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Attenuation of maitotoxin-induced cytotoxicity in rat aortic smooth muscle cells by inhibitors of Na^+/Ca^{2+} exchange, and calpain activation ☆

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ABSTRACT

The highly potent marine toxin maitotoxin (MTX) evoked an increase in cytosolic Ca^{2+} levels in fura-2 loaded rat aortic smooth muscle cells, which was dependent on extracellular Ca^{2+}. This increase was almost fully inhibited by KB-R7943, a potent selective inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger (NCX). Cell viability was assessed using ethidium bromide uptake and the alamarBlue cytotoxicity assay. In both assays MTX-induced toxicity was attenuated by KB-R7943, as well as by MDL 28170, a membrane permeable calpain inhibitor. Maitotoxin-evoked contractions of rat aortic strip preparations *in vitro*, which persist following washout of the toxin, were relaxed by subsequent addition of KB-R7943 or MDL 28170, either in the presence of, or following washout of MTX. These results suggest that MTX targets the Na^+/Ca^{2+} exchanger and causes it to operate in reverse mode (Na^+ efflux/Ca^{2+} influx), thus leading to calpain activation, NCX cleavage, secondary Ca^{2+} overload and cell death.

1. Introduction

Maitotoxin (MTX) is a potent marine toxin produced by the dinoflagellate *Gambierdiscus toxicus,* and is a principal toxin of ciguatera seafood poisoning (Yasumoto, 2000). The toxin evokes massive Ca^{2+} influx in excitable and non-excitable cells, which triggers phosphoinositide breakdown (Berta et al., 1988; Gusovsky et al., 1989; Soergel et al., 1992), arachidonic acid release (Choi et al., 1990), muscle contraction (Ohizumi and Yasumoto, 1983; Kobayashi et al., 1985), neurotransmitter release (Takahashi et al., 1982), and calpain activation (Wang et al., 1996; Zhao et al., 1999). MTX lacks ionophoretic activity (Takahashi et al., 1983), and does not release Ca^{2+} from intracellular stores (Gutierrez et al., 1997). Although the precise mechanisms of MTX cytotoxicity, and the Ca^{2+} entry pathways involved remain poorly understood, this toxin has been used as a unique pharmacological tool for research on calcium-dependent mechanisms and pathological mechanisms of necrosis (see for example Gusovsky and Daley, 1990). Recently, a MTX-induced cell death cascade has been proposed involving sequential changes in membrane permeability, including; (1) activation of a Ca^{2+}-permeable, non-selective cation channel (NSCC) that causes a large increase in intracellular Ca^{2+}; (2) opening of a cytolytic/oncolytic pore (COP) that permits molecules of <800 Da to enter the cell; and (3) formation of a glycine-sensitive lytic pore resulting in cell lysis and release of lactate dehydrogenase (Estacion et al., 2003; Wisnoskey et al., 2004).

Two concurrent mechanisms maintain cellular Ca^{2+} homeostasis: a high affinity, low capacity, Ca^{2+}-ATPase, which finely regulates free cytosolic Ca^{2+} $[Ca^{2+}]_i$ near its basal value of ~100 nM, and a low-affinity high-capacity

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Na^+–Ca^{2+} exchanger (NCX) that corrects more significant elevations in $[Ca^{2+}]_i$, such as those seen following vasoconstrictor or excitotoxic/cytolytic stimuli. Whereas Ca^{2+}-ATPase functions only as a Ca^{2+} extrusion system, NCX is a bidirectional ion transporter that exchanges Na^+ and Ca^{2+} in either a Ca^{2+} efflux, or influx mode, depending on membrane potential and transmembrane ion gradients. NCX plays a major role in regulating physiological and pathological responses to Ca^{2+} in a variety of cell types, including cultured rat vascular smooth muscle cells (Morel and Godfraind, 1984; Ashida and Blaustein, 1987; Nabel et al., 1988; Blaustein et al., 1992). In arterial smooth muscle, NCX is thought to contribute to Ca^{2+} extrusion from the cytosol in relaxation (Iwamoto, 2007). Two isoforms have been cloned in smooth muscle (Nakasaki et al., 1993) and RT-PCR and Western blot analyses have revealed mRNA and protein expression of NCX1 and NCX3 in cultured human pulmonary artery smooth muscle cells (Zhang et al., 2005).

The initial MTX trigger is thought to be activation of a NSCC, which is permeable to Na^+ and K^+, but has a low permeability to Ca^{2+} (Schilling et al., 1999; de la Rosa et al., 2007). MTX may also increase $[Ca^{2+}]_i$ via various other Ca^{2+} entry pathways following depolarization, including L-type voltage-sensitive Ca^{2+} channels (VSCCs), which are the predominant Ca^{2+} channel type in vascular smooth muscle (Sanders, 2001). Thus, the question arises as to whether the massive Ca^{2+} influx induced by MTX that results in cell death occurs via NSCCs, VSCCs, receptor-operated Ca^{2+}channels, NCX operating in reverse mode, or via some other as yet uncharacterized pathway. In addition to increasing $[Ca^+]_i$ levels, MTX also evokes Na^+ influx in several cell types, for example in HEK cells as demonstrated using the sodium-binding compound benzofuran isophalate (Schilling et al., 2006). Increased intracellular Na^+ levels would favor reversal of NCX to reverse mode (Ca^{2+} influx).

In the present study, we have used KB-R7943, a potent and selective inhibitor of the reverse mode of NCX (Iwamoto et al., 1996; Iwamoto, 2004, 2007), to assess the contribution of NCX to MTX-evoked Ca^{2+} influx. We have also used the calpain inhibitor MDL 28170, which prevents calpain-induced cleavage of NCX (Bano et al., 2005, 2007; Araujo et al., 2007). Our data offers strong evidence to support the view that NCX is a primary target of MTX, and that the toxin causes an early and rapid reversal of the NCX from Ca^{2+} efflux (forward mode) to Ca^{2+} influx (reverse mode), effectively converting NCX into a Ca^{2+} entry pathway. Furthermore, MTX also activates calpain, a protease that cleaves various substrates including NCX.

