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Interactions of maitotoxin with voltage-sensitive calcium channels in cultured neuronal cells

(calcium flux/channel blockers/dihydropyridines)

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ABSTRACT **The dinoflagellate toxin maitotoxin (MTX) stimulated $^{45}Ca^{2+}$ uptake in cultured NG108-15 neuroblastoma × glioma cells. Depolarizing stimuli (e.g., 50 mM K^+) produced an immediate stimulation in Ca^{2+} uptake, whereas that produced by MTX occurred only after a lag period of about 2 min. MTX did not stimulate Ca^{2+} uptake into fibroblasts. Both 50 mM K^+- and MTX-stimulated Ca^{2+} uptake was blocked by organic calcium channel antagonists (nitrendipine, D-600, diltiazem) at very low concentrations. Cd^{2+} was also a potent blocker. The novel dihydropyridine BAY K8644 enhanced Ca^{2+} uptake in the presence of 50 mM K^+ but had no effect in 5 mM Ca^{2+}. However, in the presence of MTX, BAY K8644 stimulated Ca^{2+} uptake in 5 mM K^+. The effects of MTX were not blocked by tetrodotoxin but were decreased in Na^+-free medium. MTX did not stimulate Na^+ uptake into NG108-15 cells and did not alter [^{3}H]nitrendipine binding to rat brain cortical synaptosomes. It is concluded that MTX may alter the voltage dependence of calcium-channel activation.**

One of the most important aspects of research on voltage-sensitive sodium channels (VSNaC) has been the discovery and development of chemical probes that selectively alter different facets of channel function (1). Thus, toxins such as tetrodotoxin (TTX), batrachotoxin (BTX), and scorpion venom have been shown to interact with separate sites on the channel molecule (2). These toxins have been invaluable in studies directed at isolating the channel molecule and in understanding its properties at a molecular level. Clearly, similar agents would be helpful in advancing our understanding of other channels, such as those for potassium and calcium.

Maitotoxin (MTX) is a water-soluble toxin of unknown structure isolated from the poisonous dinoflagellate *Gambierdiscus toxicus* (3). It is one of the entities responsible for the syndrome known as ciguatera poisoning, which is common in tropical and subtropical areas. The purified toxin has a positive inotropic effect (4), contracts smooth muscle (5, 6), stimulates transmitter release from sympathetic neurons (6) and cultured PC12h pheochromocytoma cells (7, 8), and releases prolactin from primary pituitary cultures (9). It has been suggested that MTX is an "activator" of voltage-sensitive calcium channels (VSCC) (5–9). However, no experimental data have been obtained that directly examine the effect of MTX on VSCC.

We have previously demonstrated that several cell lines including the neuroblastoma × glioma hybrid NG108-15 express a type of VSCC when grown under the appropriate conditions (10). The pharmacology of these channels has been characterized in detail (10). They appear to be extremely similar to those occurring in smooth muscle. Thus, they are blocked by nanomolar concentrations of dihydropyridines, such as nifedipine, and are activated by the novel dihydropyridines BAY K8644 and CGP 28392 (10, 11). In the present series of studies, we have investigated the interaction of MTX with VSCC in these cultured neuronal cells. The results indicate that the toxin may indeed have a direct effect on VSCC.

METHODS AND MATERIALS

MTX Preparation. Dickey *et al.* (12) have prepared four toxins from cultures of *G. toxicus* designated GT 1–4. GT 1–3 are lipid-soluble toxins. GT-4 is a water-soluble fraction containing MTX. Cultures of *G. toxicus* were pelleted and cells were extracted by refluxing in boiling aqueous methanol. The methanol extracts were concentrated and subjected to liquid/liquid partitioning followed by cold acetone precipitation of the toxic components. The crude water soluble extract was further purified by sequential treatment on columns of silicic acid, DEAE cellulose, and Sephadex G-15-120. Eluting solvents, in order, consisted of chloroform/methanol, chloroform/methanol/water, and water, respectively. For further details, see Dickey *et al.* (12).

