes.

Experimental Procedures

Materials. MTX was purified to homogeneity from *G. toxicus* as described (1). Nifedipine, trifluoperazine, prostaglandin E_2, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, phorbol-12-myristate-13-acetate, and Nonidet P-40 were from Sigma (St. Louis, MO); forskolin, ionomycin, and A23187 from Calbiochem-Behring (San Diego, CA); calmidazolium from Boehringer Mannheim (Mannheim, FRG); diltiazem from Marion Laboratories, Inc. (Kansas City, MO); methoxyverapamil (D600) from Knoll AB (Ludwigshafen am Rhein, FRG); W-7 from Rikaken Co. Ltd. (Nagoya, Japan); and rolipram (ZK 62711) from A. G. Schering Laboratories (Berlin, FRG). Ryanodine was kindly provided by Dr. B. Witkop (National Institutes of Health, Bethesda, MD) and TMB-8 by Dr. D. J. Triggle (State University of New York, Buffalo, NY). [^{3}H]Inositol (12–17 Ci/mmol), [γ-^{32}P]ATP, and [^{3}H] inositol 1-mono-, 1,4-bis-, and 1,4,5-trisphosphate standards for HPLC were from New England Nuclear (Boston, MA). No commercial standard was available for [^{3}H]inositol-1,3,4-trisphosphate when these studies were completed. The [^{3}H]cyclic AMP assay kit, quin2, monoclonal antibody anti-protein kinase C, and affinity-purified ^{125}I-protein A were from Amersham (Arlington Heights, IL). Other reagents were from standard commercial sources.

Cell culture. NCB-20 cells, derived as a hybrid of neuroblastoma and Chinese hamster embryonic brain cells, were kindly provided by Dr. D. Chuang (National Institute of Mental Health, Washington, DC). PC12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were kindly provided by Dr. G. Guroff (National Institutes of Health). NCB-20 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). PC12 cells were grown in Dulbecco's modified Eagle's medium with 6% fetal calf serum, 6% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Both cell lines were kept at 37° in an atmosphere enriched in CO_2.

Phosphoinositide breakdown. The day before the experiment, the cells were transferred from 150-cm^2 culture flasks and subcultured in 6-well dishes (35-mm diameter wells) with medium containing 10 μCi/ml [^{3}H]inositol (14–17 Ci/mmol). The procedure was essentially as described by Chuang (24) for NCB-20 cells. [^{3}H]Inositol-labeled cells were washed twice with buffer A (118 mM NaCl, 4.7 mM KCl, 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) with 3 or 5 mM $CaCl_2$ for PC12 or NCB-20 cells, respectively. The cells were then incubated in buffer A containing 60 mM LiCl (osmolarity maintained by reducing NaCl to 58 mM), for 20 min. Although it was reported (24) that 60 mM LiCl was required for maximal stimulation of phosphoinositide breakdown by carbamylcholine in NCB-20 cells, 10 mM LiCl was sufficient to attain maximal responses with MTX (data not shown). In experiments with varying concentrations of calcium, after the cells were washed, they were incubated with fresh buffer A containing LiCl and the indicated amount of calcium for 20 min before the addition of other agents. Agents were then added and incubations were carried on for 30 min at 37°. Cells were scraped from the plates and transferred to 1.5-ml microfuge tubes. After centrifugation for 1 min, the supernatant was discarded and 1 ml of 6% trichloroacetic acid was added. The tubes were vortexed and centrifuged for 1.5 min and the supernatant was applied to anion-exchange columns (Bio-Rad AG 1X8, 100–200 mesh, formate form) or extracted for HPLC determination of [^{3}H]inositol phosphates (see below). Separation and elution of inositol phosphates by anion-exchange chromatography was performed as described by Berridge *et al.* (25). Results are expressed as (cpm in [^{3}H]inositol phosphate/cpm in lipids) × 10,000 or as percent of control.

HPLC determination of [^{3}H]inositol phosphates. Protocol A was as follows. Trichloroacetic acid extracts from cells were extracted four times with 2 ml of water-saturated diethyl ether. The aqueous phase was neutralized to pH 7 with NaOH and applied to the HPLC column. The separation procedure was as described (26). A Whatman SAX Partisphere column (5-μm particle size) was utilized. The elution solvent was a continuous gradient of 0.01 to 1.75 M ammonium phosphate (in 30 min) followed by an isocratic elution (1.75 M ammonium phosphate) for 30 min. Samples were collected every 0.5 min and assayed for radioactivity. Retention times of standards are indicated in Fig. 1.

Protocol B was as follows. Incubations were stopped by aspirating the buffer and adding 400 μl of 5% perchloric acid to each well followed by 10 μl of phytate hydrolysate prepared according to the method of Wreggett *et al.* (27) and 10 μl of a mixture of adenosine and adenosine phosphates (2 mM concentrations of adenosine, AMP, ADP, and ATP).

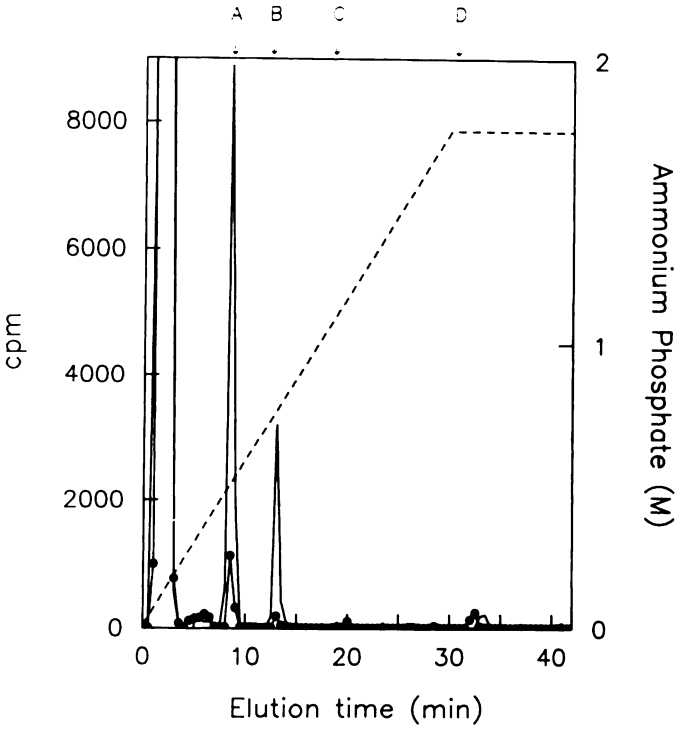

Fig. 1. A typical HPLC separation of [^{3}H]inositol phosphates in PC12 cells after stimulation with 0.5 ng/ml MTX (Protocol A). Cells were incubated with buffer A (with LiCl) for 20 min and then MTX (*solid line*) or buffer (*black dots*) was added and incubations were carried out for 30 min. *Arrows* indicate the retention times of standards: *A*, [^{3}H]inositol-1-monophosphate; *B*, [^{3}H]inositol-1,4-bisphosphate; *C*, [^{3}H]inositol-1,4,5-trisphosphate; *D*, [^{3}H]inositol-1,3,4,5-tetrakisphosphate. *Dotted line* represents ammonium phosphate concentration. For sample preparation and HPLC conditions, see Experimental Procedures, Protocol A. This experiment was repeated four times with similar results. Each experiment was carried in triplicate.