2. Material and methods

2.1. Drugs

All salts and reagents used were of analytical grade and were obtained from Sigma (St. Louis, MO). MTX purchased from Wako (Richmond, VA) was dissolved in methanol and stored at −20 °C. Ethidium bromide (Sigma) was dissolved in distilled H_2O. 2-[2-[4-(4-Nitrobenzyloxy) phenyl] ethyl] isothiourea mesylate (KB-R7943, Tocris, Ellisville, MO), carbobenzoxy-valinyl-phenyl-phenylalaninal, MDL 28170 (Calpain inhibitor III, Calbiochem, La Jolla, CA), and nifedipine (Sigma) were dissolved in DMSO and aliquots were stored at −20 °C until use. Vehicle to buffer concentrations did not exceed 0.1%.

2.2. Rat aortic smooth muscle cell culture

Rat aortic smooth muscle cells (Cell Applications Inc., San Diego, CA) were cultured in rat smooth muscle cell growth medium augmented with 10 IU/ml penicillin, 10 μg/ml, streptomycin (Sigma) and 0.25 μg/ml Fungizone (Gibco) in 75 cm^2 tissue culture flasks (Corning) in a humidified 5% CO_2 atmosphere until ready to sub-culture or harvest. Cells at 60–80% confluence between passages 10 and 15 were used for assays.

2.3. Changes in plasma membrane permeability

MTX-evoked increases in membrane permeability were measured using the vital dye ethidium bromide. This dye, which is normally excluded from the cell cytoplasm, gains access to the cell interior following pore formation where it binds to nucleic acids resulting in increased fluorescence. Rat aortic smooth muscle cells (5×10^5 cells/ml) were incubated in a mechanically stirred 2 ml cuvette at 37 °C in HEPES-buffered saline pH 7.4 containing (mM) NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, D-glucose 10, and HEPES 10, containing 10 μM ethidium bromide and challenged with MTX in the presence or absence of inhibitors. Fluorescent changes were measured using a Deltascan fluorimeter and Felix 32 software (Photon Technology International, Trenton, NJ) at excitation and emission wavelengths of 360 and 580 nm, respectively. After baseline extrapolation by linear fit and its subtraction, responses were normalized and expressed as a percent of the response to complete cell permeabilization by digitonin 10 μM.

2.4. Microscopic imaging of ethidium bromide uptake

Rat aortic smooth muscle cells were seeded on collagen-coated 25 mm round glass cover slips (Harvard Apparatus, Quebec, Canada) at a density of 1×10^5 cells per cover slip and allowed to equilibrate overnight before treatment. On the day of treatment, cells were pre-incubated with fresh medium or medium containing 10 μM KB-R7943 or MDL 28170 for 30 min. After incubation, the cover slips were transferred to a holder for microscopic imaging. Ethidium bromide (1 μg/ml) was added prior to image acquisition. Collection of data was performed using a Wave-FX spinning disk confocal microscope (Quorum Technologies, Guelph, Canada) with a laser excitation wavelength of 491 nm and an emission filter of 593 nm at a magnification of 400 ×. Images were collected at 5 min intervals for 30 min, with MTX being added after the first time point.

2.5. alamarBlue cytotoxicity assay

To assess the toxicity of MTX on rat aortic smooth muscle, cells were seeded in 12 well culture plates and treated at 60–80% confluence with various concentrations of MTX diluted in medium only, or in medium containing inhibitors for 30 min at 37 °C, after which it was aspirated and replaced with fresh medium. Cells were returned to the incubator for 24 h, and then assessed for viability using the alamarBlue (AccuMed International Inc., Westlake, OH) cytotoxicity assay. Dye was added to cultures (10% v/v) and incubated for 3–4 h. The supernatant was transferred to a 96 well titer plate and fluorescence was measured on a BioTek FL600 micro plate fluorescence reader using an excitation wavelength of 530–560 nm and an emission wavelength of 590 nm. Cell viability was calculated and plotted as percent of control cells.

2.6. Contractility studies

In conducting this research, the authors adhered to the "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation" published by the Canadian Council on Animal Care. Aortic strips were prepared from the thoracic aorta of male Sprague-Dawley rats (150–200 g) according to the method described by Furchgott (1960) and mounted under 1 g of tension in 5 ml organ baths containing Krebs-Henseleit solution of the following composition (mM); NaCl 116, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, maintained at 37 °C and continuously aerated with 95% O_2/5% CO_2. Ca^{2+} free Krebs was prepared by omitting Ca^{2+} and adding EGTA 0.1 mM. One end of each tissue preparation was anchored, and the other attached by thread to a Harvard apparatus (South Natick, MA) smooth muscle transducer for auxotonic recording. Tissues were allowed to equilibrate for 60 min prior to evoking drug responses, and all strips were initially challenged with K^+ 60 mM to test for viability. Responses to drugs were displayed on an MP150 system (BIOPAC Systems Inc., Santa Barbara, CA).

2.7. Measurement of calcium uptake

Cells were harvested, counted and re-suspended at a density of 5×10^5 cells/mL in a HEPES buffer solution containing (mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1, HEPES 10 and D-glucose 10, pH adjusted to 7.4 with NaOH. Various concentrations of KB-R7943 (1, 3, 5, and 10 μM) were added to the cells prior to calcium uptake measurement. The fluorescent indicator fura-2 AM (Molecular Probes Inc., Eugene, OR 3 μM) was added and cells incubated at 37 °C in 5% CO_2 for 30 min. Cells were spun down, washed in fresh HEPES buffer in the presence or absence of KB-R7943, incubated for an additional 15 min, spun down and re-suspended once more. Fluorescence was recorded in a mechanically stirred cuvette using excitation wavelengths alternating between 340 and 380 nm, with an emission wavelength of 510 nm. The ratio between excitation and emission was charted, with 50 pM MTX added at 600 s, 10 μM digitonin added at 1500 s, and 4.5 mM EGTA/NaOH added at 1700 s.

2.8. Data analysis

All experiments were carried out in cells from at least 3 independent cultures, and results are presented as means ±SEM. EC_{50} values were calculated using Graph Pad Prism version 5.00 for Windows (Graph Pad Software San Diego, CA), using non-linear regression and variable slope. Statistical evaluations were performed with the Students *t*-test. Differences between means were considered significant at P values <0.05.

