Maitotoxin converts the plasmalemmal Ca\(^{2+}\) pump into a Ca\(^{2+}\)-permeable nonselective cation channel

William G. Sinkins, Mark Estacion, Vikram Prasad, Monu Goel, Gary E. Shull, Diana L. Kunze, and William P. Schilling

Department of Physiology and Biophysics, and Neuroscience, and Rammelkamp Center for Education and Research, Case Western Reserve University School of Medicine, Cleveland; and the Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio

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Sinkins WG, Estacion M, Prasad V, Goel M, Shull GE, Kunze DL, Schilling WP. Maitotoxin converts the plasmalemmal Ca\(^{2+}\) pump into a Ca\(^{2+}\)-permeable nonselective cation channel. Am J Physiol Cell Physiol 297: C1533–C1543, 2009. First published September 30, 2009; doi:10.1152/ajpcell.00252.2009.—Maitotoxin (MTX), or MTX, isolated from the “red-tide” dinoflagellate Gambierdiscus toxicus, is one of the most potent toxins known and is one of the causes of Ciguatera seafood poisoning (9). MTX (Mr ~3,500 Da) at subnanomolar concentrations causes a profound increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in every cell examined to date, but the molecular identity of the channels involved remains unknown. A clue came from studies of a structurally related marine toxin called palytoxin (PTX). PTX binds to the plasmalemmal Na\(^+\)-K\(^+\)-ATPase; patch clamp; small interfering RNA; fura-2

Maitotoxin, or MTX, isolated from the “red-tide” dinoflagellate Gambierdiscus toxicus, is one of the most potent toxins known and is one of the causes of Ciguatera seafood poisoning (9). MTX (Mr ~3,500 Da) at subnanomolar concentrations causes a profound increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). Early studies revealed that MTX is not an ionophore (51). Furthermore, MTX-induced rise in [Ca\(^{2+}\)]\(_i\) is dependent on Ca\(^{2+}\) influx and does not reflect release of Ca\(^{2+}\) from internal stores (11, 46, 58). The rise in [Ca\(^{2+}\)]\(_i\) has been seen in all cells examined to date including bovine aortic endothelial cells (BAECs) (11, 57), mouse pancreatic \(\beta\)-cells (59), human skin fibroblasts (46), rat insulinoma cells (49), human SH-SYSY neuroblastoma cells (55), rat PC-12 cells (5), rat C6 glioma cells (31), HL-60 cells (32), human embryonic kidney (HEK) cells (48), THP-1 monocytes (48), BAC1 macrophages (54), and BW5147.3 lymphoma cells (48) to name a few. MTX-induced responses are also observed in sea urchin eggs (40), Xenopus oocytes (4), crayfish neurons (35), and insect myocytes (28). Originally, MTX was thought to be a specific activator of voltage-gated Ca\(^{2+}\) channels since the rise in [Ca\(^{2+}\)]\(_i\) was dependent on the presence of extracellular Ca\(^{2+}\) and could be attenuated by organic and inorganic Ca\(^{2+}\) channel antagonists (50). However, it was subsequently discovered that MTX activates a Ca\(^{2+}\)-permeable, nonselective cation channel in both excitable and nonexcitable cells (4, 6, 8, 10, 27, 31, 46, 59). In nonexcitable cells, activation of these channels by MTX allows the influx of Ca\(^{2+}\) and subsequent secondary effects such as activation of phospholipase C and release of arachidonic acid. In excitable cells, MTX-induced activation of nonselective cation channels causes membrane depolarization, activation of voltage-gated channels and secondary effects such as contraction of cardiac and smooth muscle, and the release of neurotransmitters from nerve terminals. Ultimately, MTX causes Ca\(^{2+}\) overload-induced necrotic cell death characterized by the rapid staining of the nucleus with propidium iodide and the release of large macromolecules such as lactate dehydrogenase (Mr 160 kDa) (11, 12, 54, 57). The channels activated by MTX have been recorded at the single channel level using the patch-clamp technique. MTX activates a 12-pS channel in cell-attached patches from pig cardiac myocytes with 50 mM Ca\(^{2+}\) or Ba\(^{2+}\) in the pipette (25). The channels were predominantly permeable to Ca\(^{2+}\) and Ba\(^{2+}\) but also passed Na\(^+\), K\(^+\), and Cs\(^+\). A similar 14-pS channel was observed in rat myocytes examined in outside-out patch configuration (13). A 40-pS nonselective cation channel activated by MTX in cell-attached mode was found in renal epithelial cells (8). The channel was only present when MTX was applied to the outside surface; i.e., channels were never observed upon MTX application to inside-out patches. A 16-pS channel was observed in guinea pig ventricular myocytes in cell-attached mode with MTX in the pipette solution (36). Interestingly, the channels remained active following excision of the patch into inside-out configuration. The ability of MTX to activate channels in cell-attached and excised patches demonstrates that the effect of MTX is membrane delimited and is strongly supportive of the hypothesis that these channels are directly activated by MTX via interaction with the extracellular surface of the cell. However, the identity of the channels activated by MTX remains unknown.

