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Ca^{2+} release induced by myotoxin *a*, a radio-labellable probe having novel Ca^{2+} release properties in sarcoplasmic reticulum

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1 Myotoxin *a* (MYTX), a polypeptide toxin purified from the venom of prairie rattlesnakes (*Crotalus viridis viridis*) induced Ca^{2+} release from the heavy fraction (HSR) but not the light fraction of skeletal sarcoplasmic reticulum at concentrations higher than 1 μM, followed by spontaneous Ca^{2+} reuptake by measuring extravesicular Ca^{2+} concentrations using the Ca^{2+} electrode.

2 The rate of $^{45}Ca^{2+}$ release from HSR vesicles was markedly accelerated by MYTX in a concentration-dependent manner in the range of concentrations between 30 nM and 10 μM, indicating the most potent Ca^{2+} releaser in HSR.

3 The Ca^{2+} dependency of MYTX-induced $^{45}Ca^{2+}$ release has a bell-shaped profile but it was quite different from that of caffeine, an inducer of Ca^{2+}-induced Ca^{2+} release.

4 $^{45}Ca^{2+}$ release induced by MYTX was remarkable in the range of pCa between 8 and 3, whereas that by caffeine was prominent in the range of pCa, i.e., between 7 and 5.5.

5 MYTX-induced $^{45}Ca^{2+}$ release consists of both early and late components. The early component caused by MYTX at low concentrations (30–300 nM) completed within 20 s, while the late component induced by it at higher concentrations (>0.3 μM) was maintained for at least 1 min.

6 Both the components were almost completely inhibited by inhibitors of Ca^{2+} release such as Mg^{2+}, ruthenium red and spermine.

7 $^{45}Ca^{2+}$ release induced by caffeine or β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) was completely inhibited by high concentrations of procaine. Procaine abolished the early component but not the late one, suggesting that at least the early component is mediated through Ca^{2+}-induced Ca^{2+} release channels.

8 On the basis of these results, the character of Ca^{2+} release induced by MYTX was quite different from that caused by caffeine or AMP-PCP, suggesting that MYTX induces Ca^{2+} release having novel properties in HSR. MYTX is the first polypeptide Ca^{2+} inducer and has become a useful pharmacological tool for clarifying the mechanism of Ca^{2+} release from skeletal muscle SR.

Keywords: Myotoxin *a*; skeletal muscle; sarcoplasmic reticulum; Ca^{2+} release; caffeine; procaine; excitation-contraction coupling

Introduction

The contractile state of skeletal muscle is determined by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Rüegg, 1986). Muscle cells maintain a high gradient of Ca^{2+} across not only the plasma membrane but also the sarcoplasmic reticulum membrane (SR) (Endo, 1977; Martonosi, 1984). SR has an ATP-dependent Ca^{2+} pump that accumulates Ca^{2+} into its lumen to reduce $[Ca^{2+}]_i$ below 0.1 μM and to maintain the Ca^{2+} gradient (Ebashi, 1991). It has been generally accepted that excitation of the plasma membrane evokes the depolarization of the transverse tubular membrane that leads to Ca^{2+} release from SR through a putative Ca^{2+} release channel (Schneider, 1981; Block *et al.*, 1988). This is a major process in excitation-contraction coupling. One possible candidate for the machinery of the physiological process is the Ca^{2+}-induced Ca^{2+} release channels that have been recently purified by using the plant alkaloid ryanodine, as a biochemical probe and extensively characterized (Hymel *et al.*, 1988; Smith *et al.*, 1988; Lai *et al.*, 1988; Saito *et al.*, 1988; Wagenknecht *et al.*, 1989). Application of specific drugs that affect the Ca^{2+} releasing mechanism is a useful approach to achieve a better understanding of the molecular mechanism of this release. Ryanodine (McPherson & Campbell, 1993) and 9-methyl-7-bromoeudistomin D (MBED), the most powerful caffeine-like Ca^{2+} releaser (Seino *et al.*, 1991; Fang *et al.*, 1993) have provided us with useful information. However, little information has accumulated about the physiological mechanism of the signal transduction between the transverse tubular system and SR (Schneider & Chandler, 1973; Schneider, 1981).

