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Ca^{2+} channel activating action of maitotoxin in cultured brainstem neurons

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Abstract

The actions of maitotoxin were studied using cultured brainstem cells and adrenal chromaffin cells. Maitotoxin induced a profound increase in the Ca^{2+} influx into cultured brainstem cells after a brief lag period. The maitotoxin-induced Ca^{2+} influx was suppressed by various voltage-dependent Ca^{2+} channel blockers such as Co^{2+}, Mn^{2+}, verapamil and diltiazem. Maitotoxin-catecholamine release in brainstem cells initiated to increase after a lag period of about 1 min and the increase continued even at 4 min after treatment, while in the adrenal chromaffin cells the release started after an about 1-min lag period to attain a maximum within first 2-min and gradually decrease thereafter. These results suggest that maitotoxin acts on Ca^{2+} channels to increase the Ca^{2+} influx, accompanied by enhancement of catecholamine release in the brainstem cells with a different temporal profile from that in the adrenal chromaffin cells.

Keywords: Maitotoxin; Ca^{2+} channel; Brainstem cell; Ca^{2+} influx; Catecholamine release; Adrenal chromaffin cell

1. Introduction

The concentration of intracellular free calcium ($[Ca^{2+}]_i$) is an evolutionarily conserved signal for the regulation of many aspects of cellular function. In both excitable and nonexcitable mammalian cells, $[Ca^{2+}]_i$ plays a crucial role in regulating complex Ca^{2+} signals and differentiated functions, such as excitability, secretion of hormones and release of transmitters, synaptic plasticity, gene expression, and apoptosis (Miller, 1991; Berridge, 1997). The increase in $[Ca^{2+}]_i$ can be due to calcium entry from the extracellular space, through channels in the plasma membrane or from intracellular stores of calcium (mainly endoplasmic reticulm, ER) (Dutta, 2000).

Maitotoxin is a non-peptide toxin produced by toxic, dinoflagellate, *Gambierdiscus toxicus*. A low concentration of maitotoxin induces a profound increase in Ca^{2+} influx into various kinds of cells and initiates a number of Ca^{2+}-related cellular functions, including hormonal release from secretory cells, positive inotropic effect on heart, contraction of skeletal and smooth muscles and prolongation of the duration of Ca^{2+}-dependent action potential of rat heart and insect skeletal muscles (Miyamoto et al., 1984; Taglialatela et al., 1990; Sauviat, 1997; Obara et al., 1999). Maitotoxin induces Ca^{2+} influx not only into excitable cells but also into non-excitable cells (Gusovsky and Daly, 1990; Columbo et al., 1992; Murata et al., 1992; Watanabe et al., 1993). The toxin per se is unlikely to be an ionophore, so a possibility is that maitotoxin directly stimulates widely distributed Ca^{2+} permiant channels (Takahashi et al., 1983). In neuroblastoma–glioma hybrid cells and certain pituitary tumor cells, maitotoxin-elicited Ca^{2+} influx is blocked at least in part by dihydropyridines that are selective for type L voltage-dependent Ca^{2+} channels (Gusovsky and Daly, 1990). By contrast, in other cell types such as smooth muscle BC3H1 cells (Sladeczek et al., 1988) and synaptosomes (Ueda et al., 1986), these Ca^{2+} channel antagonists have no effect on

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maitotoxin-elicited Ca^{2+} influx. The results demonstrate that maitotoxin can lead to both activation of dihydropyridine-sensitive L-type Ca^{2+} channels and of other Ca^{2+} channels that are not sensitive to dihydropyridines (Gusovsky and Daly, 1990).

In catecholamine-containing tissues, maitotoxin causes the release of catecholamines from PC12 cells and the releasing action is markedly suppressed by various Ca^{2+} channel blockers, such as divalent cations, dihydropyridines, and phenylalkylamines (Takahashi et al., 1983). Maitotoxin also induces the norepinephrine release from adrenergic nerve terminals in guinea pig vas deference (Kobayashi et al., 1985). These results suggest that maitotoxin in part acts on Ca^{2+} channels of various kinds of cells and increases the Ca^{2+} permeability of these cell membranes. In this study, we examined the effect of maitotoxin on the catecholamine release from cultured brainstem cells and adrenal chromaffin cells and compared the action of the toxin on them.

2. Materials and methods

2.1. Materials

Maitotoxin was extracted and purified as described previously (Takahashi et al., 1982). As for the experiment for cultured bovine adrenal chromaffin cells, maitotoxin was purchased from Wako (Osaka, Japan). Monoclonal antibody to tyrosine hydroxylase (PCTH-7) was kind gift of Dr. Hiroshi Hatanaka. Monoclonal antiglial fibrillary acidic protein was obtained from Amersham (Buckinghamshire). Rhodamine-conjugated goat immunoglobulin G fraction to mouse immunoglobulins, Cappel Laboratories (Cochranville, PA). Dulbecco's modified Eagle's and Ham's F12 media, and horse serum were purchased from Gibco Laboratories (Grand Island, NY). Precolostrum newborn calf serum was obtained from Mitsubishi-Kasei Chemical Industry Co., Ltd. (Tokyo). Trypsin (1:250) was purchased from Difco Laboratories (Detroit, MI). The following materials were obtained from the companies indicated: l-[7-^{3}H]norepinephrine (specific activity 46.7 Ci/mmol), [^{45}Ca]Cl_2 and [^{22}Na]Cl, New England Nuclear (Boston, MA); poly-D-lysine (Molecular weight; 30,000–150,000), deoxyribonuclease I and verapamil, Sigma Chemical Co. (St. Louis, MO); tetrodotoxin, Sankyo (Tokyo); diltiazem, Tanabe (Osaka); nicardipine, Yamanouchi (Tokyo): All other chemicals were reagent grade.

