

The Mechanism of Maitotoxin-Induced Elevation of the Cytosolic Free Calcium Level in Rat Cerebrocortical Synaptosomes

Eiki Satoh*, Toshiaki Ishii and Masakazu Nishimura

Department of Pharmacology, University of Obihiro School of Veterinary Medicine, Obihiro 080-8555, Japan

Received June 19, 2000 Accepted November 7, 2000

ABSTRACT—The present study was conducted to elucidate the mechanism of the maitotoxin (MTX)-induced increase in intrasynaptosomal free calcium level ($[Ca^{2+}]_i$). The MTX (1 ng/ml)-induced increase in $[Ca^{2+}]_i$ was partially inhibited by the omission of extracellular Ca^{2+} (Ca^{2+}_e) or the addition of verapamil, but not by adding nifedipine, ω -agatoxin IVA, ω -conotoxin GVIA and ω -conotoxin MVIIC. An increase in $[Ca^{2+}]_i$ in the absence of Ca^{2+}_e was sensitive to procaine, TMB-8, genistein and verapamil, but not to ryanodine and U-73122. These results may suggest that MTX increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} entry through voltage-independent nonselective cation channels and Ca^{2+} release from stores through a phospholipase C- γ 1-mediated pathway in rat cerebrocortical synaptosomes.

Keywords: Maitotoxin, Synaptosomes, Cytosolic free calcium

Maitotoxin (MTX), a water-soluble polyether (MW: 3424) isolated from the marine organism *Gambierdiscus toxicus*, is one of the most potent toxins known (1). This toxin increases calcium (Ca^{2+}) content and/or Ca^{2+} flux in a wide range of cell types (2, 3). Recent reports indicate that MTX primarily activates voltage-independent, nonselective cation channels (3–6). MTX is also known to activate phosphoinositide hydrolysis (2, 3, 7, 8). Recently, it has been proposed that MTX separately activates Ca^{2+} influx and phosphoinositide hydrolysis in rabbit ciliated tracheal epithelium and human astrocytoma cells (7, 8). In rat brain synaptosomes, MTX increases intrasynaptosomal Ca^{2+} and depolarizes synaptosomal plasma membranes through the inflow of extracellular Ca^{2+} (Ca^{2+}_e) (9). The identity of the MTX-activated channels and the role of the intervening biochemical events (i.e., phosphoinositide hydrolysis) leading to an increase in the intrasynaptosomal free Ca^{2+} level ($[Ca^{2+}]_i$), however, remain unknown. The aim of this study is to elucidate the mechanism of MTX-induced increase in $[Ca^{2+}]_i$ using rat cerebrocortical synaptosomes.

Synaptosomes (P_2 B fraction) were prepared using the method described by Hajós (10) from the cerebral cortices of male and female Wistar rats (200–300 g).

The following materials were used: fura 2-AM and HEPES (Dojindo Lab., Kumamoto); MTX, verapamil hydrochloride, nifedipine, ω -agatoxin IVA, ω -conotoxin

GVIA, ω -conotoxin MVIIC, ryanodine, U-73122 and genistein (Wako Pure Chemicals, Osaka); procaine hydrochloride, EGTA and TMB-8 (Sigma, St. Louis, MO, USA); SKF-96365 (Calbiochem, La Jolla, CA, USA); and rhodamine 6G (Tokyo Kasei, Tokyo). Sankyo (Tokyo) generously donated the tetrodotoxin (TTX).

The levels of $[Ca^{2+}]_i$ were measured by monitoring the intensity of fura 2 fluorescence as described by Komulainen and Bondy (11).

Membrane potentials were measured by monitoring the intensity of rhodamine 6G fluorescence using the method described by Aiuchi et al. (12). An increase in the ratio of fluorescence intensity (measured as f_s/f_i ; f_i and f_s are the intensity of fluorescence in the absence and in the presence of synaptosomes, respectively) indicates depolarization of synaptosomal membranes.

Synaptosomal protein levels were quantified by using a kit from Bio-Rad (Richmond, CA, USA), as described by Bradford (13).

The medium contained the following: 125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 6 mM glucose, 1 mM $CaCl_2$ and 25 mM HEPES. The final pH was adjusted to 7.4 with NaOH.

Statistical significance of differences was assessed by Student's *t*-test.

