

Maitotoxin Induces Calpain Activation in SH-SY5Y Neuroblastoma Cells and Cerebrocortical Cultures

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Maitotoxin (MTX) is a highly potent marine toxin that activates both voltage-sensitive and receptor-operated calcium channels in the plasma membrane. This results in calcium overload that rapidly leads to cell death. We now report that maitotoxin (0.1–1 nM) induces calpain activation in both SH-SY5Y neuroblastoma cells and fetal rat cerebrocortical cultures. MTX-induced calpain activation was confirmed by the presence of autolytic fragmentation of both subunits of calpain. Secondly, the formation of calpain-produced α -spectrin breakdown products (150 and 145 kDa) was observed. We were also able to detect intracellular hydrolysis of a peptide substrate (succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin) by activated calpain in MTX-treated cells. Calpain inhibitors (calpain inhibitor I, MDL28170 and PD150606) inhibited spectrin breakdown and SLLVY-AMC hydrolysis in MTX-treated SY5Y cells. Our results suggest that (i) calpain is activated as a result of the maitotoxin-induced calcium influx; and (ii) coupling with the *in situ* calpain assays, maitotoxin would be a useful tool in investigating the physiologic and pathophysiologic roles of calpain in neuronal cells. © 1996 Academic Press, Inc.

Key Words: calpain; calcium-activated; maitotoxin; neuronal death; protease inhibitor; calcium channel.

Calpain is a class of cytosolic cysteine proteases activated by calcium. The predominant forms of calpain in most mammalian tissues are μ - and m-calpain, requiring low and high micromolar $[Ca^{2+}]$ for *in vitro* activity (1, 2). Both isoforms are highly abundant in neurons

(3). Calpains are heterodimers: the large subunit (80 kDa) contains a cysteine protease domain as well as a 4-EF-hand calcium-binding domain while the small subunit (29 kDa) has a glycine-rich site for membrane interaction and another cluster of 4-EF-hand structures. Many highly regulated enzymes or proteins (protein kinase C, calmodulin-binding proteins, and cytoskeletal proteins) apparently are highly susceptible to calpain (4). The growing interest in calpain is in part due to its contribution to neuronal death in excitotoxicity and stroke (2, 5). Calcium buildup as a consequence of excitatory amino acid toxicity or hypoxia apparently triggers calpain activation (6). It was proposed that activated calpain, in turn, breaks down cytoskeletal proteins resulting in the loss of cell integrity and cell necrosis. More recently, calpain has also been implicated to play a role in another form of cell death: apoptosis (7–9).

Maitotoxin (MTX³) is a very potent marine toxin originated from a dinoflagellate *Gambierdiscus toxicus*, which is consumed by the surgeon fish *Ctenochateus striatus* in which the toxin accumulated (Fig. 1). MTX is known for its ability to stimulate calcium influx in both excitable and nonexcitable cells (10). Its ability to induce calcium influx is apparently mediated by both voltage-dependent calcium channels and receptor-operated calcium channels (11–13). Contraction of cardiac and smooth muscles (14, 15), stimulation of arachidonic acid release (29), phosphoinositide turnover (16, 17), and neurotransmitter release (18) have been demonstrated as a consequence of MTX treatment. These ac-

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³ Abbreviations used: MTX, maitotoxin; SY5Y, SH-SY5Y; MEM, minimum essential medium; SLLVY-AMC, succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; calpain inhibitor I, acetyl-Leu-Leu-Nle-H; MDL28170, carboxybenzyl-Val-Phe-H, SKF96365, 1-[β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl]-1H-imidazole; TCA, trichloroacetic acid; VSCC, voltage-sensitive calcium channel; SBDP, α -spectrin breakdown product; NMDA, *N*-methyl-d-aspartate.