After scraping of the wells and centrifugation, the supernatants were transferred to 1.5-ml tubes. To each tube, 350 μl of a mixture (1:1) of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine and 5 μl of phenol red were added. Tubes were vortexed and, after centrifugation, the upper layer was removed, neutralized with 0.01 N NaOH, and injected onto a Zorbax SAX column (Dupont, Wilmington, DE). The elution solvent was a gradient between 10 mM and 1.2 M ammonium phosphate. Detection of [^{3}H]-labeled material was performed with an on-line radioactivity detector (model CR; Flo-One; Radiomatic, Tampa, FL) with continuous delivery of high salt capacity Tru-Count (IN/US, Fairfield, NJ) as the scintillation fluid. Retention times of standards are indicated in Fig. 2. The retention time for [^{3}H]inositol bisphosphate coincides with that of a standard of [^{3}H]inositol-1,4-bisphosphate. However, because standards for other inositol bisphosphates were not available, the presence of other isomers that may coelute with inositol-1,4-bisphosphate cannot be ruled out.

Cyclic AMP determinations. Cells were harvested from culture flasks and washed twice with buffer A (see above). Cells were then resuspended in buffer A (about 2×10^6 cells/ml) that was supplemented with 0.1% albumin and were incubated at 37° with 1 μg/ml adenosine deaminase for 10 min. Then, the phosphodiesterase inhibitor rolipram at a concentration of 30 μM was added and the cells were incubated for another 10 min. Aliquots of the cell suspensions were then transferred to 1.5-ml microfuge tubes that contained the agents in buffer A and that had been prewarmed at 37°. The final volume was 500 μl. In experiments with varying concentrations of calcium, the cells were washed, treated with adenosine deaminase and rolipram in calcium-free buffer A, and then incubated with the indicated amount of calcium in buffer A for 10 min before the addition of other agents. Incubations were for 10 min and were stopped by transferring 400 μl to microfuge tubes preheated at 95°. After 3 min at 95°, the tubes were centrifuged for 2 min at 12,000 × *g* and cyclic AMP was determined in the supernatants by the method of Gilman (28), using a commercial [^{3}H] cyclic AMP assay kit.

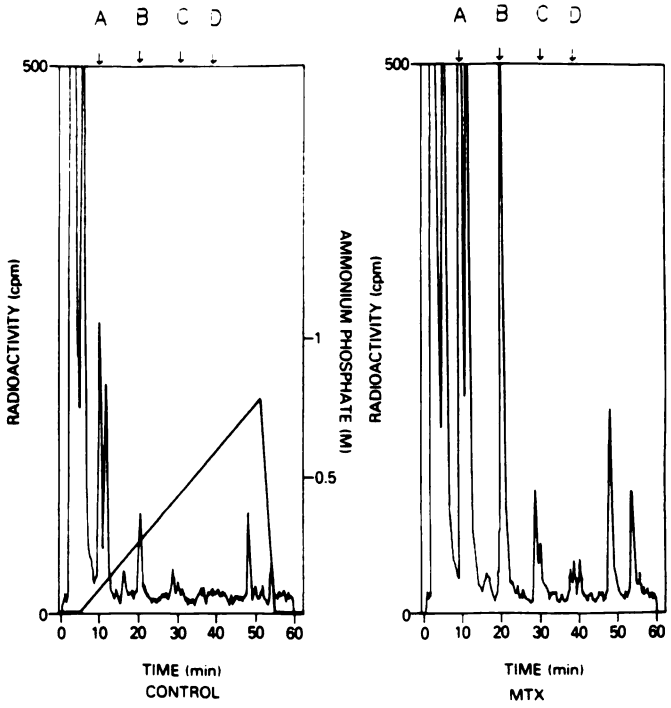

Fig. 2. A typical HPLC separation of [^{3}H]inositol phosphates in NCB-20 cells after stimulation with 0.5 ng/ml MTX (Protocol B). Cells were incubated with buffer A (with LiCl) for 20 min and then buffer (*left*) or MTX (*right*) were added and incubations were carried for 30 min. *Arrows* indicate the retention times of standards: *A*, [^{3}H]inositol-1-monophosphate; *B*, [^{3}H]inositol-1,4-bisphosphate; *C*, [^{3}H]inositol-1,4,5-trisphosphate; *D*, [^{3}H]inositol-1,3,4,5-tetrakisphosphate. The *solid line* in the *left panel* represents ammonium phosphate concentration. For sample preparation and HPLC conditions see Experimental Procedures, Protocol B. This experiment was repeated five times with similar results. Each experiment was carried in triplicate.

Measurement of Ca^{2+}-quin2 fluorescence. Cells were cultured to confluency in 150-cm^2 flasks and were harvested by shaking. Cells were then washed in buffer A and resuspended to a concentration of $1–2 \times 10^6$ cells/ml. Cells suspensions were incubated with 10 μM quin2 at 37° for 30–40 min. At the end of quin2 loading period, cell suspensions were washed, resuspended in buffer A containing the indicated amount of calcium or no calcium, and kept on ice until fluorescence determinations were performed. Aliquots (2-ml) of quin2-loaded cells were transferred to continuously stirred, temperature-controlled (36°), quartz cuvettes. Fluorescence was detected with a Perkin-Elmer LS-5 spectrofluorometer, at an excitation wavelength of 339 nm and an emission wavelength of 492 nm. In all cases, MTX-induced increases in intracellular calcium reached the level of saturation of the intracellular quin2. Thus, an accurate estimation of intracellular calcium concentration was not possible. Because quin2 is saturated at calcium concentrations of ≈1 μM, it appears that the calcium concentrations reached after MTX are 1 μM or higher.