MTX (0.1–10 ng/ml) caused the increase in $[Ca^{2+}]_i$ and membrane depolarization dose-dependently in rat cerebrocortical synaptosomes (data not shown). Removal of Ca^{2+} from the medium inhibited these effects of MTX (1 ng/ml)

*Corresponding author. FAX: +81-155-49-5402

E-mail: es@obihiro.ac.jp

by approximately 60% and 50%, respectively (data not shown). Decreasing to 6.2 mM the Na^+ in the medium or the application of TTX ($1 \mu\text{M}$) failed to alter these effects of MTX (data not shown). These results are similar to the reports of Taglialatela et al. (9) using rat whole brain. However, they suggested that MTX increased $[\text{Ca}^{2+}]_i$ through the inflow of Ca^{2+}_e because the MTX-induced increase in $[\text{Ca}^{2+}]_i$ was abolished in Ca^{2+} -free medium containing 1 mM EGTA. In the case of synaptosomes, EGTA reduces $[\text{Ca}^{2+}]_i$ by a rapid and continuous efflux of Ca^{2+} across the synaptic membrane (11). We could not measure the changes in $[\text{Ca}^{2+}]_i$ using agents that release Ca^{2+} from intracellular stores in Ca^{2+} -free medium containing 1 mM EGTA. Thus, it is not possible to measure $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free medium containing EGTA. In our Ca^{2+} -free experiments, synaptosomal preparations were suspended in Ca^{2+} -free medium containing 1 mM EGTA and subsequently re-suspended in nominally Ca^{2+} -free medium to wash out the EGTA. Then, $[\text{Ca}^{2+}]_i$ was measured in the nominally Ca^{2+} -free medium.

To identify the MTX-activated channels, the effects of MTX (1 ng/ml) on $[\text{Ca}^{2+}]_i$ and membrane potential were investigated in the presence of Ca^{2+}_e and Ca^{2+} antagonists of different classes of voltage-dependent Ca^{2+} channels. Verapamil ($200 \mu\text{M}$) inhibited both the $[\text{Ca}^{2+}]_i$ increase and depolarization induced by MTX by approximately 50% (Fig. 1: A and B). Nifedipine ($10 \mu\text{M}$), ω -agatoxin IVA (200 nM), ω -conotoxin GVIA ($1 \mu\text{M}$) and ω -conotoxin MVIIIC (500 nM) failed to modify the effects of MTX (Fig. 1: A and B). SKF-96365 ($30 \mu\text{M}$), a receptor-mediated Ca^{2+} -entry blocker, also had no effect (data not shown). The verapamil-induced inhibition may be due to a non-specific action rather than the inhibition of voltage-gated Ca^{2+} channels, since its concentration exceeds the specific range (14). Thus, the extracellular Ca^{2+} -dependent MTX-induced increase in $[\text{Ca}^{2+}]_i$ may be due to Ca^{2+} entry through voltage-independent nonselective cation channels.

To elucidate the Ca^{2+} free-insensitive effect of MTX (1 ng/ml), we tested the effects of agents that can modify the release of Ca^{2+} from intracellular stores on MTX (1 ng/ml)-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of Ca^{2+}_e . Procaine (8 mM), TMB-8 ($100 \mu\text{M}$) and genistein ($110 \mu\text{M}$, inhibitor of tyrosine kinase to induce tyrosine phosphorylation of phospholipase C- γ 1) markedly inhibited the increase in $[\text{Ca}^{2+}]_i$, but not ryanodine ($50 \mu\text{M}$) and U-73122 ($10 \mu\text{M}$, inhibitor of activation of phospholipase C- β 1 through a trimeric G protein) (Fig. 2). MTX generates inositol 1,4,5-trisphosphate (IP_3) through the hydrolysis of phosphatidylinositol by phospholipase C and IP_3 releases Ca^{2+} from intracellular stores (2). Concentrations of U-73122 and genistein were enough to induce an inhibition of IP_3 production through specific sites, respectively (7). Thus, MTX-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of