2.2. Cell culture

Rat brainstem was dissected from 16-day fetal rats (Wistar ST) and was digested with 0.25% (w/v) trypsin and 20 μg/ml deoxyribonuclease I in 1 ml of Ca^{2+} Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-PBS) for 15 min at 37 °C. Trypsinization was terminated by adding equal volume of heat-inactivated (56 °C, 30 min) horse serum and 10 ml of culture medium. After centrifugation at 150×*g* for 5 min, the tissue was dissociated into single cells by gentle pipetting and then the cell suspension was filtered through a double layer of lend paper. The cells were plated on poly-D-lysine coated 35-mm culture dish at a density of 3.5×10^{5} cells/cm^{2} and the culture were maintained at 37 °C in a CO_2 incubator (95% air/5% CO_2). Poly-D-lysine coated surfaces were prepared by allowing 1 ml of a solution of poly-D-lysine hydrobromide (0.1 mg/ml water) to stand on culture dishes for 2–3 days at room temperature, after which it was aspirated, the surface was washed twice with water and once with culture medium. The culture medium contained 90% DF medium, 5% precolostrum newborn calf serum and 5% heat-inactivated horse serum. DF medium consisted of 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesalfonic acid (Hepes) buffer, 30 nM selenium, 50 U/ml penicillin and 200 μg/ml streptomycin. The medium was changed 3 days after cell plating and hereafter every other days. Bovine adrenal glands were kindly provided by the Iwate Chikusan Center. Adrenal chromaffin cells were prepared by collagenase digestion as described previously (Tachikawa et al., 2003). The isolated cells were suspended in Eagle's minimum essential medium containing 10% calf serum, 3 μM cytosine arabinoside, and antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.3 μg/ml of amphotericin B), and were maintained in monolayer culture in 35-mm dishes at a density of 2×10^{6} cells or in 24-well plates at a density of 6×10^{5} cells. The cells were cultured at 37 °C in a CO_2 incubator (95% air/5% CO_2). Contents of epinephrine, norepinephrine and dopamine in 6×10^{5} cells plated on 24-well plates 101.9±3.5 nmol, 10.9±0.5 nmol and 0.7±0.05 nmol. Additionally, all animal procedures were designed in accordance with the Institutional Guidelines of Tohoku University, Sendai, for the care and use of laboratory animals.

2.3. Immunofluorescent staining

After washing with CMF-PBS, brainstem cells on culture dish were fixed in absolute methanol at −20 °C for 20 min and were washed with CMF-PBS (5 min×3) and treated with buffer A (0.05% Triton X-100, 0.01% timerosal and 2% chick serum in CMF-PBS) at room temperature for 20 min. Cells incubated with anti-tyrosine hydroxylase monoclonal antibody (23 μg/ml) in buffer A for 60 min at 37 °C. Cells were washed with buffer A (5 min×3) and incubated with a 1:100 diluted solution of rhodamine–goat–anti mouse immunoglobulins in buffer A (37 °C, 60 min) and after rinsing with buffer A (5 min×3), mounted with a glycerol buffer and examined using a ×40 objective on a Nikon fluorescence microscope.

2.4. Assay of ^{45}Ca influx and [^{3}H]norepinephrine release from brainstem cells

^{45}Ca influx and [^{3}H]norepinephrine release were assayed by essentially the same methods as described before except the amount of [^{3}H]norepinephrine was elevated to 4 μCi/dish and that of ^{45}Ca was increased to 0.5 and 1 μCi/ml for the maitotoxin and high K^{+} induced ^{45}Ca influx assays, respectively. The assay solution contained 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.87 mM $MgSO_4$, 5.5 mM glucose, and

50 mM Hepes, pH 7.3 at 37 °C with or without 1.2 mM ascorbic acid for [^{3}H]norepinephrine release and ^{45}Ca influx assay, respectively. For the assay in Na^+-free solutions, NaCl was substituted with 260 mM sucrose and 46 mM KCl was supplemented in the high K^+ solutions.

2.5. Isolation of [^{3}H]norepinephrine

[^{3}H]Norepinephrine release from brainstem cells by high K^+ and maitotoxin treatments was isolated according to the method of Glowinski and Iversen (1966), using columns of alumina and Dowex 50×4 (100 to 200 mesh, Na^+ from).

2.6. Assay of lactate dehydrogenase release from brainstem cells

The release of lactate dehydrogenase from brainstem cells by maitotoxin was checked by the same method as described before (Takahashi et al., 1982).

2.7. Determination of protein

The protein content of cultured brainstem cells was measured in parallel dishes by the method of Bradford using bovine plasma gamma globulin as the standard.

2.8. Measurement of catecholamine release from cultured bovine adrenal chromaffin cells

After 4 days in culture, the cells were washed with prewarmed low K^+ buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM Hepes–Tris, pH 7.4) or KRH buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 mM glucose and 0.1% BSA, pH 7.4), and then incubated for 10 min at 37 °C with high K^+ buffer (85 mM NaCl, 60 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM Hepes–Tris, pH 7.4) and maitotoxin in low K^+ buffer to evoke membrane depolarization. For assay of total catecholamine, the catecholamine secreted into the medium was extracted with 0.4 M perchloric acid and absorbed onto aluminum hydroxide (Tachikawa et al., 2003). Their amount was estimated by the ethylenediamine condensation method (Wei-Malherbe and Bone, 1952) using a fluorescence spectrophotometer (650-10S; Hitachi, Tokyo, Japan) at the excitation wavelength of 420 nm and the emission wavelength of 560 nm. At these wavelengths, both epinephrine and norepinephrine had the same fluorescence intensity. For assaying the amount of epinephrine, norepinephrine and dopamine, the incubation buffer was collected and centrifuged at 800×*g* for 10 min to remove detached cells. The resultant supernatant was acidified with perchloric acid (final concentration 0.4 N). The amount of epinephrine, norepinephrine and dopamine in the incubation buffer was quantified by high performance liquid chromatography-enhanced chemiluminescence detection as previously described (Yamakuni et al., 1998).