Myotoxin *a* (MYTX) purified from the venom of prairie rattlesnakes (*Crotalus viridis*) is a muscle toxic polypeptide composed of 42 amino acids (Fox *et al.*, 1979). Electron microscopic study has revealed that MYTX causes muscle degeneration and disturbance of the endoplasmic reticulum and muscle filaments (Cameron & Tu, 1977). In the course of our survey of natural products having Ca^{2+} releasing activity in SR, we have found that MYTX is the most powerful Ca^{2+} releaser known having novel pharmacological properties; the radio-labelled compound can be synthesized. This paper reports the detailed evidence that MYTX causes Ca^{2+} release from skeletal muscle SR. MYTX may provide a pharmacologically useful tool for resolving the molecular mechanism of Ca^{2+} release from SR.

Methods

Purification of MYTX

Myotoxin *a* (MYTX) was purified as described previously (Cameron & Tu, 1977). Crude prairie rattlesnake venom (1 g)

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was dissolved in 5–6 ml of an elution buffer consisting of 0.05 M Tris, pH 9.0, at 22°C, containing 0.1 M KCl. This was applied to a Sephadex G-50 gel filtration column (4 × 115 cm) equilibrated with the elution buffer. Fractions of 5 ml were collected at a flow rate of 0.5 ml min^{-1}. Absorbance of each fraction at 280 nm was monitored on a Shimazu UV-260 spectrometer. Appropriate tubes were pooled, and an aliquot of each fraction was used for protein determination by the method of Lowry *et al.* (1951). The Sephadex G-50 fractions were lyophilized. The lyophilized fraction was dissolved in about 4 ml of the Sephadex G-50 elution buffer, and was applied to a Sephadex C-25 cation-exchange column (1.6 × 15 cm) equilibrated with the same elution buffer. After washing the column, elution was then performed with a three-step KCl salt gradient in the 0.05 M Tris buffer. Five-ml fractions were collected, and the absorbance of each fraction at 280 nm was measured. Appropriate tubes were pooled and were dialyzed and lyophilized.

Preparation of SR vesicles from skeletal muscle

The heavy fraction of fragmented SR (HSR) was prepared from rabbit skeletal muscle by the method of Kim *et al.* (1983). Rabbits were stunned and exsanguinated. White muscle was homogenized in five volumes of 5 mM Tris-maleate (pH 7.0) and centrifuged at 5,000 *g* for 5 min. The supernatant was further centrifuged at 12,000 *g* for 30 min. The pellet was suspended in a solution containing 0.1 M KCl and 5 mM Tris-maleate and centrifuged at 70,000 *g* for 40 min. The HSR obtained was stored in the same solution at 0°C and used within 4 days.

Ca^{2+} electrode experiments

The concentration of extravesicular Ca^{2+} in the SR suspension was measured at 30°C with a Ca^{2+} electrode as described previously (Seino *et al.*, 1991). The Ca^{2+} electrode showed a Nernstian response (slope, 27–29 mV/pCa unit) in the calibration buffer containing Ca^{2+}-EGTA between pCa decreased from 6 to 4. The assay solution (final volume, 1 ml) contained 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$,

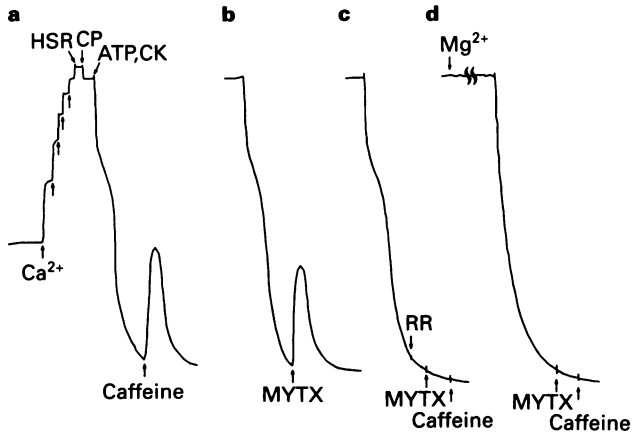


Figure 1 Ca^{2+} release induced by MYTX from skeletal muscle HSR. The concentrations of extravesicular Ca^{2+} were monitored at 30°C with a Ca^{2+} electrode in the assay solution containing 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$, 50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate (CP), 0.13 mg ml^{-1} of creatine kinase (CK) and 0.5 mM (CK) and 0.5 mM ATP. At the beginning of each experiment, 0.01 mM $CaCl_2$ was added five time stepwise as the internal standard. The reaction of Ca^{2+} uptake was started by a simultaneous addition of CK and ATP. Vertical calibration bars indicate responses for voltage change (10 mV) corresponding to 0.5 pCa unit. In (b) to (d), the traces are those only after the addition of ATP; (a) 1 mM caffeine; (b) 1 μM MYTX; (c) 2 μM ruthenium red (RR) plus 10 μM MYTX and 5 mM caffeine; (d) 4 mM $MgCl_2$ plus 10 μM MYTX and 5 mM caffeine. For abbreviations in this and other legends, please see text.