Address for reprint requests and other correspondence: W. P. Schilling, Rammelkamp Center for Education and Research, Rm. R322, MetroHealth Medical Center, 2500 MetroHealth Dr., Cleveland, OH 44109-1998 (e-mail: wschilling@metrohealth.org).
A clue to the identity of the MTX-activated channel came from studies of another marine toxin called palytoxin (PTX). PTX was originally isolated from sea corals of the genera *Palythoa* (29). It is now clear that the molecular receptor for PTX is the plasmalemmal Na⁺-K⁺-ATPase pump (NKA). Early studies showed that ouabain, a cardiac glycoside that inhibits pump function, could effectively antagonize the actions of PTX, and it was suggested that PTX may convert the NKA into a channel (16, 17). Indeed, a variety of investigators showed that PTX activates a relatively nonselective monovalent cation channel with conductance in the range of 8–14 pS (19, 23, 24, 30, 41, 42, 53, 56). PTX-induced cation fluxes were activated when the NKA was heterologously expressed in yeast, which lack an endogenous NKA activity (43, 44). Furthermore, PTX-induced single channels of 10-pS conductance were observed following reconstitution of the NKA in planar lipid bilayers following in vitro expression (19). PTX also inhibits ATPase activity associated with the NKA, although this occurs at concentrations much higher than those needed to induce channel activity (18). Together, these results provide strong evidence that the NKA is the receptor for PTX.

MTX and PTX have similar structures and both toxins produce Ca²⁺ overload-induced necrotic cell death (47) suggesting a common mechanism of action. However, preliminary studies showed that ouabain had no effect on the kinetics or magnitude of MTX-induced responses (Schilling, unpublished observations). Thus, despite similarities in structure, PTX and MTX do not affect the NKA. Given the high permeability of the MTX channels for Ca²⁺ and the structural similarity with PTX, we reasoned that MTX may activate another member of the P-type ATPase family, specifically, the plasmalemmal Ca²⁺-ATPase (PMCA) pump. Thus the purpose of the present study was to determine whether the PMCA is the receptor for MTX and to determine whether MTX converts the PMCA pump into a channel. To test this hypothesis, we examined the effect of MTX on whole cell membrane currents using the patch-clamp technique in 1) cells overexpressing the PMCA, 2) cells transfected with small interfering RNA (siRNA) to reduce expression of the PMCA and 3) cells obtained from PMCA-ablated mouse embryos. The effect of MTX on the catalytic (Ca²⁺-ATPase) activity of the PMCA in both isolated membranes and in purified PMCA preparations was also determined. The results were consistent with the hypothesis that MTX binds to and converts the PMCA into a Ca²⁺-permeable nonselective cation channel.

**MATERIALS AND METHODS**

*Solutions and reagents.* 2-(N-morpholino)ethanesulfonic acid (MES) buffered-saline (MBS) for use with Sf9 cells contained the following (in mM): 10 CaCl₂, 60 KCl, 17 MgCl₂, 10 NaCl, 4 d-glucose, 110 sucrose, 0.1% bovine serum albumin (BSA), and 10 MES, pH adjusted to 6.2 at 22°C with NaOH. The total osmolarity of MBS was ~340 mosM. HEPES-buffered saline (HBS) for use with mammalian cells contained (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 10 d-glucose, 1.8 CaCl₂, 15 HEPES, and 0.1% BSA, pH adjusted to 7.40 at 37°C with NaOH. Ca²⁺-free HBS contained the same salts as HBS without added CaCl₂. Fura-2 acetoxyethyl ester (fura-2/AM) was obtained from Molecular Probes. MTX was obtained from Wako Bioproducts and was stored as a stock solution in ethanol or in aqueous solution with 0.1% BSA at −20°C. All other salts were of reagent grade. Anti-PMCA antibodies were from the following sources: pan-PMCA mouse monoclonal 5F10 (Affinity Bioreagents, catalogue no. MA3-914); PMCA1(Upstate Biotechnology, catalogue no. 07-244); and PMCA4 (Upstate Biotechnology, catalogue no. 05-640). Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (secondary antibodies) were from GE Healthcare-Amersham, (catalog nos. NXA931 and NA934, respectively). Alexa 488-conjugated anti-rabbit IgG was from Molecular Probes (catalog no. A11034). Human PMCA1b and PMCA4b clones were generous gifts from Dr. Emanuel E. Strehler, Mayo Clinic College of Medicine.

*Cell culture.* *Spodoptera frugiperda* (SPF) cells were obtained from the American Type Culture Collection (ATCC) and were propagated in suspension culture as previously described (22, 37) using Graces insect medium supplemented with 2 mM t-glutamine, 1% penicillin-streptomycin-neomycin solution (PSN), 10% heat-inactivated fetal bovine serum (FBS), 2% yeastolate solution, and 2% lactalbumin hydrolysate solution (GIBCO). Human embryonic kidney cells (HEK), obtained from ATCC, were maintained with minimum essential medium (MEM) supplemented with 10% FBS, 1% PSN, and 2 mM t-glutamine. For passage, HEK cells were dispersed by trypsin treatment and seeded to a density of ~3 × 10⁵ cells/cm². The medium was changed every 2–3 days following seeding. BAECs were cultured as previously described (45) using Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 2 mM glutamine (complete DMEM). When grown to confluence, the cultures demonstrated contact-inhibited cobblestone appearance typical of endothelial cells.

*Isolation and culture of mouse embryonic fibroblasts.* All protocols involving the use of animals were approved by, and performed in compliance with, the University Institutional Animal Care and Use Committee guidelines. Mouse embryonic fibroblasts (MEFs) were harvested from 13.5-day-old embryos as previously described (52). Briefly, pregnant female mice were euthanized by CO₂ asphyxiation and the uterine was rapidly harvested. Embryos were isolated and the carcass of each embryo, devoid of head, tail, limbs, and liver, which were saved for genotyping, were separately incubated in 1× trypsin (Invitrogen) at 37°C for 15 min. Cells were dispersed by gentle aspiration using an 18-gauge needle, allowed to adhere, and cultured at 37°C in 5% CO₂. The culture medium included 1× high-glucose DMEM supplemented with t-glutamine, antibiotics, 10% FBS, and 50 mM β-mercaptoethanol. Cells were maintained on a 3T3 protocol and used in the P2/P3 passages. Genotyping of established cell lines, using genomic DNA extracted from head or liver of the original embryos, was carried out as previously described (38).