Ca^{2+} Uptake Studies. $^{45}Ca^{2+}$ uptake studies were carried out exactly as described by Freedman *et al.* (10). Briefly, NG108-15 cells were grown as monolayers in 5% CO_2/95% air in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were subcultured onto 60-mm tissue culture plates. To induce cellular differentiation, growth medium was supplemented with 10 μM prostaglandin E_1 and 50 μM 3-isobutyl-1-methylxanthene. Supplemented medium was replaced every 2 days or when required. 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. During the assay, tissue culture plates were supported in an open air water bath at 37°C. Cells were incubated for 5 min at 37°C. The uptake of $^{45}Ca^{2+}$ was measured for increasing periods of time in 20 mM Hepes-buffered Eagle's medium containing 135.7 mM NaCl/5 mM KCl/0.44 mM KH_2PO_4/0.34 mM Na_2HPO_4/2.62 mM $NaHCO_3$/1.3 mM $CaCl_2$/0.81 mM $MgSO_4$/5.6 mM glucose. A 50-mM KCl buffer was prepared by adjusting Na^+ reciprocally; $^{45}Ca^{2+}$ was added to give a final concentration of 1 μCi of $^{45}Ca^{2+}$ per ml (1 Ci = 37 GBq). At the indicated times, plates were aspirated, washed immediately, and inverted to allow to dry. Cells were solubilized with 5 ml of 0.2% $NaDodSO_4$, and samples were removed for estimation of $^{45}Ca^{2+}$ uptake and protein content by fluorescence (13).

Na^+ Uptake Studies. Na^+ uptake was measured by the method of O'Donnell *et al.* (14). Briefly, cells were serum-deprived for 4 hr prior to assay. For assay, the incubation

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Abbreviations: MTX, maitotoxin; TTX, tetrodotoxin; BTX, batrachotoxin; VSCC, voltage-sensitive calcium channel; VSNaC, voltage-sensitive sodium channel.

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medium contained 5 mM ouabain to inhibit the Na^+/K^+ pump and 1 μM nitrendipine. After various times of incubation, uptake was terminated by aspirating the medium and rapidly dipping the dishes 4 times in each of four 1-liter beakers containing ice-cold $MgCl_2$ (pH 7.0, with KOH). After washing and drying, cells were extracted with trichloroacetic acid. Na^+ concentration was measured in the trichloroacetic acid extract by using an atomic absorption spectrometer. Further details can be found in O'Donnell *et al.* (14) and in Owen and Villereal (15).

Binding Studies. The association and dissociation rates of specific [^{3}H]nitrendipine binding were measured in whole rat cortical synaptosomes as described (16). Synaptosomes were prepared in 0.32 M sucrose. Incubations were at 22°C for various times in Hepes/Ringer, pH 7.4, containing 1.3 mM $CaCl_2$/5.4 mM KCl/137 mM NaCl/0.8 mM $MgCl_2$/0.44 mM KH_2PO_4/20 mM Hepes. Nonspecific binding was determined with 1 μM nitrendipine.

Materials. $^{45}Ca^{2+}$ (10–40 mCi per mg of Ca) was obtained from Amersham; [^{3}H]nitrendipine (78.4 μCi/mmol) was obtained from New England Nuclear; nitrendipine and BAY K8644 were from Miles; diltiazem was from Marion Laboratories (Kansas City); D-600 was from Knoll AG (Ludwigshafen, Federal Republic of Germany). All other reagents were standard grade.