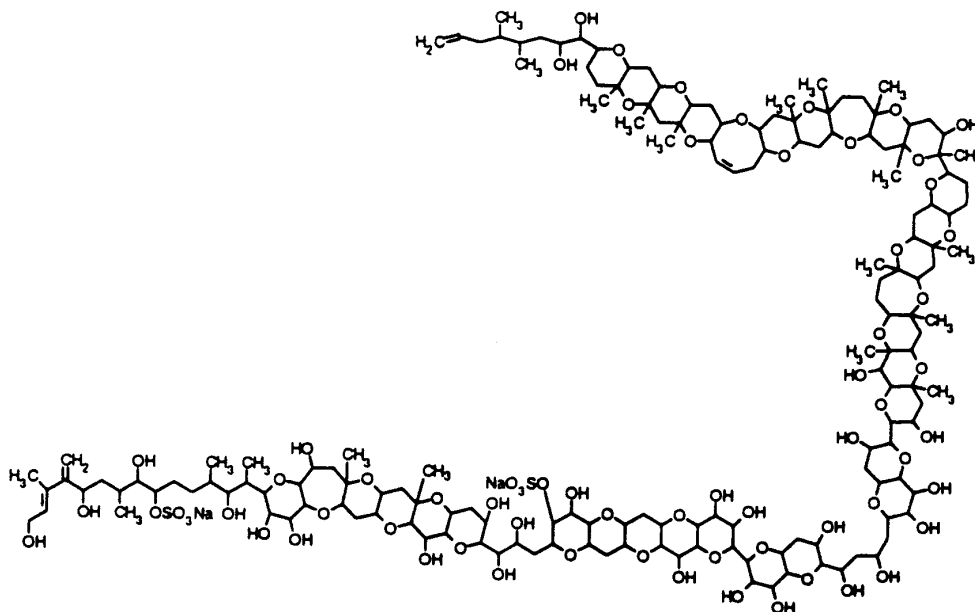


FIG. 1. Structure of maitotoxin.

tions are believed to be a result of elevated intracellular calcium levels. These observations lead us to investigate whether MTX can induce calpain activation in neuronal cells.

MATERIALS AND METHODS

Fetal rat cerebrocortical and SH-SY5Y cultures. Cortical hemispheres were sectioned from fetal rats (Sprague–Dawley) in their 18th day of gestation and were trypsin digested and triturated into single-cell suspension. Cells were pipetted into individual wells of poly-L-lysine-coated plates, yielding a final cell concentration of 200,000 cells/cm³ using Gibco's Minimum essential medium (MEM; containing 10% horse and 10% heat-inactivated fetal bovine serum). Nonneuronal cell division was halted 3 days into culture by adding 25 μ g/ml uridine and 10 μ g/ml 5-fluoro-2'-deoxyuridine (19, 20). Feedings were performed as necessary with MEM with 10% horse serum. Human neuroblastoma SH-SY5Y (SY5Y) were grown on 12-well plates to confluency (about 2 million/well) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml fungizone (amphotericin B).

MTX treatment of SY5Y and fetal cortical cultures. On the 17th–21st days postplating, the fetal cortical cultures were washed three times with serum-free medium. For the SY5Y cells, confluent cultures were washed three times with serum-free MEM. Calpain inhibitor or calcium channel blocker was added at this point for 1 h of preincubation. The cultures were then challenged with various concentrations of maitotoxin for 2.5 min to 1 h.

In situ SLLVY-AMC hydrolysis in cultures. After the cultures were washed three times with serum-free media and resuspended to 0.5 ml of medium, medium containing succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (SLLVY-AMC) or succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) (0.5 ml) was then added to achieve a final concentration of 80 μ M (21). MTX (0.1 nM) was added if desired at this point. The plates were incubated at room temperature.

Fluorescence (excitation 380 nm and emission 420 nm with slit widths set at 15 nm) was measured every 15–30 min up to 120 min with a Millipore Cytoflor 2300 fluorescence platereader.

MTX-induced calcium accumulation. After the cultures were challenged with MTX for 10 min as described above, the cultures were rapidly washed three times with 1 ml of TBS (20 mM Tris–HCl, pH 7.4, at room temperature, 155 mM NaCl). To each well, 100 μ l of water and 200 μ l of 2.5% (w/v) trichloroacetic acid (TCA) were added to lyse the cells for 30 min. The plates were sonicated in a bath for 5 min before neutralizing with 20 μ l of Tris–base and 25 μ l of Tris–HCl (pH 7.4 at 22°C). The plates were centrifuged at 4000 rpm for 5 min to pellet the debris. Aliquots of the cell lysate (250 μ l) were transferred to a 96-well plate and mixed with 10 μ l of 1 mM arsenazo III (final concentration 38 μ M) (22, 23). Standards were set up by mixing 250 μ l of mock lysis solution (containing TCA, Tris–base, and Tris–HCl but no cell extract), 38 μ M arsenazo III, and 0, 0.5, 1, 2, 5, 10, 20, or 50 nmol of calcium chloride (260 μ l). The plate was read at 650 nm for the detection of the calcium–dye complex. The total intracellular calcium contents were then calculated using a standard curve. Since total calcium accumulation instead of free calcium level was measured, nmol/million cells was used instead of molarity.