*Knockdown of PMCA1 using siRNA.* SMARTpool siRNA for PMCA1, obtained from Dharmaco, was transfected into HEK cells using the Amaxa Nucleofector with solutions and pulsing protocol recommended by the manufacturer. Dharmaco’s positive control siRNA duplex was used to optimize transfection efficiency and non-targeting siRNA (NT) was used as control. Cells were used for experimentation 72 h after transfection.

*Transient expression of the PMCA.* For Sf9 insect cells, recombinant baculovirus containing the cDNA for PMCA1b and PMCA4b under control of the polyhedrin promoter, were generated as previously described (21). For expression, Sf9 cells in Grace’s medium were plated into 100-mm plastic tissue culture dishes or onto glass coverslips (~10⁵ cell/cm²). After incubation for 30 min, an aliquot of viral stock was added (multiplicity of infection was ~10), and the cells were maintained at 27°C in a humidified air atmosphere. Cells were used for experimentation at the postinfection times indicated in the text. HEK cells were seeded onto 35-mm culture dishes and maintained until they reached 90–95% confluence. A single dish of cells was transfected with 2 μg of plasmid cDNA as previously described (12), using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the cells were dispersed with trypsin-EDTA, and reseeded onto 12-mm glass coverslips (6–9 coverslips per 35-mm dish).

*Measurement of the cytosolic free Ca²⁺ concentration.* [Ca²⁺]ᵢ, was measured in Sf9 cells using the fluorescent indicator fura-2 as previously described (45). Briefly, cells were harvested and resus-
pended at a concentration of ~2 × 10^6 cells/ml in MBS containing 2 μM fura-2/AM. After 30 min incubation at 22°C, the cell suspension was subjected to centrifugation, resuspended in an equal volume of MBS, and incubated for an additional 30 min. Aliquots from this final suspension were subjected to centrifugation and washed twice immediately before fluorescence measurement. Fluorescence was recorded in a mechanically stirred cuvette using an Amino-Bowman-2 (AB2) spectrophotofluorometer. For measurements of Ca^{2+}, excitation wavelength alternated between 340 and 380 nm every 2 s, and fluorescence intensity was monitored at an emission wavelength of 510 nm. All measurements on Sf9 cells were performed at 22°C. [Ca^{2+}] in BAECs was measured at 37°C as previously described (45).

Electrophysiological techniques. The gigaseal technique for current recording was utilized in the whole cell mode. Cells, attached to circular glass coverslips, were transferred to a perfusable recording chamber on the stage of a Nikon inverted microscope immediately before use. Unless otherwise indicated, Na+-containing Ringer solution was used as the extracellular solution for current recordings in HEK cells and contained (in mM) 160 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.2 and pCa 8). In some experiments, Na⁺ in the Ringer solution was isosmotically replaced with N-methyl-D-glucamine (NMDG). Where indicated, Ca^{2+} in the pipette solution was varied from pCa 8 to 6.3 by addition of CaCl₂. The free Ca^{2+} was calculated using the WinMaxChelator program (39). For Sf9 cell recordings, the bath solution contained (in mM) 100 Na-aspartate, 2 KCl, 4 MgCl₂, 2 CaCl₂, 80 mannitol, and 10 MES, pH 6.5, and the pipette contained 100 K-aspartate, 2 NaCl, 2 Mg-ATP, 2.9 CaCl₂, 10 EGTA, 10 HEPES (pH 7.2 and pCa 8). In some experiments, Na⁺ in the bath was replaced with NMDG. Data were obtained using an Axopatch 200A amplifier (Pacer Scientific) and sampled online using pCLAMP 8.0 software. The ground electrode was an Ag-AgCl wire connected to the bath via an agar bridge containing 150 mM NaCl. All recordings were made at room temperature (~22°C). Electrode resistances ranged from 2 to 6 MΩ and whole cell series resistances ranged from 4 to 20 MΩ. To generate current-voltage (I-V) relations, voltage ramps from −120 to +120 mV over 200 ms were repetitively applied at 15-s intervals. Otherwise indicated, the holding potential between ramps was −50 mV and the currents were not leak subtracted. All figures show representative traces corrected for liquid junction potential.

Isolation of membranes from PMCA-expressing Sf9 cells. Sf9 cells expressing PMCA1b or PMCA4b were harvested, subjected to centrifugation at 500 g for 5 min, and resuspended at a density of 5 × 10^6 cells/ml in lysis buffer containing 20 mM Tris-Cl, 5 mM EDTA, 1 mM EGTA, and protease inhibitor mixture. The cell suspension was sonicated on ice using a sonic dismembranator (Fisher) on a power setting of 2.5. The cell suspension was sonicated three times for 10 s with a 10-s rest between pulses. The cell lysate was subjected to centrifugation at 6,000 g for 10 min at 4°C. The resulting pellet was discarded, and the supernatant was centrifuged at 42,000 g for 30 min. The microsomal pellets were resuspended in lysis buffer at a protein concentration of 5–10 mg/ml and stored at −80°C until use.