3. Results

3.1. Fura 2-AM studies

Fig. 1 illustrates the rapid MTX-evoked influx of Ca^{2+} in cultured rat aortic smooth muscle cells pre-loaded with the fluorescent indicator fura-2 AM. KB-R7943, an inhibitor of NCX-mediated Ca^{2+} entry attenuated MTX

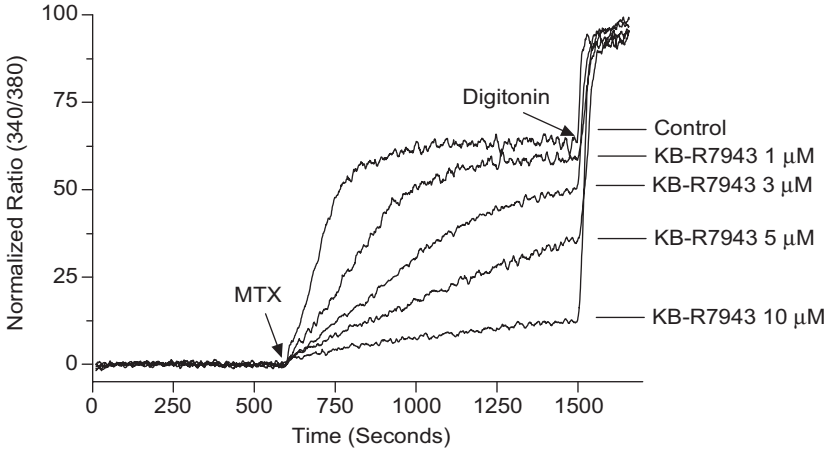

Fig. 1. The effect of KB-R7943 on MTX-evoked Ca^{2+} influx in cultured rat aortic smooth muscle cells loaded with fura-2 AM. Cells were challenged with MTX (50 pM) and digitonin 10 μM as indicated by arrows. Five representative traces from the same cell suspension are shown superimposed.

(50 pM)-evoked Ca^{2+} influx in a concentration-dependent manner with an IC_{50} of $2.5 \pm 0.5\,\mu M$.

3.2. Ethidium bromide influx: fluorimetry

MTX evoked a calcium-dependent, biphasic influx of ethidium bromide in rat aortic smooth muscle cells, in a manner similar to that reported previously using CHO-K1 cells (Lundy et al., 2004). Near-maximal influx was attained 30 min following the addition of 100 pM of MTX, and was sensitive to the receptor-mediated Ca^{2+} entry blocker SK&F 96365, and insensitive to the dihydropyridine L-type VSCC inhibitor nifedipine (1–10 μM, results not shown). MTX-evoked ethidium bromide uptake was reduced following a 15 min pretreatment and in the continued presence of KB-R7943, an inhibitor of NCX-mediated Ca^{2+} influx (Fig. 2a). A similar, but less effective reduction in ethidium bromide uptake was obtained with the calpain inhibitor, MDL 28170 (Fig. 2b).

3.3. Ethidium bromide influx: microscopy

Microscopic images of live cultured rat aortic smooth muscle cells in untreated and MTX (100 pM)-treated medium in the presence and absence of KB-R7943 10 μM or MDL 28170 (10 μM) are shown in Figs. 3 and 4,

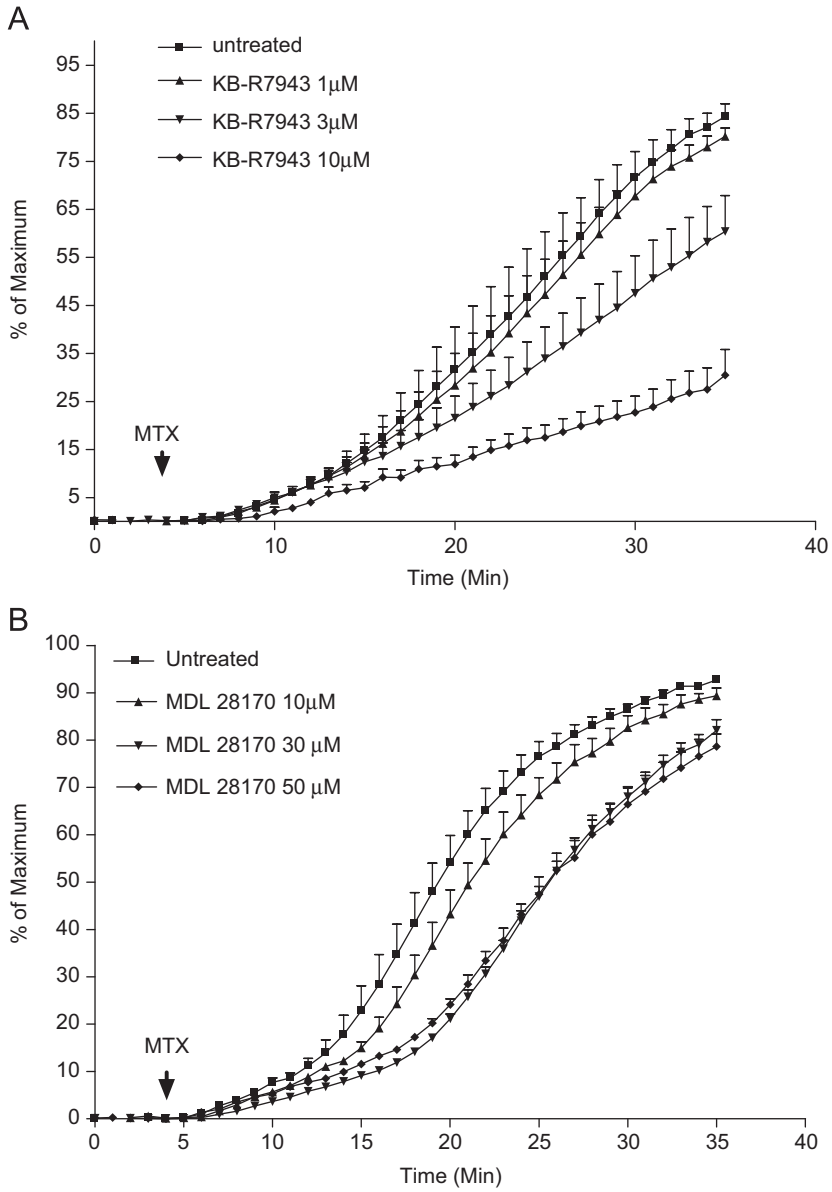

Fig. 2. Inhibitory effects of KB-R7943 (A) and MDL 28170 (B) on MTX-evoked ethidium bromide uptake in cultured rat aortic smooth muscle cells. MTX 100 pM was added as indicated (↓), both in the absence or following a 15 min pretreatment and in the continued presence of the inhibitors at the concentrations indicated. Following a 30 min MTX exposure digitonin 10 μM was added to determine maximal response. Each data point represents the mean ± SEM ($n = 4$).