Cell death measurement. After cultures were subjected to MTX treatment as described above, the plates were then washed with and maintained in normal serum-free medium (with inhibitor if desired) in an oxygenated incubator (21% O₂, 8% CO₂, 71% N₂) until 24 h after the experiment initiation. To measure cell death in SY5Y cultures, 40 μ g/ml of propidium iodide was added directly to the wells at 24 h and fluorescence of the dye–DNA complex (excitation 380 nm, emission 420 nm) was measured after 5 min with a Millipore Cytoflor 2300 fluorescence platereader.

Protein extraction. At the end point of an experiment, the medium was first removed and the attached cells were washed twice with 20 mM Tris–HCl (pH 7.4, room temperature), 155 mM NaCl, and 1 mM EDTA. Protein extraction was done as previously described (24). Briefly, cells were lysed with a lysis buffer containing 2% (w/v) SDS, 5 mM EGTA, 5 mM EDTA, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl

fluoride, 10 $\mu\text{g/ml}$ 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{g/ml}$ *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, 10 $\mu\text{g/ml}$ *N*- α -*p*-tosyl-L-phenylalanine chloromethyl ketone, and 20 mM Tris-HCl (pH 7.4) for 15 min at room temperature (300 μl). TCA [100 μl of 100% (w/v)] was added to the lysate. DNA aggregate was removed while total protein precipitate in suspension was collected into microcentrifuge tubes. The centrifuged (6000 rpm) protein pellets were washed with 1 ml of 2.5% (w/v) TCA. The final pellets were neutralized with 25 μl of 3 M Tris-base for 30–60 min and then diluted with 25 μl of water.

Electrophoresis, Western blotting, and immunostaining. The protein samples were analyzed for protein concentration with a modified Lowry assay (Bio-Rad). Samples (15 μg of protein) were run on SDS-PAGE (4–20% acrylamide) with a Tris-glycine running buffer system and transferred onto a PVDF membrane (0.2 μm) with the Tris-glycine buffer system using a semidry electrotransferring unit (Bio-Rad) at 20 mA for 1.5–2 h. The blots were probed with an anti- α -spectrin (nonerythroid; Chemicon) antibody or anti-m-calpain (a gift from T. Shearer, Oregon Health Science University), anti- μ -calpain (monoclonal antibody; a gift from J. Elce, Queen's University), anti-s-calpain (Chemicon), and a biotinylated second antibody and avidin conjugated with alkaline phosphatase. The blots were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

MTX Induces Calcium Accumulation

Maitotoxin has been reported to induce calcium influx in many cell types (10). One of our models is the neuroblastoma SH-SY5Y. These cells, which are undifferentiated and neuroblast-like, contain muscarinic receptors and can be differentiated by retinoic acid into a neurite-containing phenotype. A range of maitotoxin concentrations (from 0.01 to 30 nM, for 10 min) were tested in the SY5Y cultures to identify the optimal concentration range for evoking calcium accumulation (Fig. 2A). In these experiments, we utilized the calcium-sensitive dye arsenazo III, which when calcium-bound gives an absorbance optimum at 650 nm (22, 23). Under our conditions, total intracellular calcium accumulation within the cells was measured. This method can readily monitor calcium influx as it is insensitive to intracellular calcium store release (i.e., induced by thapsigargin) (results not shown). It was observed that MTX induced rapid calcium accumulation in a dose-dependent manner. As little as 0.1 nM could produce significant calcium influx while 1 nM gave the maximum calcium accumulation signal (Fig. 2A). With higher concentrations of MTX (3–30 nM), the calcium signal was apparently smaller; this can be attributed to physical cell loss due to cell detachment and partial cell lysis that occurred during or shortly after the MTX treatment. Using 1 nM MTX, the time course of calcium accumulation in SY5Y cells was further investigated (Fig. 2B). We confirmed that the action of MTX was very rapid and 10 min gave the maximal signal. In parallel, we also utilized fetal rat cerebrocortical cultures. A mixture of glial cells and neurons (mainly glutamatergic) was found in this primary culture. At con-