3. Results

3.1. Monolayered culture of brainstem cells of 16-day fetal rat

During the first 24 h, the dissociated brainstem cells attached to the poly-D-lysine surface and many cells have formed processes. By the 3rd to 4th day in culture, many neurites built a network throughout the culture dish (Fig. 1A). A number of glial cells seemed to be small in these culture since anti-glial fibrillary acidic protein-positive cells were less than 0.5% even 10 days after plating. Cultures were studied 4–10 days after plating since a significant number of cells stained with monoclonal antibody against tyrosine hydroxylase, a key enzyme of catecholamine synthesis, were present (Fig. 1B) and the activities of uptake and high K^+ stimulated release of [^{3}H]norepinephrine were observed throughout this period.

3.2. Maitotoxin and high K^+ induced ^{45}Ca influx into brainstem cells

Fig. 2A shows the time courses of ^{45}Ca influxes into cultured brainstem cells after the exposure to various concentrations of maitotoxin. After a brief lag period, a marked ^{45}Ca influx was caused by the treatment and continued for more than 5 min. The rate of the influx at steady state was increased and the lag period became shorter with increasing concentration of maitotoxin. The double-reciprocal plot for the rate of the influx at steady state against maitotoxin concentration fell on a straight line, from which the K_d value and the maximum rate of the influx (V_{max}) were estimated to be 1.33×10^{-7} g/ml and 0.67 nmol/s/mg protein, respectively (Fig. 2B). In the Na^+-free solution, the rate of ^{45}Ca influx induced by maitotoxin increased approximately 2 times greater than that in normal solution (compare open circles with closed circles in Fig. 2A), indicating that maitotoxin is still able to cause ^{45}Ca influx in the absence of external Na^+. ^{45}Ca influx was also induced by high K^+ treatment, however, high K^+-induced ^{45}Ca influx rapidly increased and then reached plateau a few minutes after the application and the amount of ^{45}Ca incorporated in the cells were much smaller than that by the maitotoxin treatment (Fig. 2C).

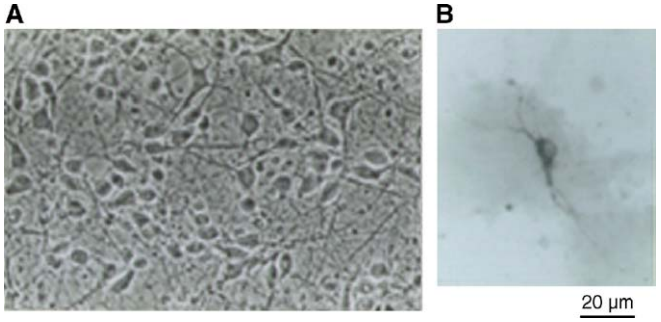


Fig. 1. Micrographs of cultured brainstem cells derived from 16-day feral rat. (A) Phase contrast micrograph taken after 8 days in culture. (B) Immunofluorescence micrograph taken after 11 days in culture using an anti-tyrosine hydroxylase monoclonal antibody. Many varicosities are visible along the neurites. Scale bar, 20 μm.

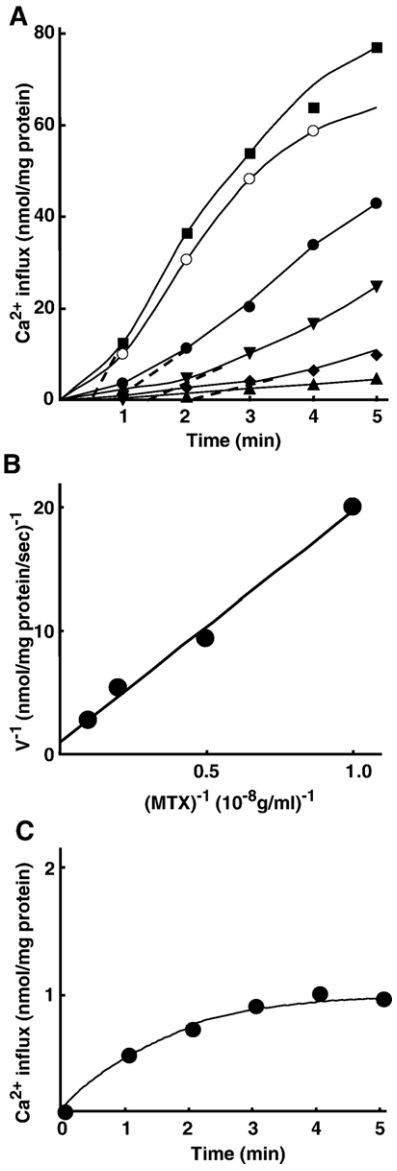


Fig. 2. ^{45}Ca influxes into brainstem cells induced by the treatment with maitotoxin and high K^+ (50.4 mM). The cells were cultured for 6 days. (A) Time courses of ^{45}Ca influx induced by 5×10^{-9} (▲), 10^{-8} (◆), 2×10^{-8} (▼), 5×10^{-8} (●, ○) and 10^{-7} g/ml (■) of maitotoxin. Open symbols show the results in the absence of external Na^+. The basal influxes in the absence of maitotoxin were 0.07 and 0.20 nmol/min/mg proteins in the presence or absence of external Na^+, respectively. (B) The double-reciprocal plot for the rate of ^{45}Ca influx at steady state estimated from (A) against maitotoxin concentration. Apparent dissociation constant of maitotoxin (K_d) and the maximum rate of Ca^{2+} influx (V_{max}) were estimated to be 1.33×10^{-7} g/ml and 0.67 nmol/s/mg proteins, respectively. (C) Time course of ^{45}Ca influx after exposure to high K^+ solution. Basal influx in the normal solution was subtracted from the data.