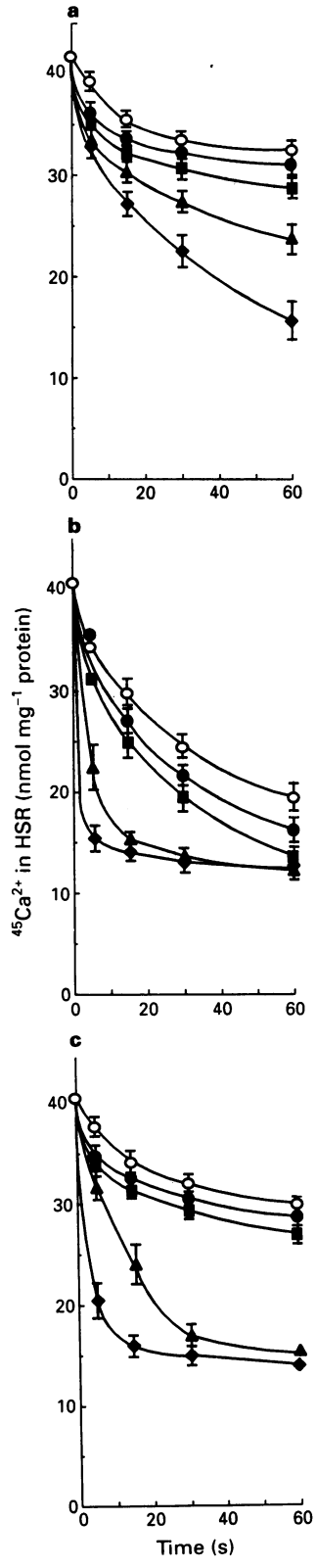


Figure 2 Stimulatory effect of MYTX on the $^{45}Ca^{2+}$ from skeletal muscle HSR at difference Ca^{2+} concentrations. The $^{45}Ca^{2+}$ content in HSR vesicles was measured at 0°C by the Millipore filtration method after 100 fold dilution of passively $^{45}Ca^{2+}$-preloaded HSR into a medium containing 90 mM KCl, 0.4 mM $CaCl_2$ with various concentrations of EGTA and 50 mM MOPS-KOH (pH 7.0) in the presence or absence of various concentrations of test substance. The initial content of $^{45}Ca^{2+}$ in HSR was obtained by adding the HSR suspension into the reaction medium containing 90 mM KCl, 5 mM $MgCl_2$, 5 mM $LaCl_3$, and 50 mM MOPS (pH 7.0). Free Ca^{2+} concentration was maintained with Ca-EGTA buffer. Values are mean with s.e.mean. ($n = 3$–4). (a) pCA 7; (b) pCa 6; (c) pCa 4. The concentrations of MYTX were 0 nM (○), 30 nM (●), 100 nM (■), 1 μM (▲), and 10 μM (◆).

50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate, 0.13 mg ml^{-1} of creatine kinase and 0.5 mM ATP. The reaction of Ca^{2+} uptake was started by the simultaneous addition of creatine kinase and ATP.

$^{45}Ca^{2+}$ release experiments

$^{45}Ca^{2+}$ release from HSR passively preloaded with $^{45}Ca^{2+}$ was measured at 0°C as described previously (Nakamura *et al.*, 1986; Kobayashi *et al.*, 1987) with slight modification. After 12-h preincubation of 20 mg ml^{-1} HSR with 5 mM $^{45}Ca^{2+}$ in a solution containing 90 mM KCl and 50 mM MOPS-KOH (pH 7.0) at 0°C, the HSR suspension was diluted with 100 volumes of an ice-cold reaction medium containing, 0.4 mM $CaCl_2$ with various concentrations of EGTA, 90 mM KCl and 50 mM MOPS-KOH (pH 7.0). For measurement of the amount of $^{45}Ca^{2+}$ in HSR at time 0 the HSR suspension was diluted with the reaction medium containing 5 mM $LaCl_3$. At an appropriate time, 5 mM $LaCl_3$ was added to stop $^{45}Ca^{2+}$. The reaction mixture was then filtered through Millipore filter (HAWP type, 0.45 μm pore size), and washed with 5 ml of a solution containing 5 mM $LaCl_3$, 5 mM $MgCl_2$, 90 mM KCl and 50 mM MOPS-KOH (pH 7.0). The amount of $^{45}Ca^{2+}$ remaining in the HSR vesicles was measured by counting the radioactivity on the washed filters.