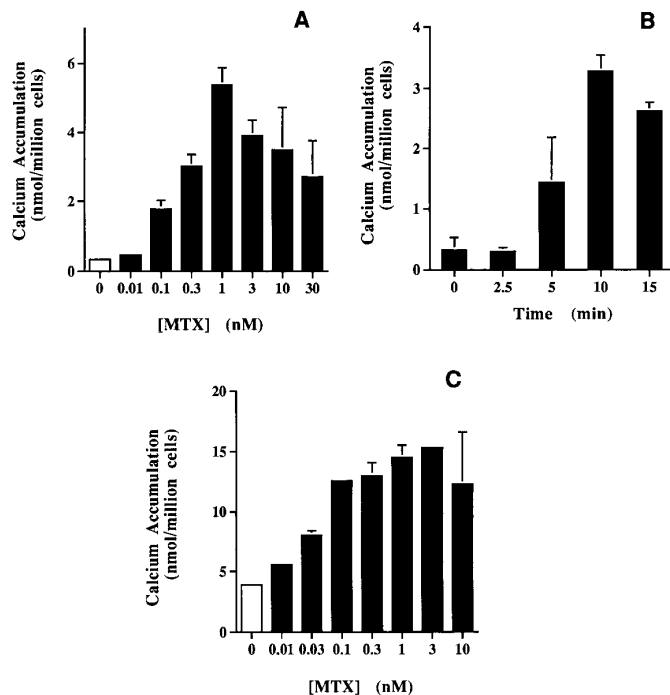


FIG. 2. Maitotoxin induces calcium accumulation in SY5Y cells and cerebrocortical cultures. Calcium accumulation following MTX challenge in neuroblastoma SY5Y cells (A, B) and in fetal rat cortical cultures (C). Total intracellular content was measured by arsenazo III binding after exposing the cultures to no treatment (open bars) or various concentrations of MTX (0.01 to 10 nM) (solid bars) for 10 min (A, C) or 1 nM MTX for 2.5 to 15 min (solid bars) (B). Data are means \pm SD, $n = 3$.

centrations from 0.1 to 3 nM, MTX (10 min) produced significant calcium accumulation.

The mode of MTX-induced calcium accumulation in SY5Y was also investigated. When we used an external medium that was calcium-free, the calcium accumulation-induced MTX was totally abolished (Fig. 3). This suggests that calcium accumulation in MTX-treated cells was a direct result of calcium influx across the plasma membrane, consistent with a previous report (10). It has also been suggested that rather than forming a calcium ionophore in cell membrane, MTX operates through endogenous calcium channels from both the voltage-sensitive (VSCC) and the receptor-operated subclasses (11, 12). A number of calcium channel blockers were thus tested. The L-type VSCC-inhibiting nifedipine and the N-type VSCC-inhibiting SNX185 (conotoxin MVIIA analog) both produced no significant effect (25) (Fig. 3). On the other hand, the nonselective calcium channel blocker SKF96365 produced a robust and significant reduction of MTX-induced calcium accumulation, consistent with previous reports (11, 12). In parallel, we also used 10 μM A23187 instead of MTX on the SY5Y cells. The calcium accumulation signal

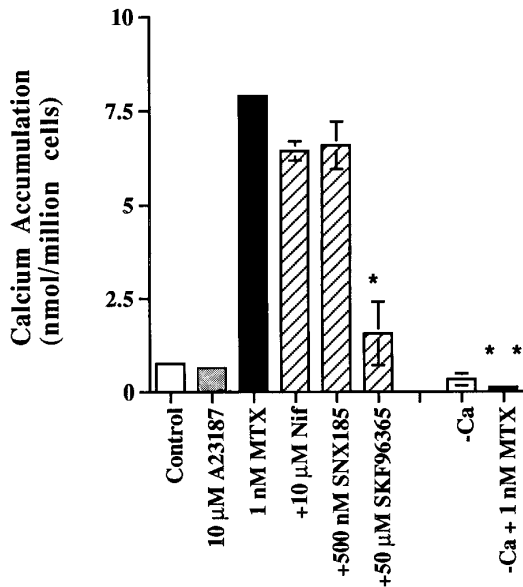


FIG. 3. Effect of extracellular calcium and various calcium channel blockers on maitotoxin-induced calcium accumulation in SY5Y cells. SY5Y cultures were subjected to no challenges (open bars), 10 μ M A23187 (shaded bar), or 1 nM MTX for 10 min in the absence (solid bar) or presence of 10 μ M nifedipine, 500 nM conotoxin MVIIA analog SNX185, or 50 μ M SKF96365 (crossed bars). In parallel, cultures in calcium-free medium were either untreated (-Ca; open bar) or treated with 1 nM MTX (-Ca + MTX; solid bar) for 10 min. Data are means \pm SD, $n = 3$. Data significantly different from MTX alone are indicated by * ($P < 0.002$, Student's t test) or ** ($P < 0.0005$).

was not detectable in A23187-treated cells, probably due to calcium leaking out during the cell-washing stage (not the case with MTX since it works on unidirectional calcium channels) (Fig. 3).