Biochemical purification of PMCA. Because the PMCA1b isoform proved difficult to purify, the biochemical purification and enzymatic assays were performed using PMCA4b. PMCA4b was purified as previously described with minor modifications (15, 34). Briefly, Sf9 cells expressing PMCA4b were harvested, pelleted, and washed in a buffer containing 20 mM HEPES (pH 7.4) and 130 mM KCl. After the wash, the cells were pelleted and resuspended in lysis buffer containing 20 mM HEPES (pH 7.4), 130 mM KCl, 500 μM MgCl₂, 100 μM CaCl₂, 1 mM PMSF, and 1.8% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS). After 1 h on ice, lysates were cleared by centrifugation at 50,000 g for 60 min. Cleared lysates were applied to a calmodulin-agarose affinity column (bed volume = 1 ml) equilibrated with column buffer containing 10 mM HEPES (pH 7.4), 130 mM KCl, 1 mM MgCl₂, 100 μM CaCl₂, 0.5 mg/ml phosphatidylcholine, and 0.1% CHAPS. The loaded column was rotated overnight at 4°C. The column was then washed with at least 20 volumes of ice-cold column buffer. Fractions (0.2 ml) were eluted with column buffer in which Ca^{2+} and Mg^{2+} were removed and replaced with 2 mM EDTA and 5% glycerol. All operations with the affinity column were performed in a 4°C cold room. Elution fractions were assayed for ATPase activity. In some preparations, CHAPS was replaced with Triton-X100 and C₁₂E₅ as previously described (60), which yielded PMCA4b preparations with catalytic activity essentially identical to those obtained using CHAPS. Purified PMCA preparations were stored in single-use aliquots at −80°C.

Measurements of PMCA catalytic activity. Ca^{2+}-ATPase activity was measured as follows. An aliquot of membrane preparation or purified PMCA4b preparation was added to a reaction cocktail containing 120 mM KCl, 1 mM MgCl₂, 30 mM HEPES (pH adjusted to 7.2 with KOH), 10 μM ouabain, 1 mM thapsigargin, 2 mM Na₃, and either 200 μM CaCl₂ or 2 mM EGTA. After a 15-min preincubation period, 1 μM ATP was added. The reaction was initiated by addition of Mg₂-ATP to a final concentration of 2 mM. The reaction was terminated by addition of ice-cold 20% trichloroacetic acid. The samples were centrifuged at 14,000 g for 5 min, and the inorganic phosphate in the supernatant was determined by the colorimetric assay of Fiske and Subbarow (14). Control experiments showed that the ATPase assay was linear over the time and protein concentrations used in the present study. Ca^{2+}-ATPase activity were defined as the activity in the presence of Ca^{2+} minus that observed in EGTA. In some experiments, the pH of the reaction cocktail was adjusted to 8.5 using a 1:1 mixture of HEPES and Tris buffer, but all other reaction conditions were the same. Where indicated, MTX was present in the reaction cocktails during the preincubation and reaction periods.

Immunoblots. Membrane proteins were fractionated by SDS-PAGE and electrotransferred to PVDF membrane (100 V for 1 h) in Tris-glycine buffer. Blots were probed with PMCA antibody and detected, following incubation with HRP-conjugated anti-rabbit IgG, by SuperSignal West Pico chemiluminescent substrate (Pierce).

Immunofluorescence. HEK cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min. The coverslips were briefly rinsed in PBS and subsequently incubated with blocking solution containing 3% IgG-free BSA (Vector Laboratories), 10% normal donkey serum, and 0.1% Triton-X 100 for 1 h at room temperature. The coverslips were incubated with the primary antibody overnight at 4°C. After being washed three times for 5 min with PBS at room temperature, the slides were incubated with Alexa 488- or Alexa 594-conjugated anti-rabbit IgG by SuperSignal West Pico chemiluminescent substrate (Pierce).

Statistical treatment of the data. Because of the limited supply and high cost of MTX, the Ca^{2+}-ATPase assays were only performed two to three times in triplicate. All other experiments were performed at least three times. Where indicated, mean values were compared using the paired Student’s t-test with P < 0.05 considered significant.

RESULTS

Overexpression of PMCA in Sf9 insect cells. In preliminary experiments we found that the effect of MTX on both BAECs and HEK cells was unaffected by ouabain (Fig. S1, see online supplemental material), a cardiac glycoside known to block the cellular response to PTX (for example see Refs. 3 and 47). These results suggested that the NKA pump was not the target for MTX and prompted us to examine the effect of this toxin on the PMCA. To begin to test the hypothesis that the PMCA is the receptor for MTX, we functionally expressed the PMCA1b clone in Sf9 insect cells by using recombinant...
baculovirus. In this heterologous system, PMCA expression is under control of the polyhedrin promoter. This promoter turns on relatively late in the baculovirus life cycle (37). As seen in Fig. 1, addition of MTX (1 nM) to Sf9 cells infected with PMCA1b baculovirus for 14 h had little or no effect on [Ca\(^2+\)]\(_i\), relative to cells infected with baculovirus containing the human B2 bradykinin receptor (BK cells), which serves as the infection control. However, the response to MTX in the PMCA1b-expressing cells was increased over control at 20, 28, and 40 h postinfection time. Similarly, PMCA1b protein expression, as determined by Western blot analysis of Sf9 cell lysates, increased in parallel as a function of postinfection time (Fig. 1B, inset); at 14 h PMCA1b protein was barely detectable, but protein increased in a time-dependent fashion from 20 to 40 h postinfection time. Thus PMCA1b expression is appropriate for a protein under control of the polyhedrin promoter and correlates with MTX-induced change in [Ca\(^2+\)]\(_i\). PMCA immunoreactivity was not observed in control BK cells. Thus this antibody apparently does not cross-react with the insect PMCA. As an additional control, we examined the effect of PTX on [Ca\(^2+\)]\(_i\), in Sf9 cells expressing the PMCA1b. PTX (100 nM) had essentially no effect on [Ca\(^2+\)]\(_i\), in PMCA1b-expressing cells at 28 h postinfection time (Fig. 1C). Together, these results demonstrate that the effect of MTX on [Ca\(^2+\)]\(_i\), is specific and related to expression of the PMCA.