[^{3}H]-ryanodine binding assay

[^{3}H]-ryanodine binding was examined as described previously (Inui *et al.*, 1987) with modification. HSR was incubated with 10 nM [^{3}H]-ryanodine at 37°C for 1 h in a solution containing 0.3 M sucrose, 1 M NaCl, 10 μM $CaCl_2$, 2 mM DTT, 0.1 mM *p*-APMSF and 20 mM Tris-HCl (pH 7.4). The amount of [^{3}H]-ryanodine bound was determined by membrane filtration through Whatman filters (GF/B). Nonspecific binding was determined in the presence of 10 μM unlabelled ryanodine.

Measurement of (Ca^{2+}-Mg^{2+})ATPase activity

(Ca^{2+}-Mg^{2+})ATPase reaction was carried out at 37°C in a medium of 0.1 M KCl, 20 mM Tris-maleate, pH 7.5, 2 mM $MgCl_2$, and 2 mM ATP. ATPase activity was determined from the amount of phosphate liberated, which was measured by the method of Chan *et al.* (1986).

Free Ca^{2+} concentration

Free Ca^{2+} concentration was maintained by using Ca^{2+}-EGTA buffer (0.2 mM $CaCl_2$ plus various concentrations of

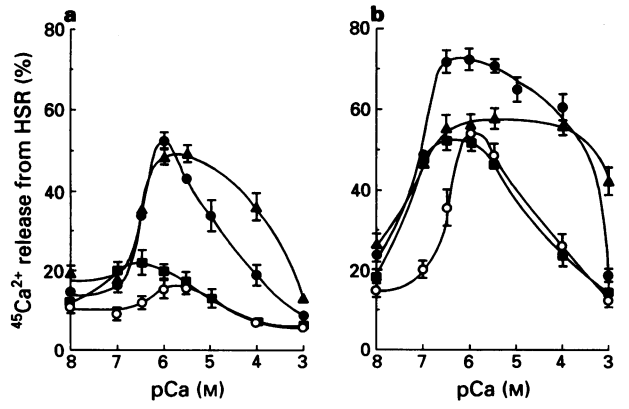


Figure 3 Effect of free Ca^{2+} concentrations on $^{45}Ca^{2+}$ release induced by several drugs from skeletal muscle HSR. $^{45}Ca^{2+}$ release from HSR for 5 s (a) and 1 min (b) was measured. Experimental protocols were similar to those described in Figure 2. Values are mean with s.e.mean. ($n = 4$). Control (○); 1 μM MYTX (●); 1 mM caffeine (■); 100 μM AMP-PCP (▲).

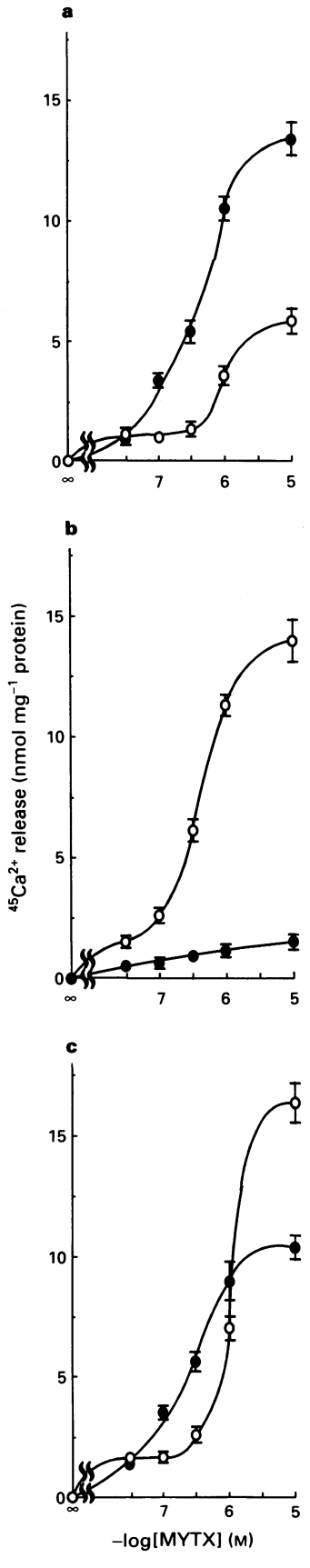
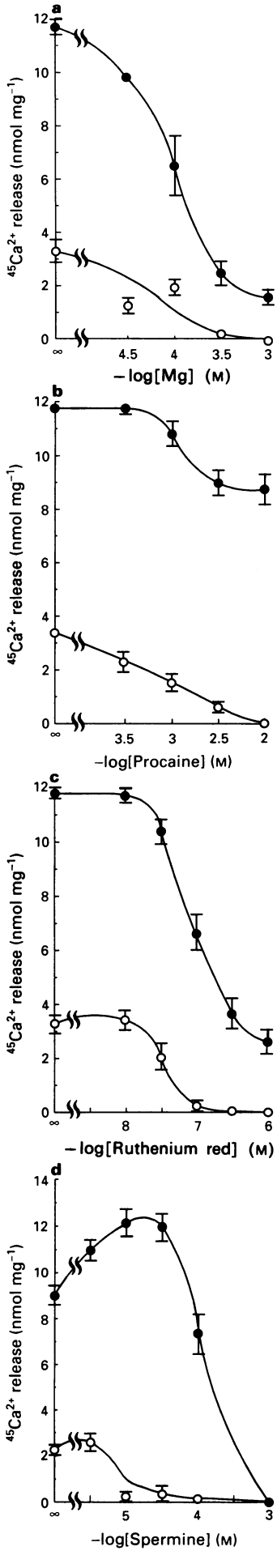