RESULTS

In NG108-15 cells, depolarizing stimuli (e.g., 50 mM K^+ or BTX) produce an immediate increase in net Ca^{2+} influx (10). This effect is shown in Fig. 1, where cells were depolarized by 50 mM K^+. Fig. 1 also illustrates the effect of MTX (100 ng/ml). It can be seen that the toxin also stimulated net Ca^{2+} influx. However, the effect of MTX only became apparent after a lag period of about 2 min. MTX-stimulated Ca^{2+} influx then increased with time and reached levels at least as great as those produced by 50 mM K^+ by the end of the assay period (15 min). MTX did not inhibit the effects of 50 mM K^+ over the same time period when the two agents were added simultaneously. Concentrations of MTX >10 ng/ml were effective in stimulating Ca^{2+} influx (Table 1). At concentrations above 300 ng/ml, very large increases in Ca^{2+} uptake were observed. However, this effect appeared nonspecific, as it could not be blocked by organic and inorganic calcium channel blockers (see below). The concentrations of MTX required to stimulate release of transmitter from PC12h cells and to contract the vas deferens are somewhat lower than the concentrations found to be effective in NG108-15 cells (3–8). However, we have found that our preparation of MTX contracts dog mesenteric artery strips at lower concentrations (>1 ng/ml). Presumably, therefore, different preparations exhibit different sensitivities to the effects of MTX. In contrast to the effects observed in NG108-15 cells, neither depolarizing stimuli nor MTX (100 ng/ml) increased Ca^{2+} influx into cultured 3T3 fibroblasts.

Table 1. Effect of different concentrations of MTX on $^{45}Ca^{2+}$ uptake into NG108-15 cells

Addition	$^{45}Ca^{2+}$ uptake, cpm per mg of protein per 15 min
K^+ (5 mM)	2935 ± 100
K^+ (50 mM)	6978 ± 174
K^+ (5 mM) and MTX (10 ng/ml)	2581 ± 289
K^+ (5 mM) and MTX (30 ng/ml)	3935 ± 599
K^+ (5 mM) and MTX (100 ng/ml)	6076 ± 256
K^+ (5 mM) and MTX (300 ng/ml)	8406 ± 1786

$^{45}Ca^{2+}$ uptake was measured over a 15-min incubation (n = 3).

We have previously shown that VSCC in NG108-15 cells are blocked by low concentrations of both organic and inorganic calcium-channel blockers (10). Fig. 2 shows that the effects of MTX (100 ng/ml) were also blocked by these same drugs at low concentrations. The characteristics of blockade of MTX-induced Ca^{2+} uptake by drugs differed somewhat from blockade of depolarization-induced uptake. Thus, MTX-induced Ca^{2+} uptake was more sensitive to the effects of D-600 and diltiazem than was 50 mM K^+-induced uptake. Moreover, the shape of the nitrendipine inhibition curve for blockade of MTX-induced Ca^{2+} uptake differed from that for nitrendipine blockade of 50 mM K^+-induced Ca^{2+} uptake (Fig. 2). Cd^{2+} was as powerful an inhibitor of the effect of MTX as it was for the effect of depolarizing stimuli.

We have previously demonstrated that the dihydropyridines BAY K8644 and CGP 28392 can enhance Ca^{2+} influx into NG108-15 cells (11). The effects of these agents are observed only in the presence of depolarizing stimuli. Thus, both drugs enhance the effects of 50 mM K^+ or BTX but have no effect at resting membrane potentials (5 mM K^+). The enhancing effects of these "agonist" dihydropyridines are blocked by "antagonist" dihydropyridines, such as nitrendipine, in a competitive fashion. It was most interesting to note (Table 2) that in the presence of MTX, BAY K8644

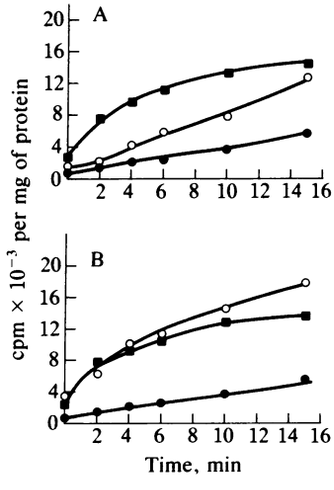

FIG. 1. Time course of $^{45}Ca^{2+}$ uptake into NG108-15 cells. ●, 5 mM K^+; ■, 50 mM K^+; (*A*) ○, 5 mM K^+/MTX (100 ng/ml); (*B*) ○, 50 mM K^+/MTX (100 ng/ml). Points are means of duplicate incubations. The experiment was done 3 times with similar results.