Fig. 3A shows the time courses of ^{45}Ca influxes induced by 5×10^{-8} g/ml maitotoxin in various Ca^{2+} concentrations. The lag period became shorter and the rate of the influx at steady state was increased with increasing the concentration of $CaCl_2$. The double-reciprocal plot for the rate of the influx at steady state against $CaCal_2$ concentration fell on a straight line and K_d value for $CaCl_2$ was estimated to be 3.75 mM. The initial rate of high K^+-induced ^{45}Ca influx was also increased with increasing $CaCl_2$ concentration and K_d value of 1.1 mM was estimated from the double-reciprocal plot (Fig. 3B).

3.3. Inhibitory effects of Ca^{2+} channel blockers on high K^+- and maitotoxin-induced ^{45}Ca influxes

Fig. 4 shows the effects of various drugs on the ^{45}Ca influx into brainstem cells induced by high K^+(open bars) and maitotoxin (hatched bars). Various voltage-dependent Ca^{2+} channel blockers such as Co^{2+}, Mn^{2+}, verapamil, diltiazem markedly suppressed both the ^{45}Ca influxes induced by high K^+ and maitotoxin. The inhibitory effects of these Ca^{2+} channel blockers on the maitotoxin-induced ^{45}Ca influx were also observed in Na^+-free solution and the ^{45}Ca influx induced by 5×10^{-8} g/ml maitotoxin was suppressed by the treatment of Co^{2+} (5×10^{-3} M), verapamil (10^{-4} M) or diltiazem (10^{-4} M) by 73%, 57% and 40%, respectively. Also maitotoxin-induced

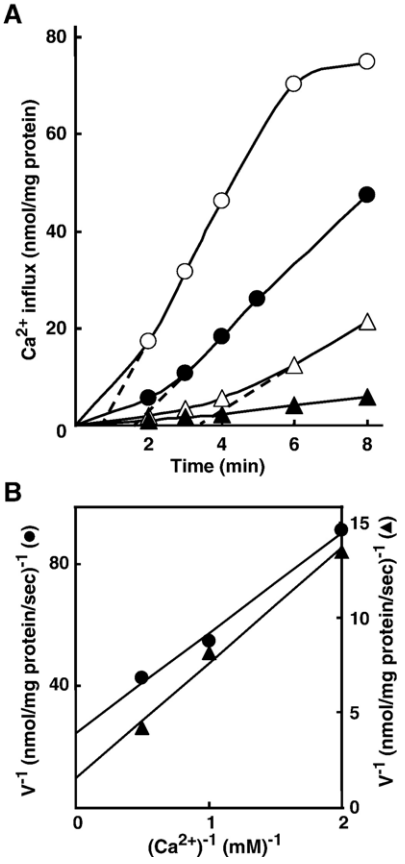


Fig. 3. ^{45}Ca influxes into brainstem cells induced by 5×10^{-8} g/ml maitotoxin at various Ca^{2+} concentrations. The cells were cultured for 5 days. (A) Time courses of ^{45}Ca influx induced by 5×10^{-8} g/ml maitotoxin at 0.3 (▲), 0.5 (△), 1.0 (●) and 2.0 mM (○) $CaCl_2$. (B) The double-reciprocal plots of the rate of high K^+ (50.4 mM) (●) and maitotoxin (5×10^{-8} g/ml) (▲) stimulated ^{45}Ca influxes into brainstem cells. The rate at initial and steady state were plotted for high K^+ and maitotoxin-induced Ca^{2+} influxes, respectively. The initial rate of ^{45}Ca influx was estimated by subtracting the basal influx over 30 s in the standard salt solution (5.4 mM K^+) from the total influx over 30 s in the high K^+ solution (50.4 mM K^+). The results for high K^+ treatment are the mean of triplicate measurements and the standard mini error was less than 20%. K_d values of 1.1 and 3.75 mM were estimated for high K^+ and maitotoxin treatment, respectively, from the plots.

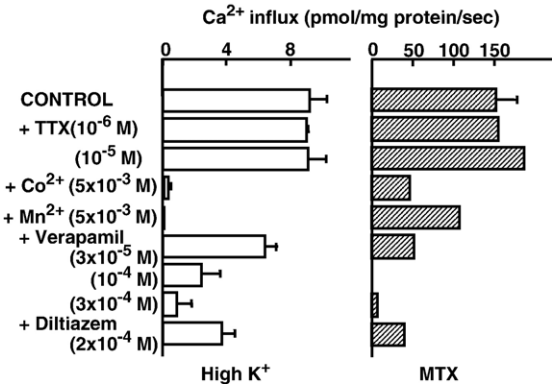


Fig. 4. Effects of several agents on the ^{45}Ca influxes into brainstem cells induced by high K^+ and maitotoxin treatment. The cells were cultured for 5 days. The initial and steady rate of ^{45}Ca influxes was measured for high K^+ (open bars) and maitotoxin (hatched bars) treatments, respectively. The initial rate was estimated by subtracting the basal influx over 1 min in the standard salt solution from the total influx over 1 min in the high K^+ (50.4 mM) solutions. The steady state rate was calculated from the time courses between 2 and 3 min after the maitotoxin treatment. The results for high K^+ treatments are the mean of duplicate measurements.