Figure 4 Concentration-dependent acceleration of $^{45}Ca^{2+}$ release from skeletal muscle HSR by MYTX. $^{45}Ca^{2+}$ release was measured at pCa 7 (a), 6 (b) and 4 (c). Experimental protocols were the same as those described in Figure 2. $^{45}Ca^{2+}$ release was carried out using HSR vesicles during 5 s (○) and 1 min (●) after dilution in the absence (control) or presence (experimental condition) of MYTX (up to 10 μM). The difference between control and experimental conditions is plotted. Values are mean with s.e.mean. ($n = 3$–4).



EGTA). The free Ca^{2+} was estimated using a microcomputer programme taking into account the binding constant for Ca-EGTA, pH, and the concentrations of K^+, Mg^{2+}, and nucleotides present (Sillen & Martell, 1964; 1971).

Materials

The sources of materials used in this work were as follows: crude venom of prairie rattlesnake from Miami Serpentarium Laboratories; procaine HCl and AMP-PCP from Sigma; ryanodine from S.B. Penick Company; $^{45}CaCl_2$ (0.70 Ci mmol^{-1}) and [^{3}H]-ryanodine (60 Ci mmol^{-1}) from Du-Pont New England Nuclear; MBED was prepared by the method described previously (Kobayashi *et al.*, 1988). All other chemicals were of analytical grade.

Results

Ca^{2+} release experiments with a Ca^{2+} electrode

The effect of MYTX on the Ca^{2+}-mobilizing activity of SR can be visualized clearly by monitoring extravesicular Ca^{2+} concentrations of HSR directly with a Ca^{2+} electrode (Nakamura *et al.*, 1986; Seino *et al.*, 1991). When the Ca^{2+} concentration was reduced to submicromolar levels, the apparent Ca^{2+} uptake slowed. As shown in Figure 1, the addition of 1 mM caffeine or 1 μM MYTX to Ca^{2+}-filled HSR caused an immediate Ca^{2+} release followed by a Ca^{2+} reuptake. The rate of Ca^{2+} reuptake was almost the same as that before the addition of caffeine or MYTX. Pretreatment of HSR with 2 μM ruthenium red (Figure 1c) or 4 mM $MgCl_2$ (Figure 1d) blocked the effect of 10 μM MYTX and 5 mM caffeine. In the light fraction of SR (LSR), however, neither MYTX (0.1–10 μM) nor caffeine (0.5–5 mM) caused Ca^{2+} release (data not shown).

$^{45}Ca^{2+}$ release from HSR

Effects of MYTX on $^{45}Ca^{2+}$ release from HSR were investigated by the Millipore filtration method. Figure 2 shows the time courses of change in the $^{45}Ca^{2+}$ content in HSR evoked by various concentrations of MYTX at three different free Ca^{2+} concentrations. $^{45}Ca^{2+}$ release was markedly accelerated by MYTX, in a concentration-dependent manner at any pCa used. MYTX at concentrations of 30 nM or more caused the acceleration of Ca^{2+} release and this release was completed within 20 s at pCa 7 and pCa 4. $^{45}Ca^{2+}$ release stimulated by MYTX at concentrations higher than 1 μM maintained at least for 1 min. The Ca^{2+} dependency of ^{45}Ca release induced by MYTX, caffeine and AMP-PCP has a bell-shaped profile (Figure 3). $^{45}Ca^{2+}$ release was stimulated remarkably by MYTX and AMP-PCP in the wider range of pCa between 8 and 3, whereas that induced by caffeine was accelerated in the range of pCa, i.e., between 7 and 5.5. Figure 4 shows the concentration-response curve for MYTX in $^{45}Ca^{2+}$ release during 5 s and 1 min. MYTX caused a concentration-dependent increase in $^{45}Ca^{2+}$ release at concentrations higher than 30 nM. The first saturation was observed at concentrations around 0.1 μM. When the concentration of MYTX was further increased, the $^{45}Ca^{2+}$ release activity increased again and reached the maximum response to MYTX at 10 μM. The effects of various inhibitors on $^{45}Ca^{2+}$ release induced by