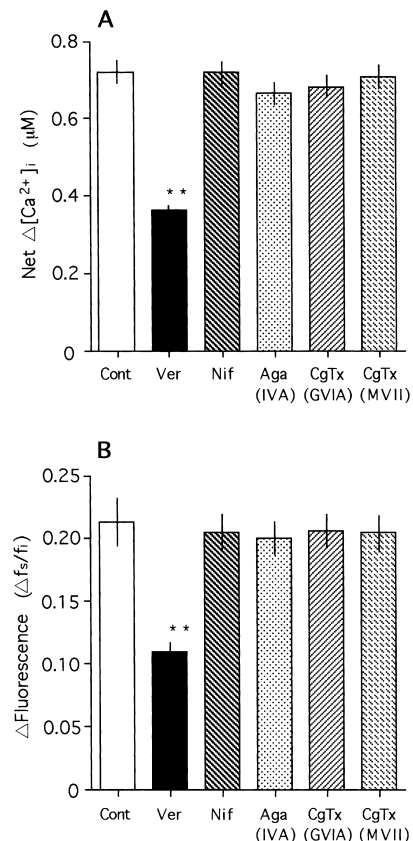


Fig. 1. Effects of various Ca^{2+} antagonists on the increase in $[\text{Ca}^{2+}]_i$ and depolarization by MTX. A: Synaptosomes preloaded with fura 2-AM were incubated for 3 min with or without MTX (1 ng/ml) in the standard medium. Verapamil (Ver, $200 \mu\text{M}$), nifedipine (Nif, $10 \mu\text{M}$), ω -agatoxin IVA (Aga IVA, 200 nM), ω -conotoxin GVIA (CgTx GVIA, $1 \mu\text{M}$) and ω -conotoxin MVIIIC (CgTx MVII, 500 nM) were added 10 min before exposure to MTX. The control (Cont) is the MTX-induced response in the absence of Ca^{2+} antagonists. The Net $\Delta[\text{Ca}^{2+}]_i$ is the difference in the increase in $[\text{Ca}^{2+}]_i$ 3 min after the addition of MTX and vehicle. B: After measurement of the fluorescence intensity (f_i) of standard medium (2 ml) containing rhodamine 6G ($0.167 \mu\text{M}$), $100 \mu\text{l}$ of a suspension of synaptosomes (6 mg of protein/ml) was added to the medium. Then, the incubation was done with or without MTX (1 ng/ml) and/or various Ca^{2+} antagonists for 10 min and the fluorescence intensity (f_s) was measured. The $\Delta\text{fluorescence}$ ($\Delta f_s/f_i$) is the difference in the ratio of fluorescence intensity (f_s/f_i) with and without MTX-treatment. The control (Cont) is the MTX-induced response in the absence of Ca^{2+} antagonists. Data are means \pm S.E.M. of values from 5 to 10 experiments. Values significantly different from the control level are indicated: ** $P < 0.01$.

Ca^{2+}_e may be mainly due to Ca^{2+} release from stores through IP_3 generated by the activation of phospholipase C- γ 1, which might imply phosphorylation on tyrosine residues (7, 15).

Verapamil (20 – $200 \mu\text{M}$) inhibited dose-dependently the effect of MTX in the presence or absence of Ca^{2+}_e (Fig. 3). In the absence of Ca^{2+}_e and the presence of verapamil, procaine or TMB-8 had no additive effect (data not

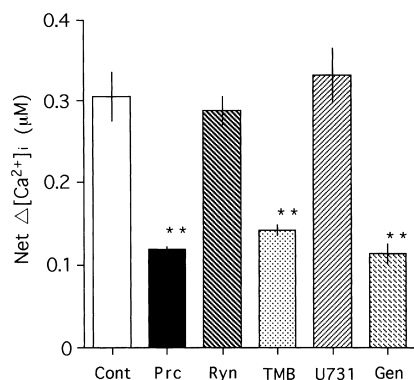


Fig. 2. Effects of procaine, ryanodine, TMB-8, U-73122 and genistein on MTX-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} . Synaptosomes preloaded with fura 2-AM were incubated for 3 min with or without MTX (1 ng/ml). Procaine (Prc, 8 mM), TMB-8 (100 μM), U-73122 (U731, 10 μM) or genistein (Gen, 110 μM) was added 10 min before exposure to MTX. Ryanodine (Ryn, 50 μM) was added at 20 min before MTX. The Net $\Delta[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 3 min after the addition of MTX or vehicle. The control (Cont) is the MTX-induced response in the absence of various agents. Data are means \pm S.E.M. of values from 5 to 10 experiments. Values significantly different from the control level are indicated: ** $P < 0.01$.