Evidence of Calpain Autolysis in MTX-Treated Cells

Calpain is known to undergo autolysis upon activation (4). We therefore examined evidence for autolysis in MTX-treated SY5Y cells, using a panel of three anti-calpain antibodies (see Materials and Methods). With a μ -calpain (large subunit)-specific antibody, we found that this subunit (80 kDa) partially autolyzed to fragments of 78 and 76 kDa in MTX-treated cells (Fig. 4). We also used an antibody that recognizes m-calpain but not μ -calpain. It also interacted with the small subunit common to both μ -calpain and m-calpain. We observed no apparent changes in the apparent size of the large subunit of m-calpain (80 kDa) in MTX-treated cells (Fig. 4). It is possible that the 80-kDa intact protein has undergone autolysis to the activated 76-kDa form, which unfortunately has an identical migration rate to the 80-kDa protein, as reported before (26, 27). The m-calpain antibody in fact also detected the small subunit (29 kDa) in control cells. It is known that the small

subunit autolyzes rapidly to a limit fragment (18 kDa) which retains calcium-binding function (26, 27). Under our conditions, the 29-kDa subunit almost completely disappeared in MTX-treated cells, but no 18-kDa band was detected. This was most likely because the 18-kDa fragment no longer contained the immunogenic site. We also used a small subunit-specific antibody and observed again that the 29-kDa intact protein was considerably reduced in MTX-treated cells (Fig. 4). Since the small subunit is used by both μ - and m-calpain, its autolysis to the activated 18-kDa form would suggest that both μ -calpain and m-calpain were probably activated in cells subjected to MTX treatment. m-Calpain has been found to translocate to membrane fraction upon activation (34). Examining whether m-calpain translocation occurs could be a means to determine whether m-calpain is activated.

Calpain-Mediated α -Spectrin Breakdown in MTX-Treated Cells

Nonerythroid α -spectrin (280 kDa) has been reported to be one of the preferred substrates of calpain. In fact, *in situ* and *in vivo* α -spectrin breakdown has been observed under conditions where endogenous calpain was activated (see Ref. 5). Typically, two fragments of 150 and 145 kDa are sequentially formed. Due to this special pattern of fragmentation, these α -spectrin breakdown products (SBDP) have been used as markers of calpain activation (5). Using an anti- α -spectrin antibody, we looked for the presence of SBDP following MTX treatment. Although challenges of 1 nM MTX for 10 min gave maximal calcium rises, we found that it took longer to get a maximal spectrin breakdown signal (1 h). We also chose to lower the MTX concentration to 0.1 nM for better cell

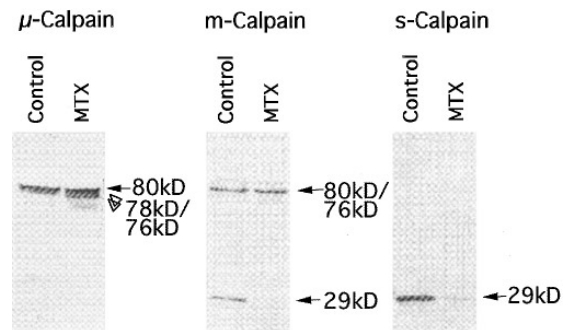


FIG. 4. Calpain autolysis in maitotoxin-treated SY5Y cells. SY5Y cultures were either untreated (control, lane 1) or treated with 1 nM MTX for 1 h (MTX, lane 2). Total cellular proteins were extracted and analyzed by PAGE (15 μ g protein/lane), electrotransferred, and then probed with various antibodies. Shown here are the developed blots. The protein the antibody was raised against was indicated. Solid arrows indicate the intact protein and autolysis products. Results shown are representative of at least three experiments.