Figure 5 Effects of representative inhibitors for Ca^{2+}-induced Ca^{2+} release on $^{45}Ca^{2+}$ release induced by MYTX from skeletal muscle HSR. Concentration-dependent effects of free Mg^{2+} (a), procaine (b), ruthenium red (c) and spermine (d) on MYTX-induced Ca^{2+} release was investigated. Data are expressed as difference between $^{45}Ca^{2+}$ release in the presence or absence of MYTX. Experimental protocols were the same as those described in Figure 2. Values are mean with s.e.mean. ($n = 3$–4). 100 nM MYTX (○); 10 μM MYTX (●).

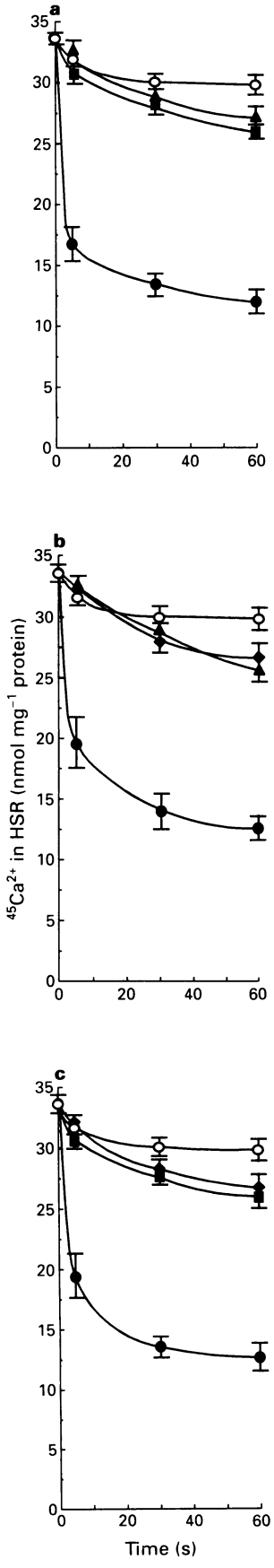


Figure 6 Interrelations among the Ca^{2+}-releasing activities of MYTX, MBED and AMP-PCP. Experimental protocols were the same as those described in Figure 2. Values are mean ($n = 4$). Control (○); 10 μM MYTX (▲); 10 μM MBED (■); 0.1 mM AMP-PCP(◆). (a), 10 μM MYTX plus 10 μM MBED (●). (b), 10 μM MYTX plus 0.1 mM AMP-PCP (●). (c), 10 μM MBED plus 0.1 mM AMP-PCP (●).

MYTX at concentrations of 100 nM and 10 μM were investigated. Figure 5a shows the effects of Mg^{2+} on $^{45}Ca^{2+}$ release triggered by MYTX. $^{45}Ca^{2+}$ release caused by MYTX at two concentrations was nearly completely inhibited by Mg^{2+} in a concentration-dependent manner. Each IC_{50} value for Mg^{2+} was approximately 100 μM. $^{45}Ca^{2+}$ release induced by caffeine or AMP-PCP from HSR was completely inhibited by procaine at a high concentration of 10 mM. As shown in Figure 5b, $^{45}Ca^{2+}$ release stimulated by 100 nM MYTX was completely inhibited by procaine (10 mM), whereas that stimulated by 10 μM MYTX was only partly inhibited. Ruthenium red caused a concentration-dependent inhibition of $^{45}Ca^{2+}$ release induced by MYTX at 100 nM and 10 μM with each IC_{50} value of about 0.1 μM (Figure 5c). $^{45}Ca^{2+}$ release caused by MYTX at 100 nM was completely inhibited by spermine at concentrations higher than 10 μM (Figure 5d). However, the effect of spermine on the release caused by 10 μM MYTX was complex. The release was potentiated by spermine at concentrations between 3 and 30 μM, whereas release was inhibited by spermine at concentrations higher than 100 μM in a concentration-dependent manner. Figure 6 shows the interrelations among the $^{45}Ca^{2+}$ releasing activities of MYTX, MBED and AMP-PCP at pCa 8. MYTX and MBED caused the maximum increase in $^{45}Ca^{2+}$ release at 10 μM. The additional application of AMP-PCP (0.1 mM) further increased the maximum response of $^{45}Ca^{2+}$ release to MYTX and MBED (Figure 6b and c). Furthermore, $^{45}Ca^{2+}$-releasing effects of MYTX and MBED are additive, suggesting that each drug potentiates $^{45}Ca^{2+}$ release from HSR through binding to the different binding sites.