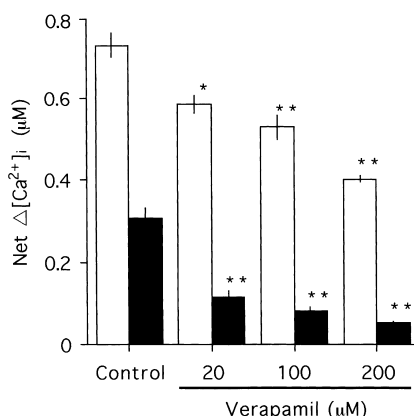


Fig. 3. Effects of verapamil on MTX-induced increase in $[Ca^{2+}]_i$ in the presence (\square) or absence (\blacksquare) of extracellular Ca^{2+} . Synaptosomes preloaded with fura 2-AM were incubated for 3 min with or without MTX (1 ng/ml). Verapamil was added 10 min before exposure to MTX. The Net $\Delta[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 3 min after the addition of MTX or vehicle. Control is the MTX-induced response in the absence of verapamil. Data are means \pm S.E.M. of values from 5 to 10 experiments. Values significantly different from the respective control level are indicated: * $P < 0.05$, ** $P < 0.01$.

shown). It is suggested that verapamil may inhibit Ca^{2+} release from stores through membrane stabilizing action because the agent inhibited membrane depolarization partially and an increase in $[Ca^{2+}]_i$ by the same degree regardless of the presence or absence of Ca^{2+}_e (Figs. 1B and 3). This is supported by the report that blockade of ^{45}Ca entry into depolarized synaptosomes by relatively high concentrations

of phenylalkylamines may result from a nonspecific membrane effect (14).

In conclusion, MTX causes an increase in $[Ca^{2+}]_i$ through mainly two pathways in rat cerebrocortical synaptosomes. One is Ca^{2+} inflow and the other is Ca^{2+} release from stores.

REFERENCES

- Yokoyama A, Murata M, Oshima Y, Iwashita T and Yasumoto T: Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. *J Biochem* **104**, 184–187 (1988)
- Gusovsky F and Daly JW: Maitotoxin: a unique pharmacological tool for research on calcium-dependent mechanisms. *Biochem Pharmacol* **39**, 1633–1639 (1990)
- Escobar LI, Salvador C, Martinez M and Vaca L: Maitotoxin, a cationic channel activator. *Neurobiology* **6**, 59–74 (1998)
- Estacion M, Nguyen HB and Gargus JJ: Calcium is permeable through a maitotoxin-activated nonselective cation channel in mouse L cells. *Am J Physiol* **270**, C1145–C1152 (1996)
- Leech CA and Habener JF: Insulinotropic glucagon-like peptide-1-mediated activation of non-selective cation currents in insulinoma cells is mimicked by maitotoxin. *J Biol Chem* **272**, 17987–17993 (1997)
- Schilling WP, Sinkins WG and Estacion M: Maitotoxin activates a nonselective cation channel and a P2Z/P2X₇-like cytolytic pore in human skin fibroblasts. *Am J Physiol* **277**, C755–C765 (1999)
- Venant A, Dazy AC, Diogene G, Metzeau P and Marano F: Effects of maitotoxin on calcium entry and phosphoinositide breakdown in the rabbit ciliated tracheal epithelium. *Biol Cell* **82**, 195–202 (1994)
- Nakahata N, Yaginuma T and Ohizumi Y: Maitotoxin-induced phosphoinositide hydrolysis is dependent on extracellular but not intracellular Ca^{2+} in human astrocytoma cells. *Jpn J Pharmacol* **81**, 240–243 (1999)
- Tagliatela M, Canzoniero LMT, Fatatis A, Renzo GD, Yasumoto T and Annunziato L: Effect of maitotoxin on cytosolic Ca^{2+} levels and membrane potential in purified rat brain synaptosomes. *Biochim Biophys Acta* **1026**, 126–132 (1990)
- Hajós F: An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93**, 485–489 (1975)
- Komulainen H and Bondy SC: The estimation of free calcium within synaptosomes and mitochondria with fura-2; comparison to quin-2. *Neurochem Int* **10**, 55–64 (1987)
- Aiuchi T, Daimatsu T, Nakaya K and Nakamura Y: Fluorescence changes of rhodamine 6G associated with changes in membrane potential in synaptosomes. *Biochim Biophys Acta* **685**, 289–296 (1982)
- Bradford MM: Rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72**, 248–254 (1976)
- Godfraind T, Miller R and Wibo M: Calcium antagonism and calcium entry blockade. *Pharmacol Rev* **38**, 321–416 (1986)
- Nakahata N, Ohkubo S, Ito E, Nakano M, Terao K and Ohizumi Y: Comparison of maitotoxin with thromboxane A₂ in rabbit platelet activation. *Toxicon* **37**, 1375–1389 (1999)