Partial purification of cytosolic and membrane protein kinase C. The procedure described by Thomas *et al.* (29) was followed with minor modifications. Confluent monolayers of cells were washed and incubated with buffer A in the presence or absence of MTX (0.5 ng/ml) for 10 min at 37°. The buffer then was aspirated, the cells were washed once with calcium-free buffer A, and 5 ml of buffer B was added (20 mM Tris·HCl, pH 7.5, 2 mM EGTA, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cells were disrupted in a glass-glass homogenizer, and the homogenate was centrifuged at 15,000 × *g* for 20 min. The supernatant represents the cytosolic fraction. The pellet was resuspended in 5 ml of buffer C (20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and was disrupted in a glass-glass homogenizer. Nonidet P-40 was added to a final concentration of 1%. After 30 min at 4°, the suspension was centrifuged at 15,000 rpm for 20 min. The supernatant constituted the membrane fraction. Cytosolic and membrane fractions were partially purified by DEAE-cellulose chromatography (DE52; Whatman). Fractions eluted with 100 mM NaCl were collected and concentrated with Centricon 30 filters. From the concentrated fractions, which contained similar amounts of proteins, aliquots were taken for protein kinase C assays (5–10 μl) and for electrophoresis and immunoblotting (10–40 μl).

Protein kinase C assay. Essentially, the assay described by Thomas *et al.* (29) was followed with minor modifications. The reaction mixture contained 20 mM Tris·HCl, pH 7.5, 1 mM $CaCl_2$, 6.25 mM magnesium acetate, 125 μM ATP, 250 μg/ml histone type IIIS, and 10,000 cpm/μl [γ^{32}P]ATP, in 45 μl. Activity was measured either in the presence of 5 mM EGTA or in the presence of 10 μg of phosphatidylserine and 0.2 μg of 1,2-diolein. Reactions were initiated by the addition of 5–10 μl of partially purified cytosolic and membrane protein extracts and were carried for 5 min at 30°. Reactions then were stopped by transferring 20 μl of the mixture to strips of phosphocellulose paper (Whatman P81). Papers were washed three times with 80 mM phosphoric acid, once with acetone, and once with petroleum ether. After drying, the papers were mixed with scintillation fluid (Hydrofluor) and radioactivity was determined by liquid scintillation counting.

SDS-PAGE and Immunoblotting. Aliquots of partially purified protein extracts were subjected to SDS-PAGE on 10% polyacrylamide gels (30) and were subsequently electroblotted onto nitrocellulose sheets (31). Nonspecific binding was prevented by a preincubation of sheets with Blotto (Advanced Biotechnologies, Columbia, MD) overnight. The nitrocellulose sheets were then incubated with a commercially available anti-protein kinase C monoclonal antibody (1:100 dilution) for 4 hr at room temperature. After three washes, a second antibody, anti-mouse IgG from rabbit (Cappel, Malvern, PA) was

applied (1:200) for 2 hr. After three washes, the nitrocellulose sheets were incubated with ^{125}I-Protein A (Amersham) for 45 min. Blots were washed, dried, and subjected to autoradiography at −70° for the times indicated in the figures. A mixture of ^{14}C-labeled protein standards (BRL, Gaithersburg, MD) was run in every gel in order to determine molecular weights. These were myosin (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), and α-chymotrypsinogen (25,700).

Results

MTX (0.5 ng/ml) stimulated the formation of [^{3}H]inositol phosphates in both NCB-20 and PC12 cells. Typical HPLC separations using either Protocol A or B are illustrated in Figs. 1 and 2. A time-course study (Fig. 3) indicates that, at 30 min, the time point used to evaluate phosphoinositide breakdown throughout this study, MTX stimulated the formation of inositol mono-, bis-, tris- (both 1,4,5- and 1,3,4- isomers), and tetrakisphosphate in PC12 cells. [^{3}H]Inositol mono- and bisphosphate levels increased linearily with time, whereas [^{3}H] inositol-1,3,4-tris-, -1,4,5-tris- and tetrakisphosphate rapidly reached plateau values (Fig. 3, legend).

MTX was completely ineffective in stimulating phosphoinositide breakdown in the absence of calcium in both cell lines (Fig. 4, A–C, and 5, A–C). Increasing free extracellular calcium resulted in a biphasic response in the stimulation of [^{3}H]inositol phosphate formation by MTX. After reaching a maximum, the responses then were reduced in magnitude with further increases in extracellular calcium (Fig. 4, A–C, and 5, A–C). Maximal responses on stimulation of phosphoinositide breakdown by MTX were obtained at 1.5 mM calcium for PC12 cells and at 2.5–4.5 mM for NCB-20 cells. The concentration of free extracellular calcium did not significantly affect [^{3}H]inositol phosphate formation in either cell line under control conditions (Figs. 4A and 5A). MTX-induced stimulation of phosphoinositide breakdown was not affected by calcium channel antagonists nor by other agents that inhibit calcium actions or mobilization (Table 1).

MTX (0.5 ng/ml) markedly potentiated forskolin-induced accumulation of cyclic AMP in PC12 cells (Fig. 5D). In NCB-20 cells, MTX at the same concentration inhibited prostaglandin E_2-induced accumulation of cyclic AMP (Fig. 4D). The effects of MTX on cyclic AMP accumulation in PC12 and NCB-20 cells were eliminated when calcium was omitted from the incubation buffer (Figs. 4D and 5D). The magnitude of the effects of MTX on cyclic AMP accumulation reached a maximum at 1.5 mM calcium for PC12 cells and at 2.5–4.5 mM calcium for NCB-20 cells (Figs. 4D and 5D). MTX-elicited effects on cyclic AMP accumulation could not be mimicked by the calcium ionophore A23187, in either PC12 or NCB-20 cells (Fig. 6). Furthermore, the calcium channel blocker nifedipine at 10 μM did not affect MTX-elicited effects on cyclic AMP accumulation in either cell line (Fig. 6). Organic calcium channel blockers, like methoxyverapamil and nifedipine, do block MTX-elicited calcium-dependent transmitter release in PC12 cells (1, 4).

The MTX-induced increases in intracellular calcium concentration were measured by increases in fluorescence in quin2-loaded cells (Fig. 7). The MTX-elicited effects on intracellular calcium varied in magnitude, depending on the extracellular calcium concentration. In both cell lines, MTX did not induce changes in quin2 fluorescence in the absence of extracellular calcium (Fig. 7). In PC12 cells, addition of calcium to the medium elicited increases in fluorescence, and MTX then induced a further increase (Fig. 7A). The L-type calcium channel antagonist nifedipine (1 μM) inhibited by 70% the MTX-elicited increase in quin2 fluorescence (data not shown). In NCB-20 cells, maximal effects of MTX were attained at calcium concentrations of 5 mM (Fig. 7B). In both NCB-20 and PC12 cells, ionomycin (1 μM) elicited a similar maximal increase in fluorescence but, in contrast to the MTX-elicited