Ca^{2+} influx was less effectively inhibited than high K^+-induced Ca^{2+} influx by Mn^{2+}. On the other hand, tetrodotoxin, a specific Na^+ channel inhibitor, up to 10^{-5} M did not affect ^{45}Ca influxes by both the treatments.

It is well known that the inhibitory effects of Ca^{2+} channel blockers such as Co^{2+} and verapamil are competitively antagonized by external Ca^{2+}. The inhibitory effects of Co^{2+} (10^{-3} M) and verapamil (1.5×10^{-5} M) on the maitotoxin (5×10^{-8} g/ml)-induced ^{45}Ca influx were decreased from 84.7% to 33.9% and from 49.5% to 15.2%, respectively, by increasing in the external Ca^{2+} concentration from 1 to 5 mM (data not shown).

The function of high K^+ sensitive Ca^{2+} channel of PC12h cells, clonal rat pheochromocytoma cells, and NG108-15 cells, a neuroblastoma × glioma hybrid cells, are suppressed by dihydropyridine derivatives, such as nicardipine and nitrendipine, at nM order concentration and the actions of

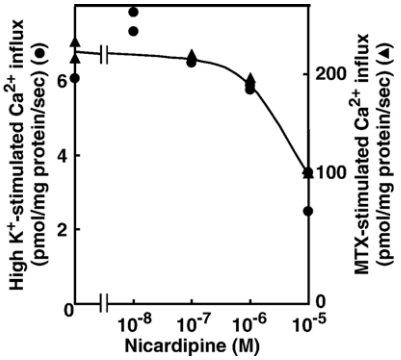


Fig. 5. The dose–inhibitory response curve for nicardipine on the high K^+ (●) and maitotoxin (5×10^{-8} g/ml) (▲)-induced ^{45}Ca influxes into brainstem cells. The cells were cultured for 6 days. The initial and steady rate of ^{45}Ca influxes were estimated for high K^+ and maitotoxin treatment, respectively, as described in the legend for Fig. 4.

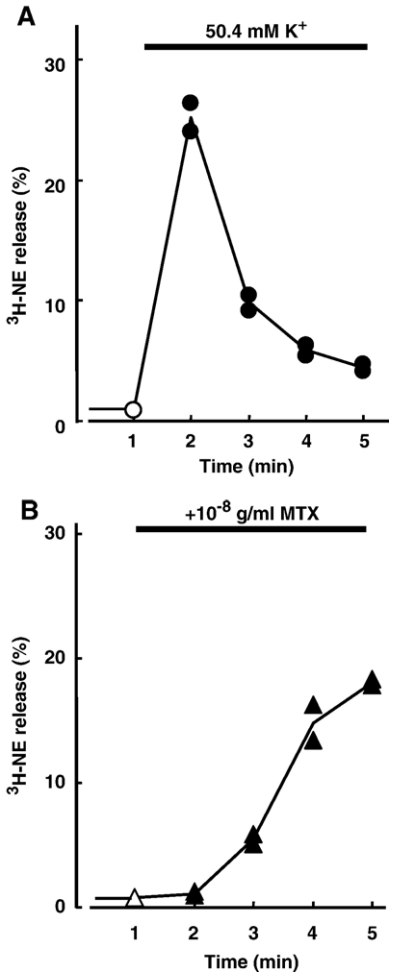


Fig. 6. The time-dependent changes of [^{3}H]norepinephrine release from brainstem cells. [^{3}H]Norepinephrine release from brainstem cells induced by high K^+ (50.4 mM) (A) and maitotoxin (10^{-8} g/ml) (B) were assayed. The cells were cultured for 6 days. After 1-min preincubation, brainstem cells were incubated with high K^+ or maitotoxin-containing solutions for the indicated time. The assay solution was changed at 1-min intervals. The amount of [^{3}H] norepinephrine released in the solution is expressed as percent of total [^{3}H] norepinephrine stored in the cells at the beginning of each period. Each point represents the data obtained by one culture dish. The total count of [^{3}H]norepinephrine uptaken into the cells at the beginning of experiment was 11497 ± 563 cmp/dish.

maitotoxin on these cells were also inhibited by them at the same concentration range. On the other hand, the function of high K^+ sensitive Ca^{2+} channel of neurons are rarely suppressed by these drugs even at 10^{-6} M. Therefore, if maitotoxin induces Ca^{2+} influx by acting on Ca^{2+} channels, it can be expected that nicardipine shows only weak inhibitory effect on the maitotoxin-induced Ca^{2+} influx into brainstem cells. Fig. 5 shows the dose–inhibitory curves for nicardipine in the high K^+ and maitotoxin-induced ^{45}Ca influxes into brainstem cells. Nicardipine scarcely inhibited both the high K^+- and maitotoxin-induced Ca^{2+} influx into brainstem cells and only 7% and 12% of inhibition were observed at 10^{-6} M. These inhibitory effects were increased to about 50% by increasing the concentration of nicardipine up to 10^{-5} M.

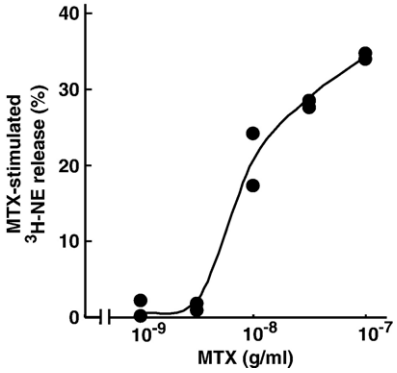


Fig. 7. The dose–response curve for maitotoxin in the [^{3}H]norepinephrine release from brainstem cells. The cells were cultured for 11 days. The amount of [^{3}H]norepinephrine released over 6 min is expressed as a percent of total [^{3}H]norepinephrine stored in the cells at the beginning of the experiment. The basal release of 5.74 ± 0.16% was subtracted from each value. The total count of [^{3}H]norepinephrine uptaken into the cells at the beginning of experiment was 28014 ± 1033 cmp/dish.