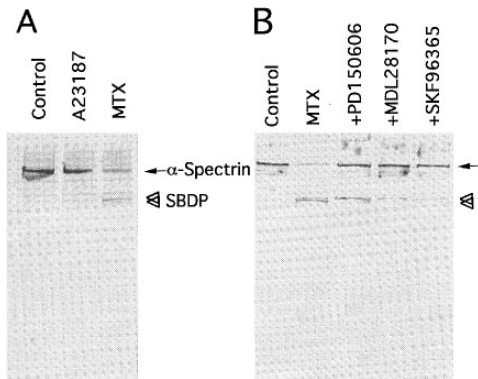


FIG. 5. Maitotoxin induces calpain-mediated α -spectrin breakdown in SY5Y cells. In (A), cultures were either untreated (control) or subjected to $20 \mu\text{M}$ A23187 or 1 nM MTX for 1 h. In (B), cultures were either untreated (control) or treated with 0.1 nM MTX for 1 h in the absence (MTX) or presence of an inhibitory agent ($10 \mu\text{M}$ MDL28170, $20 \mu\text{M}$ PD150606, or $30 \mu\text{M}$ SKF96365, with a 1-h preincubation) as indicated. Total cellular proteins were extracted and analyzed by PAGE ($15 \mu\text{g}$ protein/lane), electrotransferred, and then probed with various antibodies. Shown here are the developed blots. The arrows indicated the positions of intact α -spectrin (280 kDa) while the open arrowheads indicate the two sequentially formed spectrin breakdown products (SBDP; 150 and 145 kDa). Results shown are representative of at least three experiments.

tolerance. In the untreated cells, the intact α -spectrin band was clearly detected (Fig. 5A). In MTX-treated cells, it was observed that (i) the intact α -spectrin band intensity was markedly reduced (each lane was loaded with an equal amount of protein) and (ii) the SBDP bands (150 and 145 kDa) were formed (Fig. 5A). Again, we used A23187 in parallel and observed only a faint band around 150 kDa after 1 h of A23187 treatment. We have found that with longer duration of A23187, the signal of SBDP did get stronger (data not shown). Thus, it appears that MTX is capable of raising calcium levels more rapidly than A23187. To confirm that the SBDPs were a result of the proteolysis by calpain, we utilized two calpain inhibitors: an active-site-targeting peptidic calpain inhibitor (Z-Val-Phe-H; MDL28170) and a highly selective calcium domain-targeting mercaptoacrylate (PD150606) (5, 28). We observed that at $10 \mu\text{M}$ MDL28170 and $20 \mu\text{M}$ PD150606, the 145-kDa fragment was completely abolished, leaving only the 150-kDa fragment. It was also noted that the intact α -spectrin was almost as intense as in the control lane, reflecting that spectrin was protected from proteolysis by these agents. As it has been noted previously in A23187-treated Molt-4 cells (28), due to the uncompetitive nature of PD150606 with respect to calpain substrate, inhibition of α -spectrin cleavages by calpain was only partial (Fig. 5B). In fact, we sometimes observed a more intense 150-kDa fragment in PD150606/MTX-treated cells than in MTX-treated cells (Fig. 5B). Since the 145-kDa SBDP was a further slower-forming

cleavage product of the 150-kDa fragment, reduction of the 145-kDa fragment thus resulted in more 150-kDa fragment accumulated. In parallel, in calcium channel blocker SKF96365-treated cells, MTX-induced SBDPs were considerably reduced (Fig. 5B). In this case, the mechanism of intervention was different from that of the calpain inhibitors: instead of inhibiting calpain activity directly, SKF96365 functioned by attenuating the total cellular calcium elevation. In keeping with that, both SBDPs (150 and 145 kDa) were reduced but not totally abolished (Fig. 5B). Neither the calpain inhibitors nor SKF96365 has any effects on the intact α -spectrin signal in control cells (results not shown).