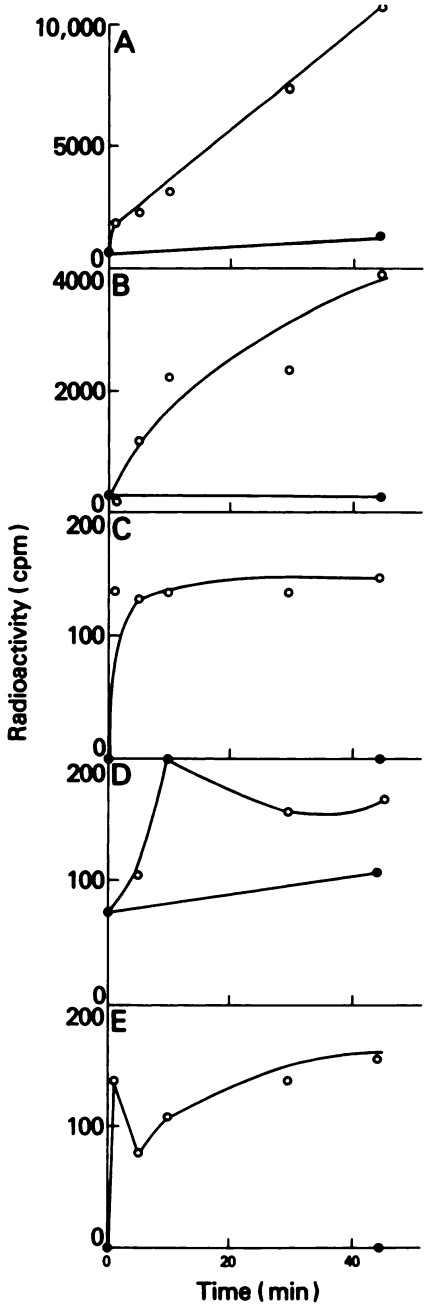

Fig. 3. Time course for MTX stimulation of [^{3}H]inositol phosphate formation in PC12 cells. Cells were incubated for the indicated time in buffer A in the presence (○) or absence (●) of 0.5 ng/ml MTX. Graphs represent cpm in [^{3}H]inositol monophosphate (A), [^{3}H]inositol bisphosphate (B), [^{3}H]inositol-1,3,4-trisphosphate (C), [^{3}H]inositol-1,4,5-trisphosphate (D), or [^{3}H]inositol tetrakisphosphate (E). For sample preparation and HPLC conditions, see Experimental Procedures. The results are from a representative experiment. In other three experiments both inositol trisphosphate isomers and inositol tetrakisphosphate reached plateau values at approximately the same times after MTX application.

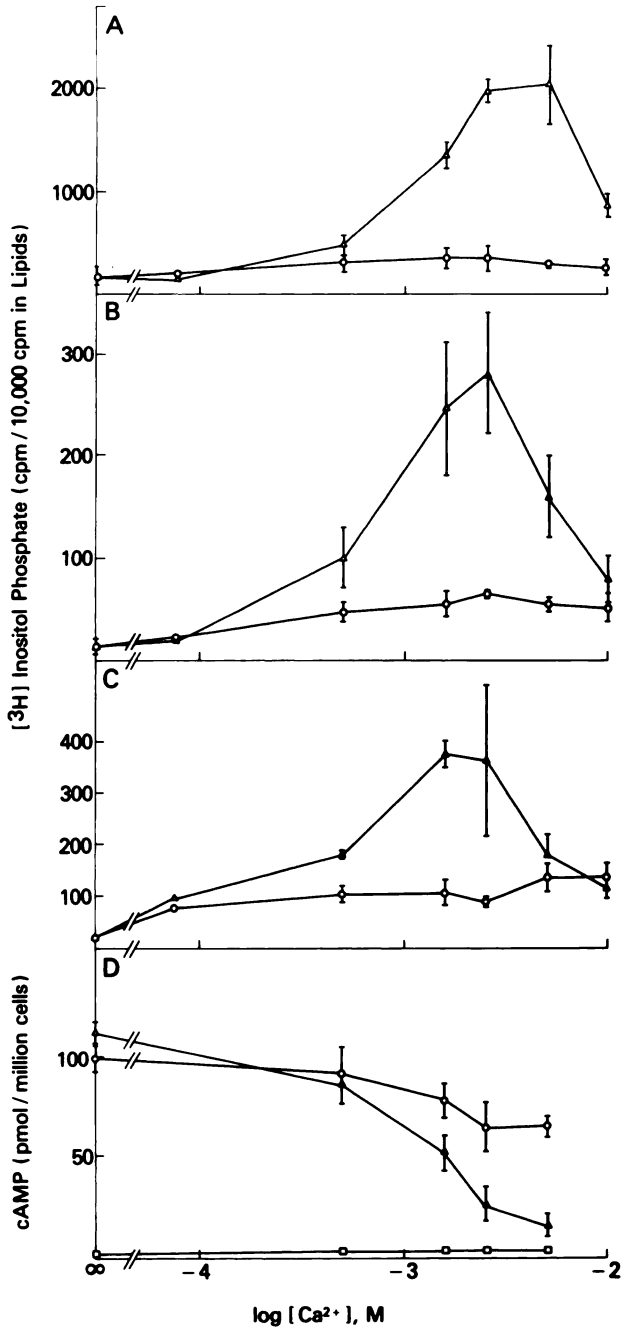

Fig. 4. Calcium-dependent effects of MTX on [^{3}H]inositol phosphate formation and cyclic AMP accumulation in NCB-20 cells. A, B, and C, Stimulation of [^{3}H]inositol phosphate formation in the presence (Δ) or absence (O) of 0.5 ng/ml MTX. A, [^{3}H]Inositol monophosphate; B, [^{3}H] inositol bisphosphate; and C, [^{3}H]inositol trisphosphate. Separation of [^{3}H]inositol phosphates was performed by anion-exchange chromatography as described in Experimental Procedures. D, Inhibition of prostaglandin E_2-induced accumulation of cyclic AMP in the presence (Δ) or absence (O) of 0.5 ng/ml MTX. Results are means (± standard errors) of three independent experiments, each one performed in triplicate. *[Ca^{2+}]* represents free calcium concentrations. For conditions and assays, see Experimental Procedures.