3.4. Maitotoxin-induced [^{3}H]norepinephrine release from cultured brainstem cells

Cultured rat brainstem cells take up and store the applied [^{3}H]norepinephrine. Fig. 6 shows the time-dependent changes of [^{3}H]norepinephrine release from brainstem cells induced by high K^+ (50.4 mM) and by maitotoxin (10^{-8} g/ml). The rate of the release of [^{3}H]norepinephrine by high K^+ was attained maximum within first 1-min and gradually decreased thereafter. On the other hand, the rate caused by maitotoxin was increased gradually after lag period of about 1 min. About 75% and 90% of the radioactive materials released from brainstem cells by high K^+- and maitotoxin-treatments, respectively, were recovered in norepinephrine fraction. Lactate dehydrogenase, a cytoplasmic marker, was not released by treatment with maitotoxin (10^{-8} g/ml) for 8 min (less than 0.1% of the total cell content/min; data not shown). Fig. 7 shows the amount of [^{3}H]norepinephrine released during 6 min by the addition of various concentrations of maitotoxin. Maitotoxin (3×10^{-9}–10^{-7} g/ml) induced a dose-dependent increase in the amount of [^{3}H]norepinephrine released.

Fig. 8 shows the effects of external Ca^{2+} on the high K^+ (A)- and maitotoxin (B)-induced [^{3}H]norepinephrine release from brainstem cells. The resting level of release of [^{3}H]norepinephrine was not changed by varying the Ca^{2+} concentration from 0 to 10 mM. Both high K^+ (50.4 mM) and maitotoxin (10^{-8} g/ml) failed to induce a release of [^{3}H]norepinephrine in the absence of external Ca^{2+}. The high K^+-induced [^{3}H]norepinephrine release increased with increasing Ca^{2+} concentrations from 0.1 to 3 mM and attained maximum around 3 mM of Ca^{2+} concentration range. The Ca^{2+} concentration required for half maximal effects (ED_{50}) was about 0.27 mM. Maitotoxin-induced [^{3}H]norepinephrine release also increased with increasing Ca^{2+} concentration but it continued to increase even up to 10 mM Ca^{2+}, a highest concentration tested in this study.

3.5. Effects of tetrodotoxin and various Ca^{2+} channel blockers on the maitotoxin-induced [^{3}H]norepinephrine release from brainstem cells

Veratridine above 10^{-6} M induced a release of [^{3}H]norepinephrine from cultured brainstem cells in a Ca^{2+}-dependent manner. The release increased with increasing the concentration of veratridine and attained maximum above 10^{-5} M and the ED_{50} value was estimated to be about 4.6 μM (data not shown). Fig. 9 shows the effects of tetrodotoxin of various concentrations on the veratridine- and maitotoxin-induced release of [^{3}H]norepinephrine from brainstem cells.

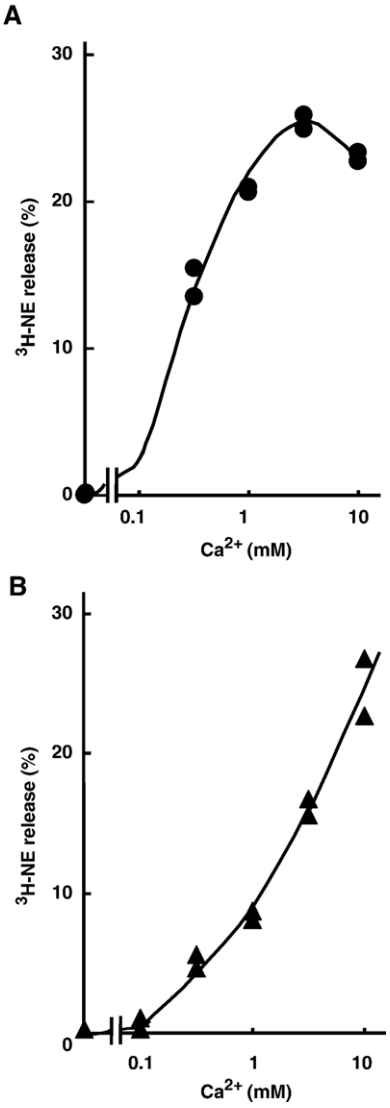


Fig. 8. The effects of high K^+ (50.4 mM) and maitotoxin (10^{-8} g/ml) on the log concentration–response curve for external Ca^{2+}. High K^+ (50.4 mM) (A) and maitotoxin (10^{-8} g/ml) (B) -induced [^{3}H]norepinephrine release from brainstem cells were assayed. The cells used in experiment of (A) and (B) were 6 and 9 days in culture, respectively. The amount of [^{3}H]norepinephrine released over 4 min are expressed as a percent of total [^{3}H]norepinephrine stored in the cells at the beginning of the experiment. The basal release (5.96 ± 0.60%) was subtracted from each value. The total count of [^{3}H]norepinephrine uptaken into the cells at the beginning of experiment was 6807 ± 338 cpm/dish (A) and 18778 ± 1029 cpm/dish (B).