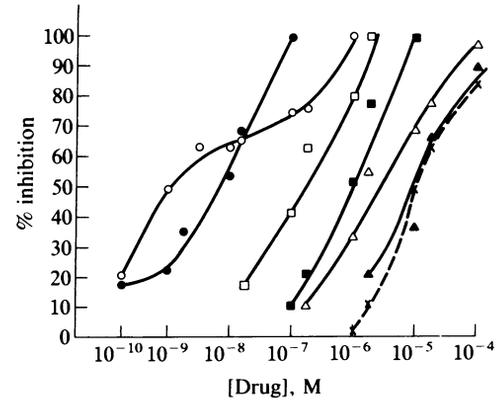

FIG. 2. Inhibitory effects of various drugs on stimulation of $^{45}Ca^{2+}$ uptake into NG108-15 cells produced by 50 mM K^+ and MTX (100 ng/ml). Incubations with MTX were carried out for 15 min. Data for 50 mM K^+ are taken from Freedman *et al.* (10). ●, nitrendipine/50 mM K^+; ○, nitrendipine/MTX; ■, D-600/50 mM K^+; □, D-600/MTX; ▲, diltiazem/50 mM K^+; △, diltiazem/MTX; ×, Cd^{2+}/MTX. Points are means of 3–9 incubations.

Table 2. Enhancement of the effect of MTX by BAY K8644

Addition	$^{45}Ca^{2+}$ uptake, cpm per mg of protein per 15 min
K^+ (5 mM)	3,200 ± 153
K^+ (50 mM)	7,667 ± 234
K^+ (5 mM) and MTX	7,133 ± 670
K^+ (50 mM) and BAY K8644	30,030 ± 2064
K^+ (5 mM) and MTX and BAY K8644	28,300 ± 2466

$^{45}Ca^{2+}$ uptake was measured over a 15-min incubation. The concentration of MTX was 100 ng/ml and concentration of BAY K8644 was 1 μM (n = 3).

was able to enhance Ca^{2+} uptake in 5 mM K^+ without the addition of a depolarizing stimulus. A dose–response curve for the effects of BAY K8644 is seen in Fig. 3. The dihydropyridine enhanced the effects of MTX over the same concentration range as it enhanced the effects of 50 mM K^+ (11). Table 3 shows that BAY K8644 only enhanced the effects of MTX at toxin concentrations that themselves stimulated net Ca^{2+} influx (>10 ng/ml).

As BAY K8644 was able to enhance the effect of MTX as it could those of a depolarizing stimulus, it seemed possible that MTX might act to depolarize cells in some fashion resulting in the opening of VSCC rather than acting on the VSCC *per se*. One way in which this could easily happen is if MTX opened VSNaC or acted as a Na^+ ionophore. We found that the action of MTX was not blocked by a high concentration of TTX (1 μM). However, replacement of Na^+ in the medium by choline clearly altered the action of MTX. Fig. 4 shows that under such conditions, MTX was still effective. However, the magnitude of its effect was clearly decreased, and the lag period associated with its action was increased. It should be noted that replacement of Na^+ by choline does not alter the effect of 50 mM K^+ at all (10). Table 4 shows that replacement of Na^+ also decreased the ability of BAY K8644 to enhance the effects of MTX.