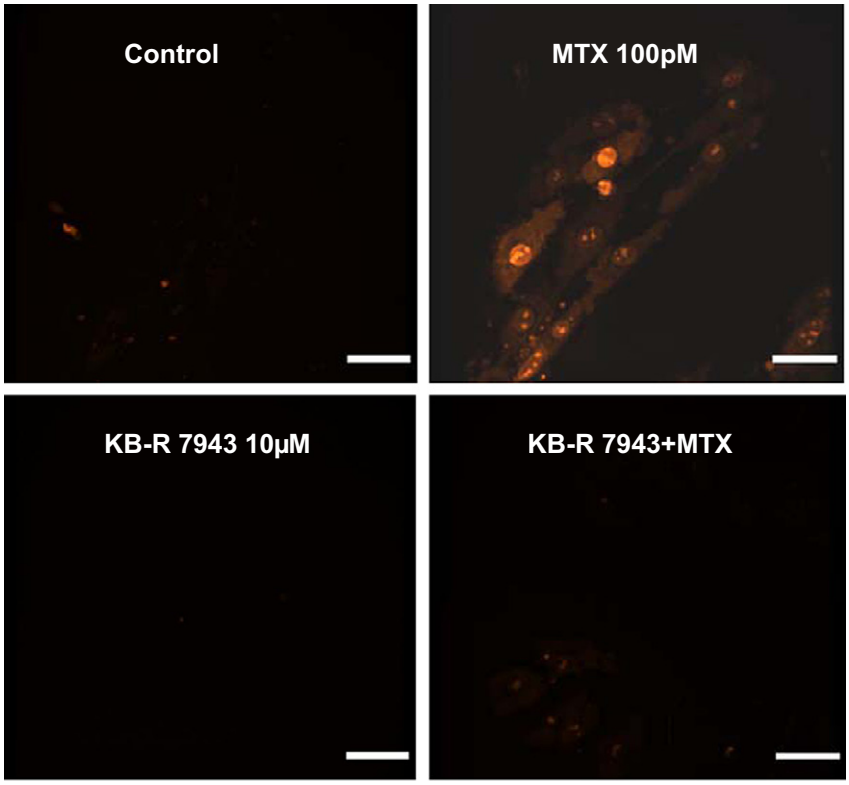

Fig. 3. Effect of the reverse mode NCX inhibitor KB-R7943 on MTX-evoked ethidium bromide uptake in cultured rat aortic smooth muscle cells. Cells were preincubated in media containing KB-R7943 10 μM at 37 °C for 30 min and challenged with MTX 100 pM. Confocal microscopy images of live cells were taken at 5 min intervals for 30 min. The images were obtained following a 30 min MTX exposure, and were corrected by subtracting background exposure at time zero. Scale bar = 20 μm.

respectively. Ethidium bromide fluorescence was clearly visible following a 30 min exposure to MTX, and was greatly attenuated following KB-R7943 or MDL 28170 pretreatment. In the absence of MTX, no fluorescence was observed following KB-R7943 or MDL 28170 treatment.

3.4. alamarBlue cytotoxicity

The viability of cultured rat aortic smooth muscle cells following exposure to various concentrations of MTX, and the protection afforded by KB-R7943 or by MDL 28170 (3–10 μM) were examined using the alamarBlue assay. The MTX concentration response curve was shifted to the right in a concentration-dependent manner suggesting a protective effect by KB-R7943 (Fig. 5a), as well as by MDL 28170 (Fig. 5b). In Fig. 5a, the control MTX EC_{50} (pM) of 57.7 ± 3.5, was increased to 170.0 ± 34.0 in the presence of KB-R7942 (3 μM), and 175.0 ± 25 in the presence of KB-R7943 (10 μM). In Fig. 5b, the control MTX EC_{50} of 70.5 ± 5.0, was increased to 135.6 ± 15.4 in the presence of MDL 28170 (3 μM), and 141.8 ± 10.2 in the presence of MDL 28170 (10 μM).

3.5. Contractility studies

Ca^{2+}-dependent, MTX-evoked contractions (tension changes) of rat aortic strips failed to relax/return to baseline following washout of MTX. This contractile state persisted despite repeated changes of the bath fluid for up to 180 min. Addition of KB-R7943 (10–30 μM) or MDL 28170 (10–30 μM) to MTX-contracted rings relaxed the tissues both in the continued presence of the toxin, or following its washout. Typically, the inhibitors caused relaxation when added after the MTX response had reached a plateau. Following repeated changes of bath fluid for 180 min, the strips were re-exposed to the inhibitors, which again relaxed the strips (in the absence of MTX). Finally, following a further 60 min wash, addition of $NaNO_2$ indicated a persistent contractile state. Typical experiments with KB-R7943 and MDL 28170 are shown in Fig. 6.

