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

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ABSTRACT—The present study was conducted to elucidate the mechanism of the maitotoxin (MTX)-induced increase in intrasynaptosomal free calcium level ([Ca\textsuperscript{2+}]). The MTX (1 ng/ml)-induced increase in [Ca\textsuperscript{2+}]i was partially inhibited by the omission of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{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\textsuperscript{2+}]i in the absence of Ca\textsuperscript{2+e} was sensitive to procaine, TMБ-8, genistein and verapamil, but not to ryanodine and U-73122. These results may suggest that MTX increases [Ca\textsuperscript{2+}]i by stimulating Ca\textsuperscript{2+} entry through voltage-independent nonselective cation channels and Ca\textsuperscript{2+} release from stores through a phospholipase C-γ1-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\textsuperscript{2+}) content and/or Ca\textsuperscript{2+} influx 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\textsuperscript{2+} influx and phosphoinositide hydrolysis in rabbit ciliated tracheal epithelium and human astrocytoma cells (7, 8). In rat brain synaptosomes, MTX increases intrasynaptosomal Ca\textsuperscript{2+} and depolarizes synaptosomal plasma membranes through the influx of extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{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\textsuperscript{2+} level ([Ca\textsuperscript{2+}])i), however, remain unknown. The aim of this study is to elucidate the mechanism of MTX-induced increase in [Ca\textsuperscript{2+}]i; using rat cerebrocortical synaptosomes.

Synaptosomes (P2B 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 TMΒ-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\textsuperscript{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/i; f and i 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 NaH2PO4, 1.2 mM MgCl2, 5 mM NaHCO3, 6 mM glucose, 1 mM CaCl2 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\textsuperscript{2+}]i and membrane depolarization dose-dependently in rat cerebrocortical synaptosomes (data not shown). Removal of Ca\textsuperscript{2+} from the medium inhibited these effects of MTX (1 ng/ml).
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 μM) 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 [Ca²⁺], through the influx of Ca²⁺, because the MTX-induced increase in [Ca²⁺] was abolished in Ca²⁺-free medium containing 1 mM EGTA. In the case of synaptosomes, EGTA reduces [Ca²⁺], by a rapid and continuous efflux of Ca²⁺ across the synaptic membrane (11). We could not measure the changes in [Ca²⁺], using agents that release Ca²⁺ from intracellular stores in Ca²⁺-free medium containing 1 mM EGTA. Thus, it is not possible to measure [Ca²⁺] in Ca²⁺-free medium containing EGTA. In our Ca²⁺-free experiments, synaptosomal preparations were suspended in Ca²⁺-free medium containing 1 mM EGTA and subsequently re-suspended in nominally Ca²⁺-free medium to wash out the EGTA. Then, [Ca²⁺] was measured in the nominally Ca²⁺-free medium.

To identify the MTX-activated channels, the effects of MTX (1 ng/ml) on [Ca²⁺], and membrane potential were investigated in the presence of Ca²⁺, and Ca²⁺ antagonists of different classes of voltage-dependent Ca²⁺ channels. Verapamil (200 μM) inhibited both the [Ca²⁺], increase and depolarization induced by MTX by approximately 50% (Fig. 1: A and B). Nifedipine (10 μM), ω-agatoxin IVA (200 nM), ω-conotoxin GVIA (1 μM) and ω-conotoxin MVIIIC (500 nM) failed to modify the effects of MTX (Fig. 1: A and B). SKF-96365 (30 μM), a receptor-mediated Ca²⁺-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 Ca²⁺ channels, since its concentration exceeds the specific range (14). Thus, the extracellular Ca²⁺-dependent MTX-induced increase in [Ca²⁺] may be due to Ca²⁺ entry through voltage-independent nonselective cation channels.

To elucidate the Ca²⁺-free-insensitive effect of MTX (1 ng/ml), we tested the effects of agents that can modify the release of Ca²⁺ from intracellular stores on MTX (1 ng/ml)-induced increase in [Ca²⁺], in the absence of Ca²⁺. Procaine (8 mM), TMB-8 (100 μM) and genistein (110 μM), inhibitor of tyrosine kinase to induce tyrosine phosphorylation of phospholipase C-γ1 markedly inhibited the increase in [Ca²⁺], but not ryanodine (50 μM) and U-73122 (10 μM), inhibitor of activation of phospholipase C-β1 through a trimeric G protein (Fig. 2). MTX generates inositol 1,4,5-trisphosphate (IP₃) through the hydrolysis of phosphatidylinositol by phospholipase C and IP₃ releases Ca²⁺ from intracellular stores (2). Concentrations of U-73122 and genistein were enough to induce an inhibition of IP₃ production through specific sites, respectively (7). Thus, MTX-induced increase in [Ca²⁺], in the absence of Ca²⁺, may be mainly due to Ca²⁺ release from stores through IP₃ generated by the activation of phospholipase C-γ1, which might imply phosphorylation on tyrosine residues (7, 15).

Verapamil (20–200 μM) inhibited dose-dependently the effect of MTX in the presence or absence of Ca²⁺ (Fig. 3). In the absence of Ca²⁺, and the presence of verapamil, procaine or TMB-8 had no additive effect (data not shown).
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.

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