Thus, it seemed possible that MTX might act at least partially as a Na^+ ionophore and depolarize cells by stimulating Na^+ influx independently of VSNaC. Therefore, we measured the effect of MTX on Na^+ influx into NG108-15 cells

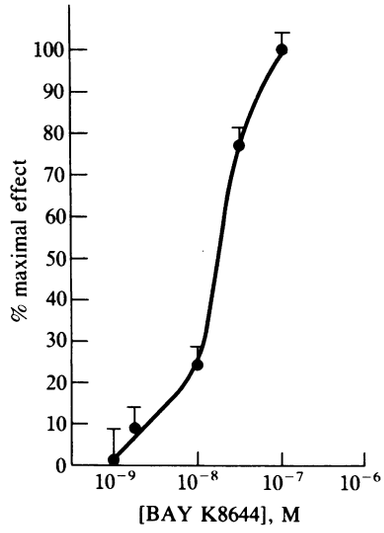

FIG. 3. Dose–response curve for the enhancing effects of BAY K8644 on MTX (100 ng/ml)-stimulated $^{45}Ca^{2+}$ uptake. Uptake was measured for a 15-min incubation period. Uptake in MTX (100 ng/ml) was 6516 ± 582 cpm × 10^3 per mg of protein per 15 min. Uptake in MTX (100 ng/ml)/BAY K8644 (1 μM) was 12,699 ± 467 cpm × 10^3 per mg of protein per 15 min (n = 3).

Table 3. Effect of BAY K8644 on $^{45}Ca^{2+}$ uptake in the presence of different MTX concentrations

Addition	$^{45}Ca^{2+}$ uptake, cpm per mg of protein per 15 min
K^+ (5 mM)	2,935 ± 100
K^+ (50 mM)	6,978 ± 174
K^+ (5 mM) and MTX (100 ng/ml)	6,076 ± 256
K^+ (5 mM) and MTX (10 ng/ml) and BAY K8644	5,585 ± 380
K^+ (5 mM) and MTX (30 ng/ml) and BAY K8644	6,890 ± 480
K^+ (5 mM) and MTX (100 ng/ml) and BAY K8644	10,600 ± 130

$^{45}Ca^{2+}$ uptake was measured over a 15-min incubation. The concentration of BAY K8644 was 1 μM (n = 3).

directly. However, we observed no stimulation of Na^+ influx by MTX (100 ng/ml) over a 10-min assay period.

These results indicate that MTX may indeed have a direct effect on VSCC. We sought evidence for such an action by measuring the effect of MTX on the kinetics of [^{3}H]nitrendipine binding to rat brain cortical synaptosomes. No effect of the toxin was observed on the rate of association or dissociation of [^{3}H]nitrendipine to its specific binding sites.

DISCUSSION

Treatment of NG108-15 cells with low concentrations of MTX clearly results in the opening of VSCC. Similar effects have also been observed in a preliminary manner in other cell lines that possess similar VSCC (unpublished observations). However, such effects are not seen in other cell lines such as fibroblasts, which lack VSCC. These observations are consistent with others in the literature showing that MTX contracts smooth muscle and stimulates neurotransmitter and hormone release (4–9). Obviously, the molecular basis of this effect is of great interest. A direct effect of the toxin on VSCC would be most interesting. However, it is also conceivable that MTX merely depolarizes cells in a nonspecific fashion, resulting in the initiation of any processes in the cell type under investigation that result from cell depolarization. We made some attempts to distinguish these possibilities. The effects of MTX are not blocked by TTX and are still apparent, albeit in reduced form, in the absence of extracel-

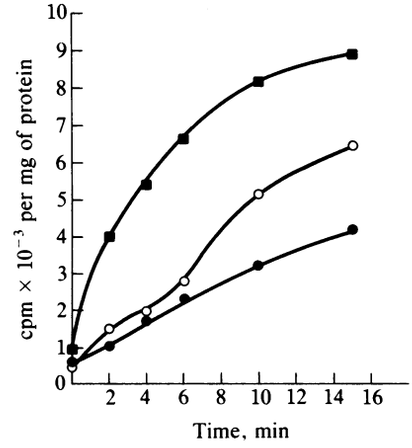

FIG. 4. Time course of $^{45}Ca^{2+}$ uptake into NG108-15 cells in medium in which all Na^+ had been replaced with choline. ●, 5 mM K^+; ■, 50 mM K^+; ○, 5 mM K^+/MTX (100 ng/ml). Points are means of duplicate incubations. The experiment was repeated 3 times with similar results. Uptake was measured over a 15-min incubation period.