4. Discussion

In this study, we examined the effects of KB-R7943, a potent, selective inhibitor of the reverse mode of NCX, and those of MDL 28170, a cell permeable calpain inhibitor, on MTX-induced toxicity in smooth muscle cells. In fura-2 loaded cells, KB-R7943 at micromolar concentrations almost completely blocked Ca^{2+} influx, indicating that NCX in the reverse mode is an early and rapid entry pathway for MTX-evoked Ca^{2+} influx, and that NCX is a primary target of MTX in these cells. A calculated EC_{50} of 2.5 μM for KB-7943 is in agreement with the previously

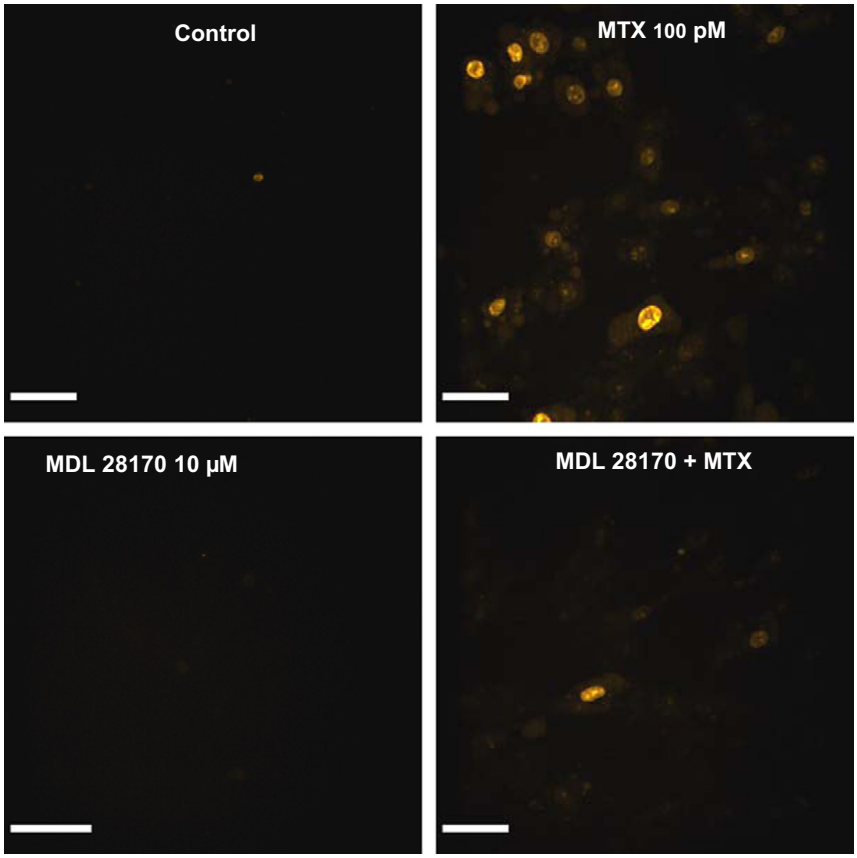

Fig. 4. Effect of the calpain inhibitor, MDL 28170, on MTX-evoked ethidium bromide uptake in cultured rat aortic smooth muscle cells. Cells were preincubated in medium containing MDL 28170 10 μM at 37 °C for 30 min and challenged with MTX 100 pM. Confocal microscopy images of live cells were taken at 5 min intervals for 30 min. The images were obtained following a 30 min MTX exposure, and were corrected by subtracting background exposure at time zero. Scale bar = 20 μm.

reported values for a variety of cell types (Iwamoto et al., 1996). Although L-Type VSCCs predominate in vascular smooth muscle (Sanders, 2001), the lack of effect of nifedipine suggests that entry via L-type VSCCs does not contribute to MTX-evoked Ca^{2+} and/or ethidium bromide influx in our experiments. In some cell types, MTX-induced Ca^{2+} influx has been reported to be at least partially blocked by dihydropyridine L-type blockers (Gusovsky and Daley, 1990), while in others neither nifedipine, agatoxinVIA, conotoxin GVIA, nor conotoxin MVIIC, which inhibit a broad spectrum of VSCC types, do not inhibit MTX-induced Ca^{2+} influx. In cells that are insensitive to VSCC inhibition, it has been suggested that Ca^{2+} may enter via voltage-independent non-selective cation channels (Satoh et al., 2001). In our studies using cultured rat aortic smooth muscle cells, MTX-induced Ca^{2+} influx and ethidium bromide uptake are dihydropyridine insensitive, suggesting that toxic Ca^{2+} entry does not occur via VSCCs, and that other Ca^{2+} entry pathways/mechanisms are operative. Sattler et al. (1998) have shown that Ca^{2+} loads entering through VSCCs in cultured cortical neurons were non-toxic as compared with similar loads entering via NCX. This finding is in accord with the "source-specific hypothesis", which proposes that the route by which Ca^{2+} ions gain access to the cytosol is the main determinant of Ca^{2+} toxicity (Sattler and Tymianski, 2000).

MTX induces a Ca^{2+}-dependent, biphasic uptake of ethidium bromide into a variety of cell types. The initial phase reflects activation of large "cytolytic/oncolytic pores" (COP) and typically occurs 2–3 min following MTX addition. The second phase occurs after a further 10–15 min, reflects cell lysis and is accompanied by LDH release (Schilling et al., 1999; Estacion et al., 2003; Lundy et al., 2004). We report here that COP are present in cultured aortic smooth muscle cells and are activated by MTX in a manner similar to that previously described in other cell types (Schilling et al., 1999; Estacion et al., 2003; Lundy et al., 2004). In addition to MTX-evoked Ca^{2+} influx, cell death as measured by both ethidium bromide uptake and the alamarBlue cytotoxicity assay was inhibited in a concentration-dependent manner by KB-R7943. This suggests that NCX in reverse mode was the entry pathway for the Ca^{2+} required to initiate ethidium bromide uptake into smooth muscle cells, resulting in cell death. Previous reports have shown that NCX reverse mode is activated by reducing extracellular Na^+ levels. If MTX activates NCX reverse mode, then lower extracellular Na^+ levels should potentiate MTX-induced Ca^{2+} entry. Indeed, Morales-Tlalpan and Vaca (2002) have examined the modulation