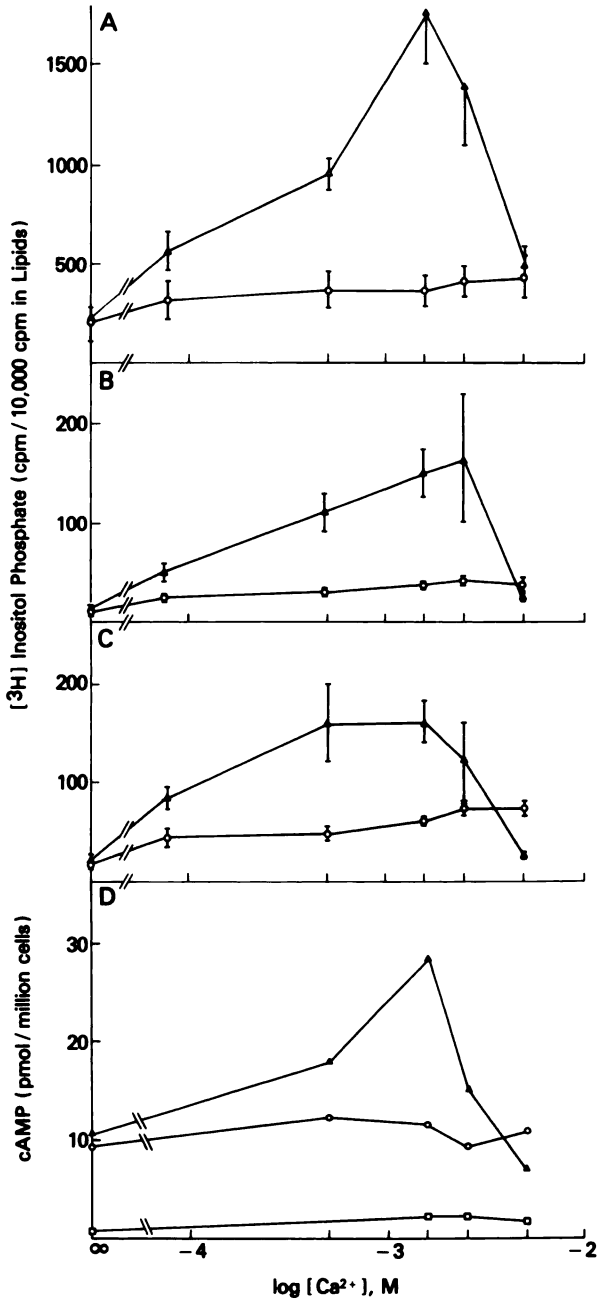

Fig. 5. Calcium-dependent effects of MTX on [^{3}H]inositol phosphate formation and cyclic AMP accumulation in PC12 cells. A, B, and C, Stimulation of [^{3}H]inositol phosphate formation in the presence (Δ) or absence (O) of 0.5 ng/ml MTX. A, [^{3}H]inositol monophosphate; B, [^{3}H] inositol bisphosphate; and C, [^{3}H]inositol trisphosphate. Separation of [^{3}H]inositol phosphates was performed by anion-exchange chromatography as described in Experimental Procedures. D, Potentiation of forskolin-induced accumulation of cyclic AMP in the presence (Δ) or absence (O) of 0.5 ng/ml MTX. Results of experiments of [^{3}H]inositol phosphate formation are means (± standard errors) of three independent experiments, each one performed in triplicate. Results on cyclic AMP formation correspond to one representative experiment performed in triplicate. This experiment was repeated three times with similar results. However, basal and stimulated cyclic AMP values varied from day to day. *[Ca^{2+}]* represents free calcium concentrations. For conditions and assays see Experimental Procedures.

response, the increase in fluorescence with the ionophore was maximal within 1 sec (data not shown).

Translocation of protein kinase C was evaluated in two ways. First, partially purified protein extracts from cytosolic and membrane fractions from PC12 and NCB-20 cells were assayed

TABLE 1

Effects of calcium channel blockers and other agents on MTX-induced phosphoinositide breakdown

Cells were incubated in buffer A (with LiCl) for 20 min and then MTX and other agents were added. Incubations were for 30 min. [^{3}H]Inositol monophosphates were analyzed by anion-exchange chromatography, as described in Experimental Procedures. Results are average of at least three separate experiments (± standard error), or are the results of individual experiments performed in triplicate.

Agents administered with MTX (0.5 ng/ml)[a]	Phosphoinositide breakdown	
	NCB-20 cells	PC12 cells
	% of MTX alone response	
Nifedipine (10 μM)	123 ± 18	92 ± 11
$LaCl_3$ (500 μM)	106 ± 33	149 ± 55
$CoCl_2$ (1 mM)	125 ± 20	103
$MnCl_2$ (1 mM)	107 ± 20	81
$CdCl_2$ (1 mM)	104 ± 22	ND[b]
Methoxyverapamil (D-600) (5 μM)	121 ± 16	ND
Diltiazem (10 μM)	ND	77

[a] A variety of other agents, which affect calcium mobilization or action, did not inhibit MTX-induced phosphoinositide breakdown. These were ryanodine, trifluoperazine, calmidazolium, TMB-8, and W-7 (all at 10 μM), in individual experiments performed in triplicate.
[b] ND, not detected.

for Ca^{2+}/phospholipid-induced histone phosphorylation (29) (Table 2). Second, aliquots of the same extracts were separated by SDS-PAGE, transferred to nitrocellulose sheets, and immunoblotted with a monoclonal antibody against protein kinase C (Fig. 8). This procedure allowed for a semiquantitative demonstration of changes in the levels of immunoreactive protein kinase C in cytosol and membranes after treatment with MTX. MTX caused a reduction in cytosolic protein kinase C activity and in blot immunoreactivity in PC12 and NCB-20 cells that were treated for 10 min with MTX (Table 2 and Fig. 8). The changes in immunoreactivity observed in PC12 after MTX treatment were comparable in magnitude to those observed in cells treated for the same length of time with the phorbol ester phorbol myristate acetate (100 nM) (Fig. 8A). In membrane fractions from both cell lines, there was an increase in protein kinase C activity (histone phosphorylation) and in immunoreactivity after MTX treatment for 10 min (Table 2 and Fig. 8).

Discussion

The marine toxin MTX has been proposed to be a direct activator of calcium channels (1, 4, 32–34). The effects of MTX on contraction of smooth muscle (32) and skeletal muscle (33), on release of catecholamines in PC12 cells (1, 4), and on release of hormones in anterior pituitary cells (34) are calcium dependent and can be inhibited with calcium channel blockers. MTX also is a very potent activator of phosphoinositide breakdown in aortic myocytes (2), NCB-20 cells (Refs. 3 and 6 and this work), PC12 cells (Refs. 4 and 6 and this work), and a variety of other cell types (5, 6). MTX stimulated the formation of all inositol phosphates in NCB-20 and PC12 cells (Figs. 1 and 2). The stimulation of phosphoinositide breakdown by MTX was dependent on extracellular calcium but could not be antagonized by either organic or inorganic calcium channel blockers in NCB-20 cells (3) (Table 1) or in PC12 cells (Table 1). The organic L-type calcium channel blocker nifedipine did block MTX-elicited increases in intracellular calcium in PC12 cells (see Results).[1] In differentiated NCB-20 cells, carbamylcholine

[1] Unpublished observations.