We also performed a similar experiment with the cerebrocortical cultures and here, instead of MDL28170, another peptidic inhibitor, calpain inhibitor I (Z-Leu-Leu-Nle-H). Almost identical results to the SY5Y experiments were obtained; the only quantitative difference was that calpain inhibitor I and SKF96365 were more effective in this case, almost completely blocking SBDP formation (Fig. 6A). Since these cultures are composed of a mixture of both glial cells and neurons, we attempted to determine the origin of the α -spectrin signals. We took advantage of a previous observation that neurons in these types of cultures are glutamatergic and are highly vulnerable to an excessive amount of glutamate or its analog *N*-methyl-D-aspartate (NMDA) while the glial cells are resistant

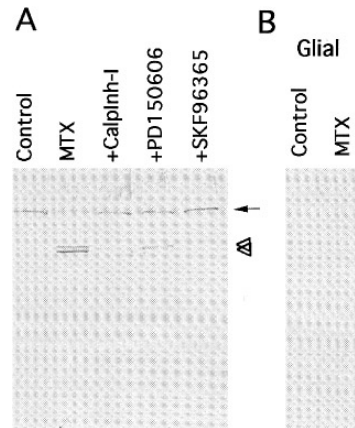


FIG. 6. Maitotoxin induces calpain-mediated α -spectrin breakdown in cerebrocortical neurons. In (A), mixed cortical cultures were either untreated (control) or treated with 1 nM MTX for 1 h in the absence (MTX) or presence of an inhibitory agent ($10 \mu\text{M}$ MDL28170, $10 \mu\text{M}$ PD150606, or $30 \mu\text{M}$ SKF96365, with a 1-h preincubation) as indicated. Total cellular proteins were extracted and analyzed by PAGE ($15 \mu\text{g}$ protein/lane), electrotransferred, and then probed with various antibodies. Shown here are the developed blots. The arrow indicated the position of intact spectrin (280 kDa) while the open arrowheads indicate the two sequentially formed α -spectrin breakdown products (SBDP; 150 and 145 kDa). In (B), a glial cells-only culture (see Materials and Methods) was subjected to either no treatment (control) or 0.1 nM MTX for 1 h (MTX). Results shown are representative of at least three experiments.

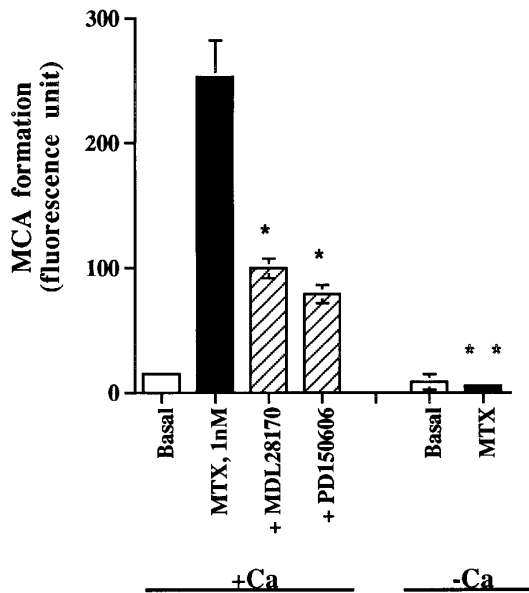


FIG. 7. *In situ* SLLVY-AMC hydrolysis by calpain in maitotoxin-treated SY5Y cells. SLLVY hydrolysis was measured in SY5Y cells without (basal; open bar) or with MTX treatment (60 min) in the absence (MTX; solid bar) or presence of 10 μ M MDL28170 or 25 μ M PD150606 (crossed bars). Basal (open bar, far right) and MTX-induced (solid bar, far right) SLLVY-AMC hydrolysis in SY5Y cells in calcium-free medium (-Ca) was also monitored. Data are means \pm SE ($n = 3$). Data significantly different from MTX alone are indicated by * ($P < 0.001$, Student's t test) or ** ($P < 0.0001$).

(20). We proceeded to pretreat the cultures with 500 μ M NMDA (for 3 h) followed by a recovery period of 72 h with medium changes. At that time, the culture dishes had only glial cells remaining. With this type of glial-only cultures, we found that in either the control or the MTX-treated cells, very little α -spectrin or its SBDP bands were detected under these conditions (Fig. 6B).

In Situ SLLVY-AMC Hydrolysis by Calpain in SY5Y Cells

In addition to examining protein degradation in cells following MTX treatment, we attempted to detect calpain activation by using a cell-permeable peptide substrate, SLLVY-AMC (21). Apparently, since SLLVY-AMC does not contain any charged residues, it can penetrate cells and presumably the intracellular concentration reached an equilibrium with the extracellular concentration (80 μ M) during the preincubation period. We then measured the fluorescence of the cleavage product 4-methylcoumarinamine, as it was liberated from the peptide. We confirmed that in resting cells, very little hydrolysis of SLLVY was detected while MTX treatment induced a marked increase in this hydrolytic activity (Fig. 7). We observed that PD150606 as well as MDL28170 partially attenuated this activity. When extracellular calcium is

removed, very little SLLVY hydrolysis remained, even in MTX-treated wells. Taken together, these data confirm that calpain was activated in MTX-treated cells.