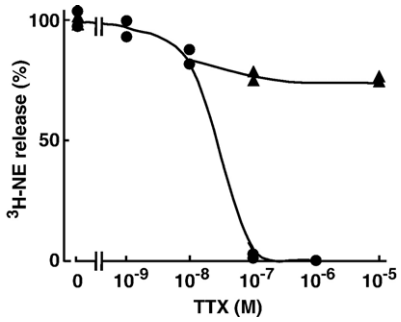


Fig. 9. The dose–inhibitory response curve for tetrodotoxin on the veratridine and maitotoxin-induced [^{3}H]norepinephrine release from brainstem cells. The cells used for the experiment of veratridine and maitotoxin-induced release was 7 and 4 days in culture, respectively. The cells were treated with tetrodotoxin 3 min before the treatment of veratridine or maitotoxin. Veratridine (●) stimulated release over 1 min and maitotoxin (▲)-stimulated release over 4 min were expressed as relative to the release in the absence of tetrodotoxin. The veratridine- and maitotoxin-induced release in the absence of tetrodotoxin were 15.0±0.4% over 1 min and 33.9±0.5% over 4 min, respectively.

Tetrodotoxin effectively suppressed the veratridine-induced release in a dose-dependent manner and IC_{50} was less than 3×10^{-8} M and the full inhibition was achieved by 10^{-7} M of tetrodotoxin. On the other hand, tetrodotoxin scarcely inhibited the maitotoxin-induced release of [^{3}H]norepinephrine from brainstem cells and only 24% of inhibition was observed even by increasing its concentration up to 10^{-5} M.

3.6. Maitotoxin-induced catecholamine release from bovine adrenal chromaffin cells

Fig. 10 shows maitotoxin induced the catecholamine release from cultured adrenal chromaffin cells in a concentration-dependent manner. The stimulatory effect of maitotoxin on the release was observed at the concentrations of 10^{-10} g/ml and reached a plateau at 10^{-8} g/ml.

Fig. 11 shows the time-dependent changes of catecholamine (epinephrine, norepinephrine and dopamine) release from adrenal chromaffin cells induced by maitotoxin (10^{-9} g/ml). The rate of the release of catecholamine by maitotoxin was attained maximum within first 2-min and gradually decreased thereafter.

4. Discussion

In the present report, we examined the action of maitotoxin on neuronal Ca^{2+} channels using the pharmacological techniques. Maitotoxin induced a profound Ca^{2+} influx into cultured brainstem cells and [^{3}H]norepinephrine release from them. The concentration of maitotoxin which induced the release was nearly the same as that caused Ca^{2+} influx. The maitotoxin-induced release of [^{3}H]norepinephrine was abolished in the absence of external Ca^{2+} and was inhibited by various Ca^{2+} channel blockers, which also suppressed the Ca^{2+} influx induced by maitotoxin. Most of the radioactive material released by maitotoxin was identified as [^{3}H]norepinephrine. The release of lactate dehydrogenase, a cytoplasmic marker enzyme, was not observed at all. From these results, it was concluded that [^{3}H]norepinephrine was released from brainstem noradrenergic neurons by exocytosis as the result of Ca^{2+} influx induced by maitotoxin.

The Ca^{2+} influx induced by maitotoxin was not affected by the treatment with tetrodotoxin and was observed even in the absence of external Na^+. These results indicate that the maitotoxin-induced Ca^{2+} influx was not caused as the result of membrane depolarization owing to external Na^+. This indication is consistent with the result that the Ca^{2+} influx induced by high-K^+ treatment, which causes the membrane depolarization, ceased a few minutes after the treatment, while the influx caused by maitotoxin continued for longer time. The dose-dependent effect on the increase in the rate of Ca^{2+} influx was saturable and the apparent K_d value of 1.33×10^{-7} g/ml was obtained from the double-reciprocal plot. This observation suggests that maitotoxin exert its action by reacting with the specific site of a limited number on the plasma membrane. The rate of Ca^{2+} influx into brainstem cells was suppressed by various Ca^{2+} channel blockers and the inhibitory action of these blockers were decreased with the increase in the external Ca^{2+} concentration. All of these properties are characteristics of voltage-dependent Ca^{2+} channels. Furthermore, there is a good correlation between the inhibitory potencies of dihydropyridine derivatives on the Ca^{2+} influx and [^{3}H]norepinephrine release induced by high-K^+ and on those by maitotoxin among various kinds of cells. Dihydropyridine derivatives at nM order concentration inhibited the high-K^+ sensitive Ca^{2+} channel function in PC12 cells and neuroblastoma x glioma hybrid cell, NG108-15 cells, whereas, it scarcely showed any suppression in neurons (Takahashi et al., 1983; Ichida et al., 1994). Actions of maitotoxin were also suppressed by dihydropyridine derivatives at nM order concentration in PC12 and NG108-15 cells but not in brainstem cells (Freedman et al., 1984). These results suggest that the Ca^{2+} mobilizing action of maitotoxin is mediated by Ca^{2+} channels of these cells.

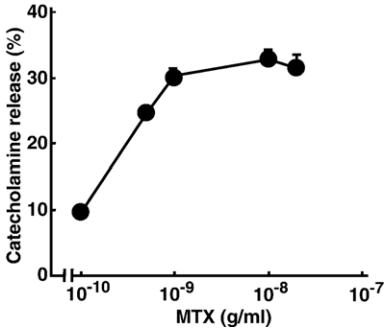
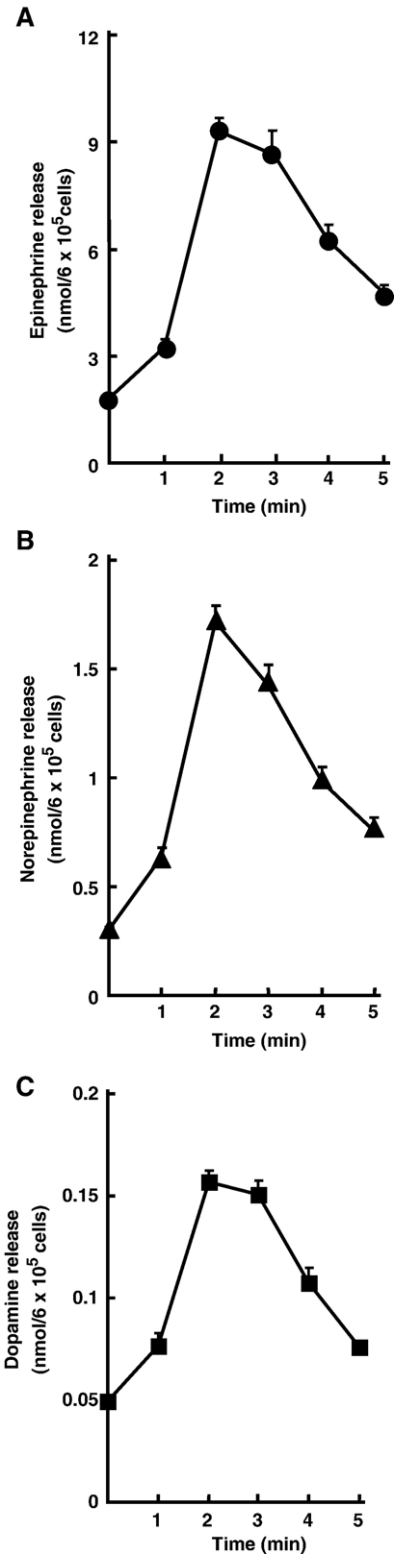