To obtain direct evidence that MTX converts the PMCA into a channel, whole cell currents were recorded in Sf9 cells expressing PMCA1b versus control BK cells. As seen in Fig. 2, addition of MTX produced a time-dependent increase in both inward and outward current. The I-V relationship was linear with K-aspartate in the pipette and Na-aspartate in the bath solution. As expected for a nonselective cation channel, replacement of Na\(^+\) in the bath with the large impermeant cation NMDG caused a substantial reduction in inward current and a shift of the reversal potential to negative values (Fig. 2, inset). The same current was observed in both control BK cells and in PMCA1b-expressing Sf9 cells. However, the magnitude of the current was approximately sixfold greater in the PMCA cells (P < 0.02, n = 6), consistent with the hypothesis that overexpression of PMCA1b gives rise to an increased number of MTX-sensitive channels.

**Effect of MTX on PMCA enzymatic activity.** To determine whether MTX inhibits the ATPase activity associated with the PMCA, membranes were isolated from Sf9 cells expressing the PMCA4b, and Ca\(^2+\)-dependent ATP hydrolysis was evaluated by measuring the release of inorganic phosphate as a function of time. The assay, which in control experiments was linear with time and protein concentration, was performed in the presence of thapsigargin, ouabain, and NaN3 to block other types of ATPase activity. Ca\(^2+\)-ATPase activity was defined as the difference of activity in the presence of 200 μM free Ca\(^2+\) versus that in the absence of Ca\(^2+\) (i.e., in the presence of EGTA). At pH 7.2, MTX produced a graded inhibition of Ca\(^2+\)-ATPase activity with an IC\(_{50}\) in the range of 3 μM (Fig. 3). Ca\(^2+\)-ATPase activity in membrane preparations from control BK cells was <3% of that observed in preparations from PMCA-expressing cells. Thus >97% of the enzymatic activity observed reflects heterologously expressed PMCA4b.

Although these results demonstrate that MTX affects the enzymatic activity of the PMCA, the concentration needed for inhibition was approximately three orders of magnitude higher than that required for elevation of [Ca\(^2+\)]\(_i\) (compare with Figs. 1 and 2). A similar phenomenon has been reported for the effect of PTX on NKA activity. Depending on the ionic conditions, PTX increases channel activity with Ki in the micromolar range (18). Furthermore, in both assays, the apparent affinity of PTX for the NKA is dramatically reduced by the presence of

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**Fig. 1.** Effect of maitotoxin (MTX) on cytosolic free Ca\(^2+\) concentration ([Ca\(^2+\)]\(_i\)) of *Spodoptera frugiperda* (Sf9) cells overexpressing plasmalemmal Ca\(^2+\)-ATPase-1 (PMCA1). Sf9 insect cells were infected with recombinant baculovirus for expression of either PMCA1 (A) or the human B2 bradykinin (BK) receptor (B). The cells were harvested and loaded with fura-2 at the postinfection times indicated to the right of each trace. Four traces are superimposed in each panel. MTX (1 nM) was added at the time indicated by the arrow. Symbols represent means ± SE values at selected time points (n = 3). B, inset: proteins from Sf9 cell lysates harvested at the postinfection times indicated below each lane were separated by SDS-PAGE and subjected to Western blot analysis. C, same protocol as in A and B with the exception that MTX or PTX was added (at the arrow) to either PMCA or BK cells.

**Fig. 2.** A, BK cells: same protocol as in Figs. 1A and 1B. Symbols represent means ± SE values at selected time points (n = 3). B, inset: proteins from Sf9 cell lysates harvested at the postinfection times indicated below each lane were separated by SDS-PAGE and subjected to Western blot analysis. C, same protocol as in A and B with the exception that MTX or PTX was added (at the arrow) to either PMCA or BK cells.
The explanation for this shift in PTX potency derives from the fact that it is the E2-P form of the NKA that exhibits the highest apparent affinity for PTX. Since K\textsuperscript{+} rapidly catalyzes the conversion of E2-P into the K\textsuperscript{+}-E1 form, the apparent affinity of the NKA for PTX decreases in the presence of elevated K\textsuperscript{+}. We reasoned that a similar phenomenon may occur for MTX action on the PMCA. In the case of the PMCA, however, two H\textsuperscript{+} bind to the E2-P form of the PMCA and are transported in exchange for Ca\textsuperscript{2+}. The prediction therefore would be that the apparent affinity of MTX should increase at higher pH if indeed the E2-P form of the pump has the highest affinity for the toxin. As seen in Fig. 3, the IC\textsubscript{50} for MTX-induced inhibition of Ca\textsuperscript{2+}-ATPase activity decreased ~10-fold from 3 \mu M to 200 nM when pH was increased from 7.2 to 8.5. In parallel experiments, the effect of PTX on PMCA catalytic activity was examined. A slight stimulation of Ca\textsuperscript{2+}-ATPase activity was observed in the presence of 1 \mu M PTX at both pH values examined (114% at pH 7.2 and 107% at pH 8.5, average of 2 experiments performed in triplicate). Thus the ability of MTX to inhibit PMCA in a pH-sensitive fashion is not shared by PTX. To further define the pH sensitivity, the response of fura-2-loaded BAECs to MTX was was examined at pH 7.4 and 8.5 (Fig. 4). The EC\textsubscript{50} for MTX-induced change in [Ca\textsuperscript{2+}], was also decreased ~10-fold upon increasing pH.