DISCUSSION

Maitotoxin has been found to evoke a number of calcium-dependent cellular processes, such as phospholipase C activation (10, 16, 17, 29). However, to our best knowledge, this study is the first demonstration that calpain is also activated in cells following MTX treatment. The evidence for calpain activation in MTX-treated cells includes (i) evidence for autolysis of both μ - and m-calpains (Fig. 4); (ii) formation of distinct spectrin breakdown products (150 and 145 kDa) (Figs. 5 and 6); (iii) *in situ* hydrolysis of peptidic substrate (SLLVY-AMC) for calpain (Fig. 7); and (iv) inhibition of both spectrin breakdown and SLLVY-AMC hydrolysis by two classes of calpain inhibitors (active-site-targeting calpain inhibitor I and MDL28170) as well as calcium binding domain-targeting mercaptoacrylate PD150606 (Figs. 5–7).

The phenomenon of spectrin breakdown in MTX-challenged cells is a intriguing one. α - and β -spectrins are important cortical cytoskeletal proteins which interact with actin, calmodulin, and annexins (30–32). Spectrin breakdown has in fact been demonstrated in both necrosis (e.g., in NMDA/glutamate toxicity to cerebrotical neurons) and apoptosis (e.g., in staurosporine or dexamethasone-treated T lymphoma cells) (20, 33). It is not difficult to visualize the potential impact of spectrin breakdown on cytoskeleton integrity. Related to this, we have previously identified that CaMBPs as a family are selectively sensitive to the attack of calpains under *in vitro* conditions (using purified calpain) (4). In fact, in addition to spectrin, MAP2 was another cytoskeletal CaMBP that was degraded in MTX-treated cells (results not shown). It is plausible that through the proteolytic modification of these proteins, calpain exerts its negative impact on cell viability following MTX treatment.

If calpain activation was important to MTX-induced cell death, one would argue that application of effective calpain inhibitors may protect cells from the MTX toxicity. In our preliminary experiments, we did not observe a significant improvement of cell survival using calpain inhibitors (unpublished results). Our interpretation is that the cytotoxic pathway induced by MTX is multifactorial and not due to calpain activation alone. Another factor is that the magnitude of the MTX challenge might have been too high (1 nM), making it almost insurmountable. Thus, it appears possible that if we attenuate the MTX challenge, the cytoprotective effect of calpain inhibition may be more readily observed.

It is important to point out that the present study established that MTX is a tool in studying the physio-

logical roles of calpain. We found that MTX was a better activator of calpain than calcium ionophore A23187 (Fig. 5A). In addition to SH-SY5Y, we note that in a number of other cell types, including a Chinese hamster ovary (CHO) cell line, MTX was also a superior calpain activator. Another technical note is that A23187 and ionomycin are highly hydrophobic compounds and have a strong tendency to adhere to plastic. They also cause ionophore-treated cells to self-aggregate (clumping), most notably in cells maintained in suspension. Often, the ionophoric effect of A23187 or ionomycin is irreversible. On the other hand, MTX appears to be much easier to use and does not cause these potential problems. We have also observed that a brief treatment (e.g., 1 h) of low concentrations (0.01–0.1 nM) of MTX could lead to transient calpain activation but did not affect long-term cell survival (results not shown). The fact that MTX is inducing calcium influx through endogenous calcium channels may explain why it is a better activator of calpain than traditional calcium ionophores. Arguably, MTX treatment may be the more “natural” way to evoke calpain activation.

This study also highlighted a number of cell-based calpain assays that can be used to monitor calpain activity *in situ*: (i) autolytic fragmentation of calpain (Fig. 4), (ii) formation of distinct spectrin breakdown products (150 and 145 kDa) (Figs. 5 and 6), and (iii) intracellular hydrolysis of SLLVY-AMC (Fig. 7). Thus, we propose here that, when used at low concentrations and in a control manner (e.g., short treatment time), MTX can be a very useful tool in investigating both the physiologic and the pathologic roles of calpain, especially in conjunction with the cell-based calpain assays described here. Indeed, such a cellular calpain assay system may also find utility in evaluating the effectiveness of novel calpain inhibitory agents in intact cells.

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