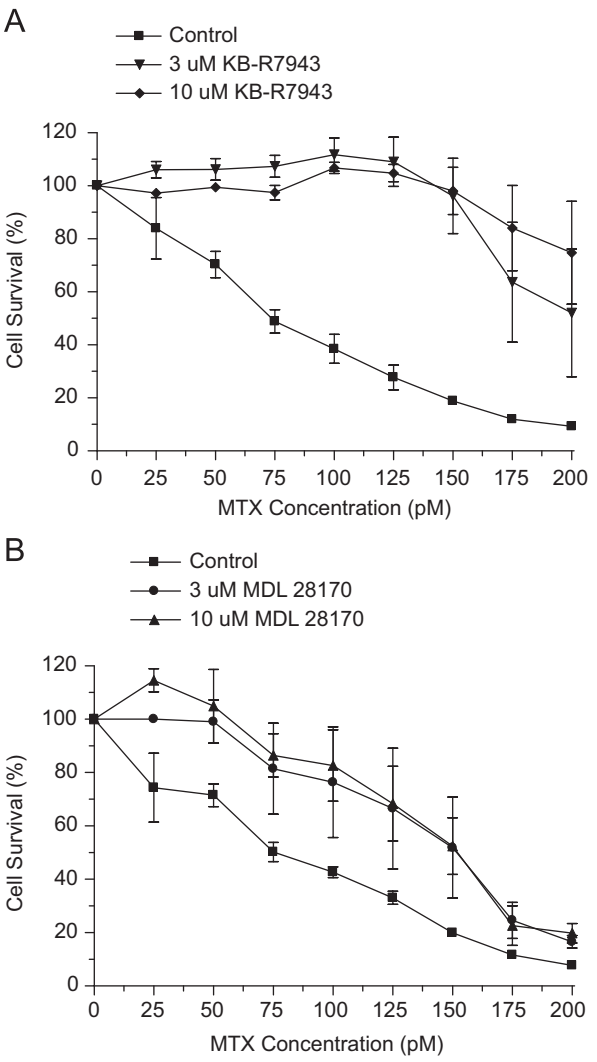

Fig. 5. The effect of KB-R7943 (A) and MDL 28170 (B) on MTX-induced cytotoxicity in rat aortic smooth muscle cultures. Cell cultures were treated with varying concentrations of MTX in culture medium containing 0, 3, or 10 μM of the inhibitors. After 30 min the culture medium was replaced with drug-free culture medium. Cell survival was determined at 24 h using the alamarBlue cytotoxicity assay. Results represent the mean ± SEM of 3 separate experiments.

of the MTX response by extracellular cations, including the role of extracellular Na^+ on the activation of MTX-induced Ca^{2+} influx. Ca^{2+} influx was shown to be markedly potentiated in the absence of extracellular Na^+, as compared with 145 mM Na^+ using fura-2 loaded CHO cells, a finding that supports Ca^{2+} entry via NCX.

KB-R7943 at 10 μM was reported to have no effect on other ion transporters, including Na^+/H^+ exchange, sarcolemmal and SR Ca^{2+} ATPases, Na^+, K^+ ATPase, and dihydropyridine sensitive Ca^{2+} uptake (Iwamoto et al., 1996; Iwamoto, 2004, 2007).

MTX-activated Ca^{2+}-dependent cysteine proteases referred to as calpains, are associated with cellular necrosis (Wang et al., 1996; Hajimohammadreza et al., 1997; Zhao et al., 1999). Ca^{2+}-activated calpains cleave NCX and thus inhibit its capability to remove calcium accumulated by excitotoxic/cytotoxic stimuli (Bano et al., 2005, 2007). Calpain inhibition in cells requires the use of membrane permeable inhibitors such as MDL 28170. MTX-induced ethidium bromide uptake was inhibited by micromolar concentrations of MDL 28170, which was, however, not as effective as, and had a slower onset than KB-R-7943 (see Fig. 6). A modest inhibition of cell death by calpain inhibition in other cell types suggests that multiple pathways may be involved in MTX-induced cytotoxicity (Zhao et al., 1999). Our results suggest that MTX-induced calpain activation in rat aortic smooth muscle is mediated by Ca^{2+} influx via reversal of NCX since the initial Ca^{2+} entry was almost fully inhibited by KB-R7943. This view is supported by a recent study using hippocampal neurons in which AMPA-receptor-mediated neurodegeneration via calpain activation involved Ca^{2+} entry via NCX in reverse mode, a scenario in which the exchanger, by serving as a pathway for the calpain activator Ca^{2+}, initiates its own demise (Araujo et al., 2007). In rat spinal cord white matter, KB-R7943 prevented the increase in calpain-mediated spectrin break down products induced by anoxia (Li et al., 2000), adding further support to the view that calpain activator Ca^{2+} enters via the reverse mode of NCX. NCX is closely coupled to other primary and secondary active transporters including, for example, the Na^+/H^+ exchanger, whose activation would cause increased intracellular sodium $[Na^+]_i$ and Ca^{2+} influx via reversal of NCX. Interestingly, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) an inhibitor of the Na^+/H^+ exchanger also inhibits MTX-induced ethidium bromide uptake in rat aortic smooth muscle cells suggesting it too is targeted by MTX (unpublished results). Furthermore, Schilling et al. (2006) have proposed that the primary active transporter Na^+ K^+ATPase in the presence of palytoxin, functions as a cation channel, with a resulting increase in $[Ca^{2+}]_i$ entering via the plasmalemmal Na^+ pump. It is possible that palytoxin and MTX share similar actions on Na^+ K^+ ATPase, and on other ion transporters including NCX.

Contractile tension in arterial rings is effectively modulated by procedures influencing Ca^{2+} influx or efflux via NCX (Ashida and Blaustein, 1987). Vascular smooth muscle relaxation results from a decrease in $[Ca^{2+}]_i$ as a result of efflux via NCX in forward mode (Iwamoto, 2007). MTX-induced contractions of isolated vascular tissue do not relax despite repeated washout of the toxin (Ohizumi and Yasumoto, 1983; Dubyak, 1986; Berta et al., 1988), suggesting that the normally operative Ca^{2+} extrusion mechanisms are unable to restore Ca^{2+} homeostasis. Inhibition of Ca^{2+} extrusion due to reverse mode NCX and/or NCX proteolysis may explain why MTX-induced contractions are persistent and irreversible following washout of the toxin. Our finding that MTX-contracted rat aortic strip preparations were relaxed by the addition of KB-R7943 is consistent with the view that blockade of Ca^{2+}entry via MTX-induced reversal of NCX allows intracellular buffering mechanisms to restore the overall balance between entry and buffering of the Ca^{2+}load, thus facilitating relaxation. MDL 28170 inhibition of calpain-induced NCX proteolysis would be expected to achieve a similar endpoint.