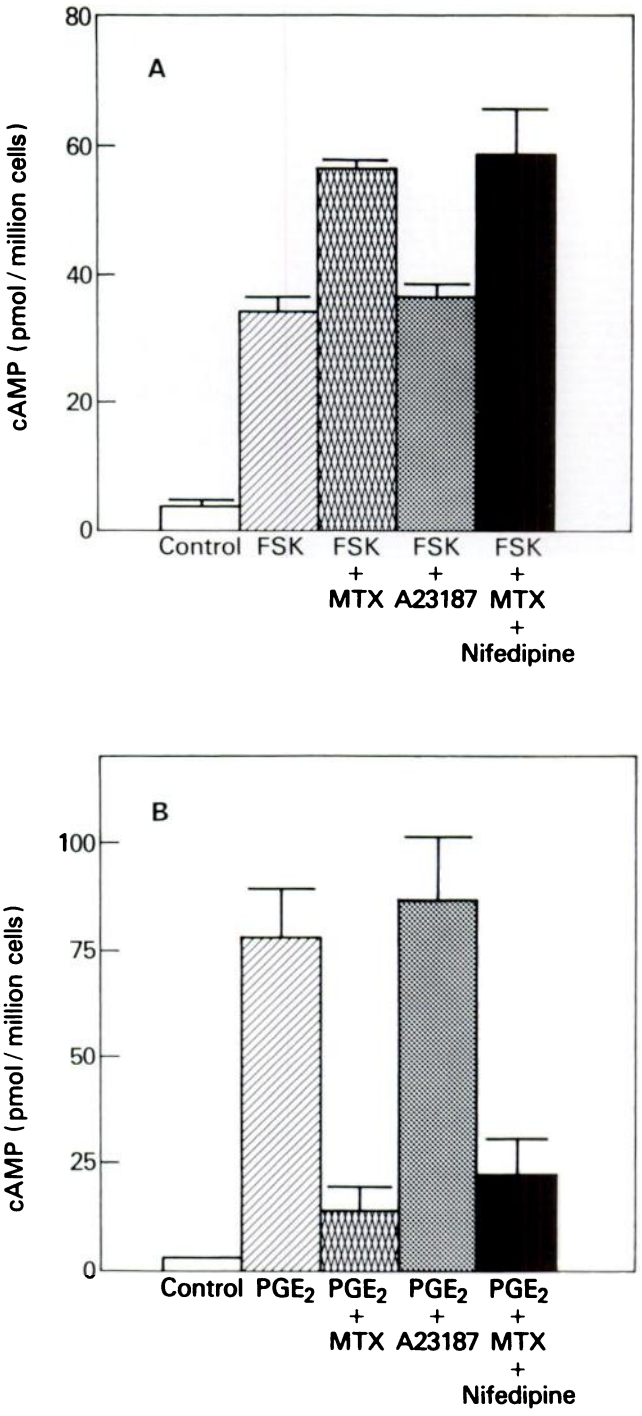

Fig. 6. Lack of effects of a calcium ionophore, A23187, and lack of inhibitory effects of nifedipine on MTX-induced effects on cyclic AMP accumulation in PC12 cells (A) and NCB-20 cells (B). Cells were incubated in HEPES buffer in the presence of forskolin (*FSK*) (10 μM) (PC12 cells), prostaglandin E_2 (*PGE_2*) (10 μM) (NCB-20 cells), MTX (0.5 ng/ml), A23187 (10 μM), nifedipine (10 μM), or combinations of these agents. For conditions and assays see Experimental Procedures. Results are means (± standard errors) of three independent experiments, each one performed in triplicate.

still stimulates phosphoinositide breakdown in the absence of extracellular calcium, whereas MTX does not (6). Thus, the calcium-dependent phospholipase C system is still functional, under conditions where MTX stimulation is dependent on extracellular calcium. The EC_{50} for MTX-induced phosphoi-

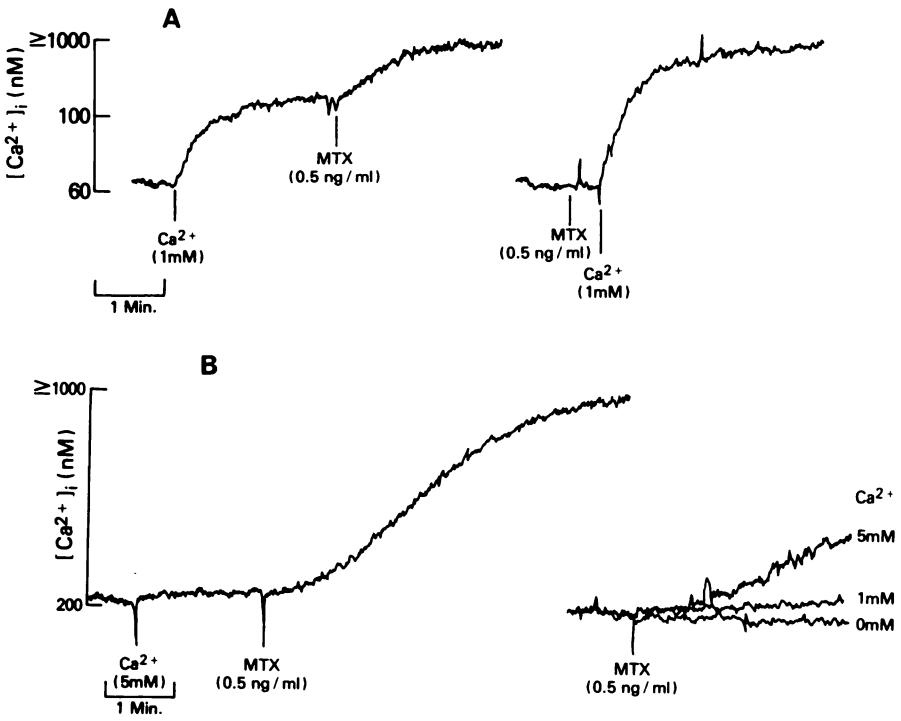

Fig. 7. MTX-induced increases in intracellular calcium concentrations determined with quin2 fluorescence in PC12 (A) and NCB-20 (B) cells. Because increases in calcium after MTX resulted in saturation of quin2, an accurate quantification of calcium levels was not possible to establish. See Experimental Procedures for details. The experiment was repeated two more times in both cell lines with identical results.

TABLE 2

Translocation of protein kinase C activity after MTX in PC12 and NCB-20 cells

PC12 and NCB-20 cells were incubated with or without 0.5 ng/ml MTX for 10 min. Cytosolic and membrane extracts were obtained and activity was determined by histone IIIS phosphorylation, as described in Experimental Procedures. Values correspond to means (± standard errors) of three to five experiments performed in duplicates.