**Fig. 3.** Effect of MTX on Ca\textsuperscript{2+}-ATPase activity. Membrane preparations were isolated from PMCA4-expressing Sf9 cells and Ca\textsuperscript{2+}-dependent ATP hydrolysis was measured as described in MATERIALS AND METHODS in the absence or presence of the indicated concentration of MTX. Assays were performed at pH 7.2 (■) or 8.5 (○). Symbols represent means ± SE (n = 2–3 for each) normalized to the value obtained in the absence of MTX. Each experiment was performed in triplicate.

**Fig. 4.** Effect of pH on MTX-induced change in [Ca\textsuperscript{2+}], in bovine aortic endothelial cells (BAECs). Four traces are shown superimposed in each panel. Fura-2-loaded BAECs were suspended in HEPES-buffered saline with pH 7.4 (A) or pH 8.5 (B), and the fluorescence was recorded as a function of time. MTX was added to each trace at the arrow at the final concentration indicated to the right of each trace. Curves are representative of 3 independent experiments.
from 7.4 to 8.5 (from ~0.2 to ~0.02 nM). These results are consistent with the hypothesis that the PMCA is the molecular target for MTX.

To further test for a direct effect of MTX on PMCA, we examined the effect of MTX on the Ca\(^{2+}\)-ATPase activity associated with purified PMCA. The procedure for purification and reconstitution of the PMCA from mammalian (33, 34) and Sf9 insect cells (15) is well established in the literature and is based on a single-step procedure using a CaM affinity column. These previous studies have shown that the PMCA can be purified to near homogeneity and that activity can be preserved by addition of the appropriate phospholipids. Briefly, membrane preparations from PMCA4b overexpressing Sf9 cells were solubilized in lysis buffer containing detergent, and the PMCA was extracted from the lysate using a CaM-Sepharose 4B column. As seen in Fig. S2 (online supplemental material), specific Ca\(^{2+}\)-ATPase activity eluted from the column was enriched approximately sixfold relative to the starting membrane fraction. In parallel experiments, we examined the effect of MTX on the Ca\(^{2+}\)-ATPase activity associated with the purified PMCA4b. At pH 7.2, MTX (500 nM) significantly inhibited Ca\(^{2+}\)-ATPase activity 36.4 \(\pm\) 8.2% \((n = 3, P < 0.03)\). This is similar to the level of inhibition found in Sf9 cell membrane preparations at this pH and concentration of MTX (see Fig. 3).

These results demonstrate that the purified PMCA is active and that MTX inhibits enzymatic activity by direct interaction with the PMCA protein itself.

**Overexpression of PMCA in mammalian cells.** To further test the hypothesis that MTX converts the PMCA into a channel, we overexpressed the PMCA1b in HEK293 cells using the bicistronic pIRES2-EGFP vector that directs the expression of the PMCA and GFP from a single mRNA. Cells expressing PMCA were identified by green fluorescence. To determine whether overexpression of the PMCA1b was associated with increased MTX-induced channel activity, whole cell membrane currents were recorded in HEK cells using the patch-clamp technique. Currents were initially recorded with normal Ringer as the bath solution and with a Cs-aspartate-based pipette solution (pCa 8). Under these ionic conditions, addition of MTX produced an increase in inward and outward currents in PMCA1b-expressing HEK cells (Fig. 5). The currents activated in a time-dependent fashion and exhibited a linear \(I-V\) relationship. Upon changing the bath solution to one in which the Na\(^+\) was replaced by NMDG, the inward current was greatly reduced and the reversal potential shifted from approximately \(-15\) to \(-60\) mV, consistent with activation of a nonselective cation channel. Similar results were obtained in GFP-only control cells, but the amplitude of the current was significantly

![Fig. 5. Effect of MTX on whole cell membrane currents in HEK cells heterologously expressing PMCA1. Left: whole cell membrane currents were recorded in human embryonic kidney (HEK) cells transfected with either pIRES-EGFP alone (●) or pIRES2-EGFP-PMCA (○). The pipette contained Cs\(^+\)-aspartate solution with Ca\(^{2+}\) buffered to pCa 8. Voltage ramps were applied every 15 s. Inward currents at \(-80\) mV are plotted as a function of time after establishment of whole cell recording. At the time indicated by the top horizontal bar in each panel, the bath solution was changed to one containing MTX (1 nM) and MTX in N-methyl-D-glucose (NMDG)-Ringer. \(I-V\) plots for each experiment are shown at the times indicated by the solid circles in main panels (a, b). Right: means \(\pm\) SE values for the peak inward current at \(-80\) mV following MTX addition for the pipette solution listed above each bar. Where indicated, ATP concentration was 4 mM. Currents in PMCA cells (open bar) were significantly greater than those in EGFP-only cells (solid bar) under all conditions \((P < 0.03)\). Values in parentheses indicate the number of cells tested for each condition. Inset: membrane proteins from control EGFP-only or PMCA-transfected HEK cells were separated by SDS-PAGE and probed for PMCA by Western blot analysis.](http://ajpcell.physiology.org/)

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reduced versus that observed in the PMCA1b-expressing HEKs (Fig. 5, left).

Previous studies on PTX have shown that currents associated with activation of the NKA are sensitive to pump ligands (2, 3). In particular, elevating intracellular Na\(^+\) and adding ATP to the pipette solution caused a dramatic increase in sensitivity to PTX. To begin to evaluate the effect of various pump ligands on the response to MTX, we recorded currents in both PMCA1b-expressing cells and GFP controls with ATP in the pipette and with higher pCa. As summarized in Fig. 5, right, adding ATP and increasing Ca\(^{2+}\) greatly increased the magnitude of the current following addition of 1 nM MTX to the bath solution. The increase was seen in both control and PMCA1b-expressing HEK cells, but under all conditions examined, the MTX-induced currents were significantly greater in PMCA-overexpressing cells.