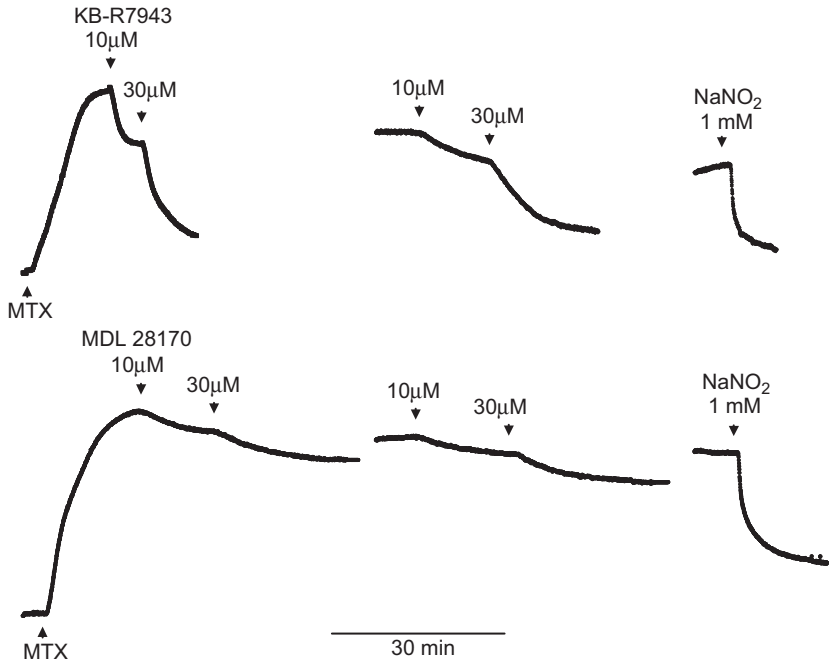

Fig. 6. KB-R7943 and MDL 28170 relax MTX-contracted rat aortic strips both in the presence of, and following washout of MTX. Top, a typical record showing from left to right; a strip contracted by MTX 100 pM and exposed to KB-R7943 (10 and 30 μM added cumulatively); strip re-exposed to KB-R7943 180 min after washout of MTX, and; after a further 60 min wash, addition of the smooth muscle relaxant $NaNO_2$ 1 mM. Bottom record, protocol repeated on another MTX-contracted strip using MDL 28170.

It is generally accepted that a rapidly developing Ca^{2+}conductance through the voltage-independent NSCC is the initial event triggering downstream biochemical events in the MTX cell death cascade (Wisnoskey et al., 2004; de la Rosa et al., 2007). Our results indicate that MTX-induced reversal of NCX contributes to a rapid entry of Ca^{2+}at a very early stage following binding of the toxin, and triggers the cell death cascade. Thus blockade of NCX reversal may provide a therapeutic target for ciguatera poisoning.

References

Ashida, T., Blaustein, M.P., 1987. Regulation of cell calcium and contractility in mammalian arterial smooth muscle: the role of the sodium–calcium exchange. J. Physiol. 392, 617–635.

Araujo, I.M., Carreira, B.P., Pereira, T., Santos, P.F., Soulet, D., Inacio, A., Bahr, B.A., Carvalho, A.P., Ambrosio, A.F., Carvalho, C.M., 2007. Changes in calcium dynamics following the reversal of the sodium–calcium exchanger have a key role in AMPA receptor mediated neurodegeneration via calpin activation in hippocampal neurons. Cell Death Different. 14 (9), 1635–1646.

Bano, D., Young, K.W., Guerin, C.J., LeFeuvre, R., Rothwell, N.J., Naldini, L., Rizzuto, R., Carafoli, E., Nicotera, P., 2005. Cleavage of the plasma membrane Na^+/Ca^{2+} exchanger in excitotoxicity. Cell 120, 275–285.

Bano, D., Munarriz, E., Chen, H.L., Ziviani, E., Lippi, G., Young, K.W., Nicotera, P., 2007. The plasma membrane Na^+/Ca^{2+} exchanger is cleaved by distinct protease families in neuronal cell death. Ann. NY Acad. Sci. 1099, 451–455.

Berta, P., Phaneuf, S., Derancourt, J., Cassanova, J., Durand-Clement, M., le Peuch, C., Haiech, J., Cavadore, J.C., 1988. The effect of maitotoxin on phosphoinositides and calcium metabolism in a primary culture of aortic smooth muscle cells. Toxicon 26, 133–141.

Blaustein, M.P., Ambesi, A., Bloch, R.J., Goldman, W.F., Juhaszova, M., Lindenmayer, G.E., Weiss, D.N., 1992. Regulation of vascular smooth muscle contractility: roles of the sarcoplasmic reticulum (SR) and the sodium/calcium exchanger. Jpn. J. Pharmacol. 58 (Suppl. 2), 107P–114P.

Choi, O.H., Padgett, W.L., Nishizawa, Y., Gusovsky, F., Yasumoto, T., Daly, J.W., 1990. Maitotoxin effects on calcium channels, phosphoinositide breakdown, and arachidonic acid release in pheochromocytoma PC12 cells. Mol. Pharmacol. 37, 222–230.

de la Rosa, L.A., Parrilla, A-P., Martinez-Martinez, A., 2007. Biochemistry of maitotoxin. In: In: Botana, L.M. (Ed.), Phycotoxins Chemistry and Biochemistry. Blackwell Publishing, Ames, IA, pp. 55–73.

Dubyak, G.E., 1986. Extracellular ATP activates phosphoinositide breakdown and Ca^{2+} mobilization in ehrlich ascites tumor cells. Arch. Biochem. Biophys. 245, 84–95.

Estacion, M., Weinberg, J.S., Sinkins, W.G., Schilling, W.P., 2003. Blockade of maitotoxin-induced endothelial cell lysis by glycine and L-alanine. Am. J. Physiol. 284, C1006–C1020.

Furchgott, R.F., 1960. Spiral cut strips of rabbit aorta for in vitro studies of responses of arterial smooth muscle. Methods Med. Res. 8, 177–186.

Gusovsky, F., Daley, J.W., 1990. Maitotoxin: a unique pharmacological tool for research on calcium-dependent mechanisms. Biochem. Pharmacol. 39, 633–1639.