	Protein kinase C activity	
	PC12 cells	NCB-20 cells
	pmol of P incorporated/min/10-μl aliquot	
Cytosol extract		
Control	6.53 ± 0.57	4.51 ± 0.15
MTX (0.5 ng/ml)	4.21 ± 0.46*	2.83 ± 0.57*
Membrane extract		
Control	3.86 ± 0.22	2.96 ± 0.04
MTX (0.5 ng/ml)	4.89 ± 0.19*	4.04 ± 0.32*

*$p < 0.05$ versus respective control.

nositide breakdown in NCB-20 (3) and PC12 cells (4) is ≈150 pM. Because stimulation of phosphoinositide breakdown by MTX is not affected by calcium channel blockers, it can be inferred that the effects of MTX on isolated organs and on release of neurotransmitters, which in most cases are inhibited by calcium channel blockers, occur through different mechanisms or sites of action than the effects of MTX on phosphoinositide breakdown.

MTX significantly stimulated phosphoinositide breakdown in PC12 cells at lower calcium concentrations than in NCB-20 cells. Thus, at 77 μM extracellular calcium, MTX induced significant breakdown of phosphoinositides in PC12 cells, whereas at this concentration of calcium MTX had no effect on breakdown of phosphoinositides in NCB-20 cells. In PC12 cells maximal stimulation was observed at 1.5 mM calcium for [^{3}H]inositol monophosphate formation, 2.5 mM calcium for [^{3}H]inositol bisphosphate formation, and 0.5 mM calcium for [^{3}H]inositol trisphosphate formation. In NCB-20 cells, maximal stimulation was observed at 4.5 mM calcium for [^{3}H] inositol monophosphate formation. 2.5 mM for [^{3}H]inositol bisphosphate formation, and 1.5 mM for [^{3}H]inositol trisphosphate formation. The formation of [^{3}H] trisphosphates in the presence of MTX in both cell lines reached maximal values at lower calcium concentrations than formation of [^{3}H]inositol monophosphate. It has been proposed that phospholipase C hydrolyzes phosphatidylinositol when calcium levels are high, whereas the hydrolysis of polyphosphoinositides has a lower calcium requirement (35). Because the experiments in PC12 and NCB-20 cells were performed under identical conditions, the differences in calcium requirements are probably either due to differences in the enzymes involved in phosphoinositide breakdown in the two cell lines, or more likely, due to the fact that access of calcium to the enzymes occurs more readily in PC12 cells than in NCB-20 cells in the presence of MTX. Calcium influx alone, however, does not seem to be sufficient for MTX to induce stimulation of phosphoinositide breakdown, because the calcium ionophores ionomycin and A23187 at 1 μM did not elicit marked stimulation of phosphoinositide breakdown in these cell lines (4, 6). MTX does not have any ionophoretic activity in liposomes or mitochondria (36).

MTX-induced breakdown of phosphoinositide in both PC12 and NCB-20 cells *must* lead not only to formation of inositol phosphates but also to formation of diacylglycerols. The latter, in concert with calcium ions, would activate protein kinase C. Preliminary results indicate that MTX activate protein kinase C. Preliminary results indicate that MTX indeed stimulates, in these cells, the formation of diacylglycerols.[2] The activation of protein kinase C by a phorbol ester in PC12 cells has been shown to enhance adenosine receptor- and forskolin-mediated stimulation of cyclic AMP accumulation (13). In agreement with the stimulatory effect of protein kinase C activation by a

[2] P. Holbrook, F. Gusovsky, and J. W. Daly, unpublished results.

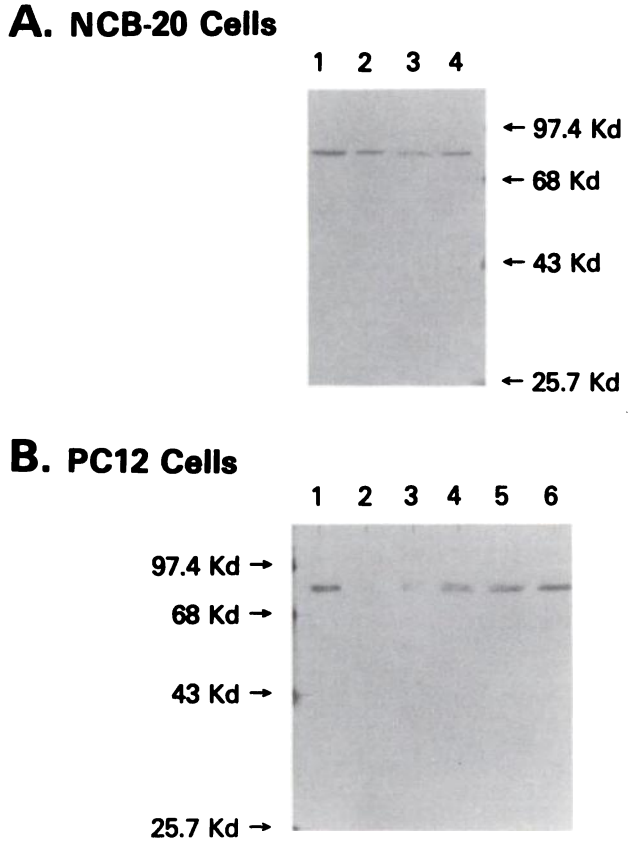

Fig. 8. Translocation of protein kinase C determined by immunoblotting. A, NCB-20 cells were incubated for 10 min with (*lanes 2* and *4*) or without (*lanes 1* and *3*) 0.5 ng/ml MTX. Cytosolic (*lanes 1* and *2*) and membrane (*lanes 3* and *4*) extracts were obtained, and SDS-PAGE and immunoblotting were performed as described in Experimental Procedures. B, PC12 cells were incubated for 10 min with no agent (*lanes 1* and *4*), 100 nM phorbol-12-myristate-13-acetate (*lanes 2* and *5*), or 0.5 ng/ml MTX (*lanes 3* and *6*). Cytosolic (*lanes 1–3*) and membrane (*lanes 4–6*) extracts were obtained, and SDS-PAGE and immunoblotting were performed as described in Experimental Procedures. The experiment was repeated three more times with similar results.