[^{3}H]-ryanodine binding to HSR

[^{3}H]-ryanodine binding to the HSR membrane was examined in the presence of unlabelled ryanodine or MYTX. MYTX (up to 10 μM) did not affect [^{3}H]-ryanodine binding to HSR, although the binding of [^{3}H]-ryanodine was inhibited by unlabelled ryanodine in a concentration-dependent manner with the IC_{50} value of approximately 15 nM.

(Ca^{2+}-Mg^{2+})ATPase activity of HSR

Volpe *et al.* (1986) reported that MYTX inhibited Ca^{2+} loading and stimulated Ca^{2+}-dependent ATPase of LSR without affecting unidirectional Ca^{2+} release. But in HSR, MYTX at concentrations up to 400 μM did not exhibit significant effect on the (Ca^{2+}-Mg^{2+})ATPase activity (Figure

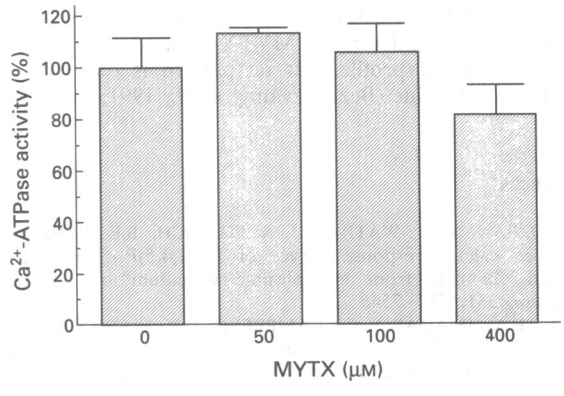


Figure 7 Effects of MYTX on Ca^{2+}-ATPase activity of skeletal muscle HSR. HSR vesicles (0.1 mg ml^{-1}) were incubated at 27°C for 5 min with varying concentrations of MYTX and A23187 (4 μM). At the end of incubation, ATPase activity was measured as described under Methods. Results are the mean ± s.e. of three experiments and are expressed as a percentage against control activity determined in the absence of MYTX (control, 1040 ± 122 nmol P_i mg^{-1} protein min^{-1}; 40 μM MYTX, 1178 ± 20 nmol P_i mg^{-1} protein min^{-1}; 100 μM MYTX, 1100 ± 118 nmol P_i mg^{-1} protein min^{-1}; 400 μM MYTX, 848.5 ± 120 nmol P_i mg^{-1} protein min^{-1}).

7). The EC_{50} value of MYTX for $^{45}Ca^{2+}$ release was about 1 μM. In addition, MYTX did not cause $^{45}Ca^{2+}$ release from LSR (data not shown). In the $^{45}Ca^{2+}$-release measurement, therefore, the effect of MYTX on the (Ca^{2+}-Mg^{2+})ATPase is excluded.

Discussion

The Ca^{2+}-induced Ca^{2+} release channels may be the machinery of the physiological process in the excitation-contraction coupling in skeletal muscle (Endo *et al.*, 1979; Ford & Podolsky, 1970; Endo, 1977). The channels have been purified using [^{3}H]-ryanodine as a specific ligand (Inui *et al.*, 1987; Hymel *et al.*, 1988; Wagenknecht *et al.*, 1989). The functions of Ca^{2+} release channels are inhibited by several inhibitors such as procaine, Mg^{2+}, ruthenium red and spermine (Palade, 1987; McPherson & Campbell, 1993). In the present study, we found that MYTX accelerated $^{45}Ca^{2+}$ release from HSR in a concentration-dependent manner at concentrations higher than 30 nM, making it the most potent inducer known of Ca^{2+} release in SR. MYTX even at high concentrations had no ionophoretic activity on the membrane of HSR because the rate of Ca^{2+} reuptake after a rapid Ca^{2+} release was almost the same as that before the addition of MYTX or caffeine. MYTX had no effect on (Ca^{2+}-Mg^{2+})ATPase even at high concentrations up to 400 μM. The Ca^{2+} mobilizing effect of MYTX were blocked by inhibitors of Ca^{2+}-release (Mg^{2+}, ruthenium red and spermine). These results suggest that MYTX induces Ca^{2+} release by affecting Ca^{2+} release channels in SR.