Gusovsky, F., Yasumoto, ., Daly, J.W., 1989. Maitotoxin, a potent, general activator of phosphoinositide breakdown. FEBS Lett. 243, 307–312.

Gutierrez, D., Diaz de Leon, L., Vaca, L., 1997. Characterization of the maitotoxin-induced influx pathway from human skin fibroblasts. Cell Calcium 22, 31–38.

Hajimohammadreza, I., Raser, K.J., Nath, R., Nadimpalli, R., Scott, E.W., Wang, K.K., 1997. Neuronal nitric oxide synthesis and calmodulin-dependent protein kinase II alpha undergo neurotoxin-induced proteolysis. J. Neurochem. 69, 1006–1013.

Iwamoto, T., 2004. Forefront of Na^+/Ca^{2+} exchanger studies: molecular pharmacology of Na^+/Ca^{2+} exchange inhibitors. J. Pharmacol. Sci. 96, 27–32.

Iwamoto, T., 2007. Na^+/Ca^{2+} exchange as a drug target-insight from molecular pharmacology and genetic engineering. Ann. NY Acad. Sci. 1099, 516–528.

Iwamoto, T., Watano, T., Shigekawa, M., 1996. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J. Biol. Chem. 271, 22391–22397.

Kobayashi, M., Ohizumi, Y., Yasumoto, T., 1985. The mechanism of action of maitotoxin in relation to Ca^{2+} movements in guinea pig and rat cardiac muscles. Br. J. Pharmacol. 86, 385–391.

Li, S., Jiang, Q., Stys, P.K., 2000. Important role of reverse Na^+/Ca^{2+} exchange in spinal cord white matter injury at physiological temperatures. J. Neurophysiol. 84, 1116–1119.

Lundy, P.M., Nelson, P.M., Mi, L., Frew, R., Minaker, S., Vair, C., Sawyer, T.W., 2004. Pharmacological differentiation of the $P2\times_7$ receptor and the maitotoxin-activated cationic channel. Eur. J. Pharmacol. 487, 17–28.

Morales-Tlalpan, V., Vaca, L., 2002. Modulation of the maitotoxin response by intracellular and extracellular cations. Toxicon 40, 493–500.

Morel, N., Godfraind, T., 1984. Sodium/calcium exchange in smooth muscle microsomal fractions. Biochem. J. 218, 421–427.

Nabel, E.G., Berk, B.C., Brock, T.A., Smith, T.W., 1988. Na^+/Ca^{2+} exchange in cultured vascular smooth muscle cells. Circ. Res. 62, 486–493.

Nakasaki, Y., Iwamoto, T., Hanada, H., Imagawa, T., Shigekawa, M., 1993. Cloning of the rat aortic smooth muscle Na^+/Ca^{2+} exchanger and tissue specific expression of isoforms. J. Biochem. 114, 528–534.

Ohizumi, Y., Yasumoto, T., 1983. Contractile response of the rabbit aorta to maitotoxin, the most potent marine toxin. J. Physiol. 327, 711–731.

Sanders, K.M., 2001. Signal transduction in smooth muscle invited review: mechanisms of calcium handling in smooth muscles. J. Appl. Physiol. 91, 1438–1449.

Satoh, E., Ishii, T., Niishimura, M., 2001. The mechanism of maitotoxin-induced elevation of the cytosolic free calcium level in rat cerebrocortical synaptosomes. Jpn. J. Pharmacol. 85, 98–100.

Sattler, R., Tymianski, M., 2000. Molecular mechanisms of calcium-dependent excitotoxicity. J. Mol. Med. 78, 3–13.

Sattler, R., Charlton, M.P., Hafner, M., Tymianski, M., 1998. Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. J. Neurochem. 71, 2349–2364.

Schilling, W.P., Wasylyna, T., Dubyak, G.R., Humphreys, B.D., Sinkins, W.G., 1999. Maitotoxin and $P2Z/P2\times_7$ purinergic receptor stimulation activates a common cytolytic pore. Am. J. Physiol. 277, C766–C776.

Schilling, W.P., Snyder, D., Sinkins, W.G., Estacion, M., 2006. Palytoxin-induced cell death cascade in bovine aortic endothelial cells. Am. J. Physiol. Cell Physiol. 291, 657–667.

Soergel, D.G., Yasumoto, T., Daley, J.W., Gusovsky, F., 1992. Maitotoxin effects are blocked by SK&F 96365, an inhibitor of receptor-mediated calcium entry. Mol. Pharmacol. 41, 487–493.

Takahashi, M., Ohizumi, Y., Yasumoto, T., 1982. Maitotoxin, a Ca^{2+} channel activator candidate. J. Biol. Chem. 257, 7287–7289.

Takahashi, M., Tatsumi, M., Ohizumi, Y., Yasumoto, T., 1983. Ca^{2+} channel activating function of maitotoxin , the most potent marine toxin known, in clonal rat pheochromocytoma cells. J. Biol. Chem. 258, 10944–10949.

Wang, K.K., Nath, R., Raser, K.J., Hajimohammadreza, I., 1996. Maitotoxin induces calpain activation in SH-SY5Y neuroblastoma cells and cerebrocortical cultures. Arch. Biochem. Biophys. 331, 208–214.

Wisnoskey, B.J., Estacion, M., Schilling, W.P., 2004. Maitotoxin-induced cell death cascade in bovine aortic endothelial cells: divalent cation specificity and selectivity. Am. J. Physiol. Cell Physiol. 287, C345–C356.

Yasumoto, T., 2000. In: Botana, L.M. (Ed.), Historic Considerations Regarding Seafood Safety, pp. 1–17.

Zhang, S., Yaun, J.X.-J., Barrett, K.E., Dong, H., 2005. Role of NA^+/Ca^{2+} exchange in regulating cytosolic Ca^{2+} in cultured human pulmonary artery smooth muscle cells. Am. J. Physiol. Cell Physiol. 228, C245–C252.

Zhao, X., Pike, B.R., Newcombe, J.K., Wang, K.K.W., Postmantur, R.M., Hayes, R.L., 1999. Maitotoxin induces calpain but not caspase-3 activation and necrotic cell death in primary septo-hippocampal cultures. Neurochem. Res. 24, 371–382.