**Overexpression of PMCA4b in HEK cells.** Because the effect of MTX on the Ca\(^{2+}\)-ATPase activity was examined on the PMCA4b isoform (see Fig. 3), we wanted to determine whether overexpression of PMCA4b would also yield an enhanced current response to MTX. As seen in Fig. 6, MTX-induced currents were significantly increased in HEK cells overexpressing the PMCA4b isoform relative to GFP controls. As discussed above, the Ca\(^{2+}\)-ATPase assays demonstrated that the apparent IC\(_{50}\) of PMCA4b isoform for MTX is shifted approximately 10-fold by a change in pH from 7.2 to 8.5. To determine whether a similar shift in affinity occurs at the whole cell current level, on-rates and off-rates were determined in PMCA4b overexpressing cells (Fig. 6D). The rate of current activation by 0.2 nM MTX was unaffected by pH, but the off-rate initiated by washout of extracellular MTX was increased 8.6-fold at pH 7.2 relative to that observed at 8.5 (\(\tau_{\text{off}}\) was 6.0 and 51.8 min at pH 7.2 and 8.5, respectively), consistent with the shift in affinity observed in the Ca\(^{2+}\)-ATPase assays and in the fura-2 measurements shown in Fig. 4.

**Effect of PMCA downregulation on the cellular response to MTX.** If indeed the PMCA is the receptor for MTX, then knockdown of the native PMCA protein should attenuate the response to MTX. For these experiments, HEK cells were transfected with siRNA directed against human PMCA1 or with control, nontargeting (NT) siRNA. Western blot analysis revealed that 72 h after transfection with PMCA1-siRNA, expression of PMCA1 protein was greatly reduced relative to the NT control (Fig. 7), but PMCA4 protein expression was unaffected (not shown). Plasmalemma-associated PMCA1 immunofluorescence was also greatly decreased by PMCA1-siRNA. Likewise, whole cell membrane currents were significantly attenuated in cells transfected with PMCA1-siRNA (Fig. 7); peak MTX-induced currents were reduced more than 75%. To determine whether the effect of siRNA knockdown was specific, whole cell currents were recorded in response to PTX. Knockdown of PMCA1 had no effect on PTX-induced membrane currents (Fig. 7, bottom right).

To further test the hypothesis that downregulation of PMCA attenuates the response to MTX, the effect of MTX was examined in two MEF cell lines isolated from mice with the following genotype: 1) PMCA1\((+/-)\), PMCA4\((+/-)\) and 2) PMCA1\((+/-)\), PMCA4\((+/-)\). The effect of MTX on whole cell currents in these two MEF cell lines is shown in Fig. 8A. Bath perfusion with MTX (0.2 nM) produced a time-dependent increase in whole cell inward current in MEFs with the PMCA1\((+/-)\), PMCA4\((+/-)\) genotype. The
I-V relationship was linear with a reversal potential near 0 mV (Fig. 8B), consistent with MTX currents recorded in Sf9 insect cells or HEK cells (see above). Importantly, MTX-induced currents recorded under identical conditions were 37.3% smaller (P < 0.001) in MEFs with the PMCA1(-/-) / PMCA4(-/-) genotype (Fig. 8D). MEFs with this genotype have reduced PMCA1 protein expression and completely lack PMCA4 protein relative to the PMCA1(+/--), PMCA4 (+/+) genotype (Fig. 8C). Together these results demonstrate that MTX-induced currents are significantly reduced by RNAi or genetic knockdown of PMCA protein expression.

Fig. 7. Effect of small interfering RNA (siRNA) on PMCA protein expression and on MTX-induced membrane currents. Top: HEK cells were transfected with either SMARTpool PMCA-siRNA or a nontargeting (NT) siRNA control (Dharmacon) using Lipofectamine 2000. Cells were maintained in culture for 72 h posttransfection before fixation and labeling with a mouse monoclonal anti-PMCA antibody. Immunofluorescence images were obtained using conventional fluorescence microscopy. For Western blot analysis, cells were harvested, solubilized in lysis buffer containing 1% Triton X-100, and subjected to SDS-PAGE. Bands were visualized by chemiluminescence assay. Each lane shows results from an individual transfection along with the actin loading-control; all bands are from the same gel. Bottom: whole cell membrane currents were recorded in HEK cells. The pipette contained Cs+-aspartate solution with Ca2+ buffered to pCa 7. Two traces are shown superimposed. Inward current was recorded at a constant holding potential of −50 mV. At the time indicated by the horizontal bar above the traces, the cells were perfused with bath solution containing MTX (1 nM). Bottom, middle: means ± SE (n = 5 cells) current at −50 mV for NT-control or PMCA-siRNA-transfected cells following MTX addition is shown. Bottom, right: PTX-induced currents at −80 mV in control and PMCA siRNA-transfected cells.

Fig. 8. MTX-induced whole cell currents in mouse embryonic fibroblasts (MEF) from PMCA4 knockout mice. Whole cell currents were recorded in symmetrical Na-aspartate solutions with 2 mM Ca2+ in the bath. A: currents at the holding potential of −60 mV were recorded before and after perfusion of the cell with bath solution containing 0.2 nM MTX (at the arrow). B: I-V relationship obtained from voltage ramps applied either before (control, solid line) or at the peak of the MTX response (dashed line). C: proteins from MEF cells with the genotype indicated below each lane were separated by SDS-PAGE and probed with anti-PMCA4, -PMCA1, or pan-PMCA antibody as indicated on left; bottom gel shows actin loading control. The ratio of band intensities (lane a divided by lane b) determined by densitometry are indicated to the right of the gel as the means ± SE (n = 3). D: means ± SE (n = 8 for each) peak MTX-induced currents recorded in MEF cells with the genotype indicated below each bar. Cells with the PMCA4(−/−) genotype exhibited significantly (P < 0.001) smaller MTX-induced currents.