Procaine is a selective inhibitor (Endo, 1977) of Ca^{2+}-induced Ca^{2+} release channels with an IC_{50} value of 1 mM (Seino *et al.*, 1991). Ca^{2+} release induced by potentiators of Ca^{2+}-induced Ca^{2+} release such as caffeine and AMP-PCP was abolished in the presence of procaine (10 mM), indicating total block of Ca^{2+}-induced Ca^{2+} release channels. $^{45}Ca^{2+}$ release induced by 100 nM MYTX was completely inhibited by 10 mM procaine (IC_{50}, 0.91 mM), whereas that by 10 μM MYTX was only partially inhibited by it (IC_{50}, 1.1 mM) (Figure 5b). Therefore, $^{45}Ca^{2+}$ release evoked by MYTX at concentrations higher than 300 nM probably has two components (Figure 2). These observations suggest that the early component inhibited by procaine is due to Ca^{2+} release through the Ca^{2+}-induced Ca^{2+} release channels, while the late one resistant to procaine possibly mediated through Ca^{2+} release channels with novel pharmacological properties. However, we cannot exclude the possibility that procaine cannot completely inhibit Ca^{2+}-induced Ca^{2+} release induced by a potent Ca^{2+} releaser. This needs further consideration.

The Ca^{2+} dependency of MYTX-induced $^{45}Ca^{2+}$ release has a bell-shaped profile, but its pattern is quite different from that of caffeine (Bezprozvanny *et al.*, 1991; Seino *et al.*, 1991). The affinity of Ca^{2+} for the channels increased in the presence of caffeine (the EC_{50} values of Ca^{2+} for $^{45}Ca^{2+}$ release in the absence and presence of 1 mM caffeine were approximately 300 and 30 nM), whereas this was not changed by MYTX. Although the Ca^{2+} dependency of MYTX-induced Ca^{2+} release was rather similar to that of AMP-PCP, the pharmacological properties of MYTX, including the procaine sensitivity, were quite different from those of AMP-PCP.

It has been reported that the ryanodine receptor protein consists of several ligand-binding domains, i.e. domains for caffeine, adenine nucleotides, ryanodine and divalent cations (Pessah *et al.*, 1987). The maximum responses of $^{45}Ca^{2+}$ release to AMP-PCP and MBED increased further in the presence of MYTX. These data suggest that MYTX binds to different sites from those of AMP-PCP and MBED/caffeine. It has been reported that [^{3}H]-ryanodine binds to Ca^{2+}-induced Ca^{2+} release channels in an open state, this binding being increased by binding of several potentiators of Ca^{2+}-induced Ca^{2+}-release to the channels (Fleisher *et al.*, 1985; McPherson & Campbell, 1993). MYTX had no effect on [^{3}H]-ryanodine binding to HSR. Imperatoxins from the scorpion *Pandinus imperator* affected [^{3}H]-ryanodine binding (Valdivia *et al.*, 1992) suggesting that their binding sites are different from those of MYTX. Furthermore, the binding of [^{125}I]-MYTX to HSR was not affected by MBED/caffeine or AMP-PCP (Ohkura *et al.*, unpublished data). On the basis of these observations, it is suggested that there are three possibilities, i.e., MYTX binds to (1) a novel binding site on Ca^{2+}-induced Ca^{2+} release channels, (2) a regulatory protein of Ca^{2+}-induced Ca^{2+} release channels, or (3) a novel type of Ca^{2+} release channels.

One of the great advantages of MYTX as a pharmacological probe is that this compound is a polypeptide and ^{125}I-labelled MYTX can be synthesized. We have recently succeeded in the synthesis of ^{125}I-labelled MYTX with a high specific radioactivity (40–70 Ci mmol^{-1}) and in the demonstration of the presence of its specific binding site on HSR (Ohkura *et al.*, unpublished data). We also found that MYTX caused Ca^{2+} release from SR of chemically skinned fibres (Furukawa *et al.*, unpublished data). MYTX is the first polypeptide Ca^{2+} inducer in SR and has become a useful pharmacological tool, not only clarifying the regulatory mechanism of Ca^{2+} release channels but also purifying a novel type of Ca^{2+} release channel or its regulatory protein.

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