phorbol ester on cyclic AMP generation in PC12 cells (13), stimulation of phosphoinositide breakdown by MTX in PC12 cells *enhanced* forskolin-elicited accumulation of cyclic AMP. The activation of protein kinase C by a phorbol ester in NCB-20 cells has the opposite effect, namely inhibition of the cyclic AMP accumulation that is elicited via prostaglandin E_2 or other receptors (23). Again, in agreement with an inhibitory effect of protein kinase C activation by a phorbol ester on cyclic AMP generation in NCB-20 cells, stimulation of phosphoinositide breakdown by MTX in NCB-20 cells *inhibited* prostaglandin E_2-elicited accumulation of cyclic AMP. The mechanisms whereby stimulation of protein kinase C can either potentiate or inhibit cyclic AMP generation in different cell types are as yet poorly understood. It has been proposed that the potentiative effects are due to a suppression of tonic inhibition to adenylate cyclase through a protein kinase C-mediated phosphorylation of G_i, the guanine nucleotide-binding protein involved in receptor-mediated inhibition of adenylate cyclase (37). Inhibitory effects of activation of protein kinase C on cyclic AMP accumulation in NCB-20 cells have been proposed to be related either to phosphorylation of receptors involved in the stimulatory signal or to phosphorylation of G_s, the guanine nucleotide-binding protein involved in receptor-mediated stimulation of adenylate cyclase (23). MTX, through stimulation of phosphoinositide breakdown in a wide range of cell types (5, 6), now provides another approach to study the involvement of protein kinase C in the regulation of cyclic AMP generation, thereby supplementing the use of phorbol esters as exogenous activators of protein kinase C.

Whereas extracellular calcium per se had no effect on forskolin-induced cyclic AMP accumulation in PC12 cells, there was a dose-dependent inhibition of prostaglandin E_2-induced cyclic AMP formation with increasing concentrations of calcium in NCB-20 cells (Fig. 4D). The mechanism of inhibition of cyclic AMP accumulation by extracellular calcium is unclear. One possibility is that elevations in external calcium ions might increase internal calcium and, thereby, activate calcium-sensitive phosphodiesterases. Calcium ions also produce a marked inhibition of accumulations of cyclic AMP elicited by forskolin in intact human platelets (38). In this case, a potent inhibitor of calcium-sensitive phosphodiesterases, namely isobutylmethylxanthine, did not significantly affect the inhibition, which was maximal at 3 mM calcium. There have been many reports on the inhibition of adenylate cyclase in membrane preparations by calcium ions, apparently through a specific inhibitory site (see Ref. 39).

In the absence of calcium, MTX had no significant effect on cyclic AMP accumulation in either cell line. The MTX-induced potentiation of forskolin-stimulated cyclic AMP accumulation in PC12 cells increased as the concentration of extracellular calcium was increased. In NCB-20 cells, MTX induced an inhibition of prostaglandin E_2-stimulated cAMP accumulation that was greater than the inhibition caused by calcium alone. In both cell lines, the effects of MTX on cyclic AMP accumulation reached a maximal value at the same concentration of calcium at which MTX induced maximal formation of [^{3}H] inositol monophosphate. MTX-induced increases in intracellular calcium alone do not appear to be sufficient to account for the effects of MTX on cyclic AMP generation. Thus, neither a calcium ionophore (A23187) nor a calcium channel blocker (nifedipine) had any effect on cyclic AMP generation (Fig. 6). Organic calcium channel blockers, such as nifedipine and methoxyverapamil, do block MTX-induced activation of calcium channels and resultant transmitter release in various preparations including PC12 cells (1, 4, 32–34), but they do not block MTX-induced stimulation of phosphoinositide breakdown (Ref. 3 and this work). MTX clearly induced increases in intracellular calcium in both cell lines (Fig. 7). This response was dependent on the concentration of calcium in the medium (Fig. 7A). In PC12 cells the time course of the increase in calcium cannot unambiguously establish whether this effect is the cause or the consequence of MTX-induced formation of inositol trisphosphate. If the breakdown of phosphoinositides by MTX is the consequence of entry of calcium, then entry must occur through the stimulation of an as yet undescribed calcium uptake phenomenon, because phosphoinositide breakdown induced by MTX is not altered by organic or inorganic calcium channel blockers (Table 1). In addition, this mechanism should be highly compartmentalized, because calcium ionophores such as A23187 and ionomycin elicit no or only slight stimulation of phosphoinositide breakdown (4, 6), nor do ionophores have any effect on cyclic AMP accumulation (Fig. 6). Because calcium per se does not appear to be sufficient to alter cyclic AMP generation, it appears likely that the other

obligatory product of phospholipase C action, namely diacylglycerols, act in concert with calcium to cause translocation and activation of protein kinase C. This activation of protein kinase C would then cause augmentation of cyclic AMP generation in PC12 cells and inhibition of cyclic AMP generation in NCB-20 cells.

Protein kinase C undergoes translocation when cells are exposed to agents that stimulate phosphoinositide breakdown or to phorbol esters, which bind and activate protein kinase C (see Ref. 29). Translocation of protein kinase C, thus, is an indication of activation of this enzyme in whole cells. Treatment of both PC12 and NCB-20 cells with MTX (0.5 ng/ml) for 10 min resulted in a decrease in cytosolic and an increase in membrane activity and content of protein kinase C (Table 2; Fig. 8). The experiments were performed after a 10-min treatment with MTX, as were the experiments on accumulation of cyclic AMP accumulation (Figs. 4D and 5D). In PC12 cells, translocation by MTX was comparable to that observed with a phorbol ester that directly activates protein kinase C (Fig. 8). Thus, it appears very likely that in these two cell lines the effects of MTX on cyclic AMP accumulation are due to protein kinase C activation by diacylglycerols.

In summary the results presented indicate that (i) MTX at picomolar concentrations can stimulate phosphoinositide breakdown in NCB-20 and PC12 cells in a manner dependent on the presence and concentration of extracellular calcium; (ii) there are different calcium requirements for stimulation of phosphoinositide breakdown by MTX in the two cell lines, with higher concentrations of calcium being required for threshold and maximal responses to MTX in the NCB-20 cells; (iii) there are marked parallels in calcium dependency for the effects of MTX on phosphoinositide breakdown and MTX-elicited effects on cyclic AMP accumulation in PC12 and NCB-20 cells; (iv) the effects of MTX on cyclic AMP accumulation are not mimicked by a calcium ionophore and are not blocked by a calcium channel blocker, suggesting that MTX-elicited increases in intracellular calcium alone are insufficient to account for effects on cyclic AMP formation; (v) the effects of MTX on cyclic AMP generation, which are potentiative in PC12 and inhibitory in NCB-20 cells, parallel the effects of phorbol esters in these two cell lines; and (vi) MTX causes, like phorbol esters, an activation and translocation of protein kinase C from cytosol to membranes. It appears likely that MTX stimulation of phosphoinositide breakdown affects cyclic AMP generation through the formation and action of diacylglycerols as endogenous activators of protein kinase C. Thus, MTX appears to represent an invaluable tool for eliciting phosphoinositide breakdown and studying the sequelae to such breakdown in a variety of cells and systems.

Acknowledgments

Drs. J. R. Cunha-Melo and M. Beaven provided invaluable counsel in the quin2 fluorescence determinations.

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