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DISCUSSION

NKA, SERCA, and PMCA are all members of the P-type ATPase family of ion transporters (20, 26). These pump proteins are thought to have two gates that restrict access of the transported ions to their binding sites within the transport pathway. During the pump cycle, these proteins assume two major conformational forms designated E1 and E2. When the pumps are in the E1 form, the inner gate is open and the ion binding sites are accessible from the cytosol. When the pumps are in the E2 form, the outer gate is open and the ion binding sites are accessible from the extracellular space (or ER lumen for SERCA). Thus, for these proteins to act as ion pumps, the two gates can never open simultaneously. PTX binds to the NKA and allows both gates to open, but the surprising result is that the simultaneous opening of both gates converts the NKA from a pump that at maximum moves 100–200 ions per second across the membrane to a cation channel that transports millions of ions per second. Thus the primary difference between pumps and channels resides not so much in the molecular architecture of the ion translocation pathway itself, but rather in the intrinsic gating properties of the protein.

MTX and PTX are both large cyclic polyether compounds that rapidly dissipate the normal ionic gradients that exist across the cell membrane, which ultimately leads to a Ca\(^{2+}\) overload-induced necrotic cell death (47). Although the toxins are structurally similar and produced the same cellular outcomes, their molecular targets are different. Specifically, the results of the present study suggest that MTX binding to the PMCA converts the pump into a Ca\(^{2+}\)-permeable nonselective cation channel. The evidence is as follows. First, overexpression of the PMCA in either Sf9 insect cells or in HEK cells produced a significant increase in MTX-induced channel activity. The whole cell currents exhibited a linear I-V relationship with a reversal potential near 0 mV. Furthermore, the reversal potential was sensitive to replacement of extracellular Na\(^+\) with the large relatively impermeant cation NMDG consistent with activation of a nonselective cation channel.

Changes in PTX-induced currents in response to alterations in pump ligands; i.e., Na\(^+\), K\(^+\), and ATP, provided important evidence that the NKA was the receptor for PTX (1-3). In the present study, the effect of MTX on whole cell currents in control and PMCA-overexpressing HEK cells was dramatically increased by elevations of [Ca\(^{2+}\)], and by inclusion of ATP in the pipette solution. Furthermore, the effect of [Ca\(^{2+}\)] was graded over the concentration range of 10–500 nM. These results provide additional evidence that the whole cell currents observed are indeed related to activation of the PMCA.

Second, knockdown of the PMCA was associated with a decrease in MTX-induced channel activity. Specifically, siRNA directed against PMCA1 dramatically reduced PMCA1 protein expression. Likewise, transfection with PMCA1-siRNA greatly reduced MTX-induced currents but had no effect on PTX-induced channel activity. A similar result was obtained in MEFs isolated from PMCA4-ablated mice; i.e., MTX-induced currents were significantly attenuated in MEFS lacking the PMCA4 protein. Thus, by both overexpression and knockdown approaches, whole cell current amplitude at a fixed concentration of MTX is directly related to PMCA protein expression level. These results are also consistent with the hypothesis that MTX can affect both PMCA1 and PMCA4 pump isoforms.

Third, PMCA enzymatic activity in isolated membrane fractions and in purified PMCA preparations was inhibited by MTX in a concentration-dependent fashion. Interestingly, the concentration required for inhibition of Ca\(^{2+}\)-ATPase activity (micromolar) was 1,000-fold greater than the concentration needed for activation of channel activity (nanomolar). Similar observations for the effect on PTX on NKA have been explained by the different apparent affinities of the toxin for the various conformational states assumed by the pump during the normal transport cycle (3, 18). In particular, elevations of extracellular K\(^+\) or the absence of ATP at the cytoplasmic surface greatly reduced the apparent affinity of the NKA for PTX. Likewise, reducing H\(^+\) concentration (increasing pH) produced a dramatic 10-fold shift in the ability of MTX to both inhibit Ca\(^{2+}\)-ATPase and stimulate channel activity, suggesting that the E2-P form of the PMCA may have the highest affinity for MTX. Importantly, the pH sensitivity provides additional support for the hypothesis that PMCA pump ligands modulate MTX affinity.

Taken together, the results of the present study clearly demonstrate that the PMCA is a target for MTX and provide strong support for the hypothesis that MTX converts the PMCA from pump to channel mode of operation. Thus MTX may prove to be a useful tool to evaluate the molecular features of the PMCA translocation pore. More importantly, these results provide additional evidence for commonality between channels and transporters with respect to transport mechanisms. Although the list of transporters that can apparently function in channel mode is expanding (7), the physiological implications of the channel mode of operation for most transporters remains unknown. The fact that high-affinity toxins have evolved specifically to trigger channel mode raises the intriguing possibility that endogenous ligands with the same properties may also exist. Finally, it is important to note that there are thousands of P-type ATPase pump units in the surface membrane and endoplasmic reticulum of most cells. If only a small fraction of these were to function as channels, the consequences for cell function and homeostasis would be disastrous. Whether-or-not the channel mode of operation of the NKA, SERCA, or PMCA plays an important role in pathological cell death remains an interesting possibility for future investigations.

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Current address for M. Estacion: Department of Neurology, Yale University School of Medicine, New Haven, CT 06510.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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