Fig. 10. The dose–response curve for maitotoxin in the catecholamine release from adrenal chromaffin cells. The cells were cultured for 4 days. Cells were incubated with various concentrations of maitotoxin in low K^+ buffer for 10 min. The amount of catecholamine release was determined by the ethylenediamine condensation method (Wei-Malherbe and Bone, 1952) using a fluorescence spectrophotometer (650-10S; Hitachi, Tokyo, Japan) at the excitation wavelength of 420 nm and the emission wavelength of 560 nm. At these wavelengths, both epinephrine and norepinephrine had the same fluorescence intensity. Catecholamine release is shown as a percentage of total cellular catecholamine content.

Previously we showed that there are at least two types of Ca^{2+} channels in PC12 cells; one is a dihydropyridine-insensitive spike-generating channel, the other is dihydropyridine-sensitive channel with sustained activation state over 30 s (Takahashi et al., 1983). The spike-generating channel of NG108-15 cell seemed to be dihydropyridine insensitive. Since the action of maitotoxin to both cells were suppressed by low concentration of dihydropyridine, it is likely that maitotoxin exert its action by acting not on spike-generation Ca^{2+} channel but on the second type of Ca^{2+} channel of these cells. On the contrary, maitotoxin affected on Ca^{2+} spikes in superior survical ganglial neurons (Freedman et al., 1984). This result may imply that maitotoxin can act on the spike-generating Ca^{2+} channel of neurons. Maitotoxin prolonged the Ca^{2+}-dependent action potential duration but did not affect the rate of rise of it. On the other hand, there is a brief lag period before occurring the Ca^{2+} influx into brainstem cells after the treatment with maitotoxin. These results may imply that maitotoxin increases the Ca^{2+} permeability by acting on Ca^{2+} channel in open state and preventing its closing. But more detailed studies including single channel analysis of Ca^{2+} channels are necessary to identify the precise site and the mechanisms of action.



The most important findings in the present study are the facts that maitotoxin-induced catecholamine release in brainstem cells initiates to increase after a lag period of about 1 min and the increase continues even at 4 min after treatment, while in the adrenal chromaffin cells the release starts after an about 1-min lag period to attain a maximum within first 2-min and gradually decrease thereafter. It has been documented that a Ca^{2+} channel-forming peptide, tricosporin-B-III, induces Ca^{2+} influx-dependent catecholamine release in cultured adrenal chromaffin cells (Tachikawa et al., 1991). Like tricosporin-B-III, maitotoxin induces Ca^{2+} influx accompanied by a transient increase in catecholamine release in PC12 cells (Takahashi et al., 1983), raising the possibility that this toxin induces Ca^{2+} influx to produce catecholamine release in the adrenal chromaffin cells as well. The different modes of action of maitotoxin in cultured brainstem cells and adrenal chromaffin cells appear to be correlated with the difference in temporal profiles of Ca^{2+} influx from via voltage-dependent Ca^{2+} channels. These findings thus suggest the presence of a difference in the Ca^{2+} channel-dependent regulatory mechanism of catecholamine release between the brainstem neurons and the adrenal chromaffin cells.

In summary, we here examined the action of maitotoxin on cultured brainstem cells and adrenal chromaffin cells. We found that maitotoxin induced an appreciable increase in the Ca^{2+} influx into cultured brainstem cells via Ca^{2+} channel which was blocked by different voltage-dependent Ca^{2+} channel blockers. Maitotoxin also induced catecholamine

Fig. 11. The time-dependent changes of catecholamine release from adrenal chromaffin cells. Catecholamine release from adrenal chromaffin cells induced by maitotoxin (10^{-9} g/ml) were assayed. The cells were cultured for 4 days. After 1-min preincubation, cells were incubated with maitotoxin in low K^+ buffer for indicated times. The assay solution was changed at 1-min intervals. (A) Epinephrine release evoked by maitotoxin. (B) Norepinephrine release evoked by maitotoxin. (C) Dopamine release evoked by maitotoxin. The amount of epinephrine, norepinephrine and dopamine was quantified as previously reported (Yamakuni et al., 1998). The values for basal release were subtracted from the data. The amount of epinephrine, norepinephrine and dopamine is expressed as nmol/6×10^5 cells.

release in the brainstem cells with a different temporal profile from that in the adrenal chromaffin cells. These findings suggest that maitotoxin may become a very useful pharmacological tool for the study of Ca^{2+} channels involved in catecholamine release in the brainstem neurons as well as in the endocrine secretary cells.

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