Table 4. Effect of BAY K8644 on MTX-stimulated Ca^{2+} uptake, in normal and Na^+-free medium

Addition	$^{45}Ca^{2+}$ uptake, cpm per mg of protein per 15 min	
	Normal	Na^+-free
K^+ (5 mM)	2,925 ± 205	3136 ± 138
K^+ (50 mM)	6,351 ± 298	6693 ± 313
K^+ (5 mM) and MTX	6,200 ± 204	4566 ± 112
K^+ (5 mM) and MTX and BAY K8644	10,488 ± 366	6555 ± 292

$^{45}Ca^{2+}$ uptake was measured over a 15-min incubation. The concentration of MTX was 100 ng/ml and concentration of BAY K8644 was 1 μM (n = 3).

lular Na^+. Moreover, MTX does not appear to stimulate Na^+ influx. Clearly, MTX could depolarize cells by other mechanisms that do not depend on Na^+. For example, the toxin could inhibit a resting K^+ current such as an M current (17). However, the data reported here make it quite likely that the VSCC themselves constitute a specific site of action for the toxin. It is possible that MTX modifies VSCC in such a way that it alters the relationship between channel opening and membrane potential. Thus, modified channels may open at resting membrane potentials. This would be analogous to the effects of BTX or veratradine on VSNaC (2). These alkaloids alter VSNaC so that they open at resting membrane potentials. We can postulate that VSCC exist in at least two states, which are in equilibrium. One state is an inactive state stabilized by antagonist dihydropyridines, such as nitrendipine (18). A second state is stabilized by agonist dihydropyridines, such as BAY K8644. In this state, there is a greater probability of VSCC opening and VSCC stay open longer (19). Addition of BAY K8644 to a cell recruits VSCC into the latter state. Normally, such VSCC do not open at resting membrane potentials. However, in the presence of MTX, these channels will open at normal resting potentials—hence, the observed ability of BAY K8644 to enhance the effect of MTX even in 5 mM K^+.

Several other features of the action of MTX also require explanation. For example, it is clear from Fig. 2 that, although MTX-activated VSCC are blocked by organic calcium channel blockers, the dose–response curves for this effect differ somewhat from those for blocking of channels opened by 50 mM K^+. Here again, the analogy with BTX is useful. It should be noted that, in addition to altering the voltage dependence of VSNaC opening, BTX also has several other effects on VSNaC (2). These include inhibition of channel inactivation and also, most interestingly, changes in the ability of ions to permeate the channel (20). It is probable that the change in channel conformation presumably induced by BTX alters several parameters simultaneously. Thus, if MTX alters the conformation of VSCC, then it is quite probable that the altered conformation would have an altered mode of interaction with various classes of channel blocking agents.

A second facet of MTX action to be explained is its dependence on the presence of Na^+. This dependence has also been observed in investigations of MTX-induced release of norepinephrine from PC12h cells (7). One possibility is that the binding of MTX to the cell or its entry into the cell is Na^+ dependent. Similar considerations may apply to the lag period associated with the action of MTX. It is possible that the binding or entry of the toxin into the cell is relatively slow. It is also possible that rather than acting on VSCC itself, MTX stimulates some cellular system—e.g., an enzyme that produces a covalent modification of the channel. Again, there is a precedent for such an action with toxins such as cholera (21) or pertussis (22).

The results reported here are consistent with the above-stated hypothesis for the action of MTX. Clearly, further investigations will be aided by elucidation of the structure of the molecule and the appropriate electrophysiological studies.

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