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Maitotoxin-activated single calcium channels in guinea-pig cardiac cells

[1]Masaki Kobayashi, *Rikuo Ochi & Yasushi Ohizumi

Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan and *Department of Physiology, School of Medicine, Juntendo University, Tokyo 113, Japan

1 In order to clarify the mechanism of Ca-dependent excitatory action of maitotoxin (MTX), the most potent marine toxin known, patch-clamp techniques were used to analyse electrophysiological effects of MTX on guinea-pig isolated cardiac myocytes

2 The whole-cell recordings showed that MTX (0.3 ng ml^{-1}) produced a sustained inward current that was enhanced by adrenaline (2 μM) and abolished by Cd^{2+} (1 mM).

3 This current was predominantly carried by Ca^{2+} or Ba^{2+} and has an almost linear current-voltage relationship.

4 In cell-attached patches, MTX added to the pipette solution activated Ca channels with novel properties. The opening events of these channels occurred as long bursts, and the channel gating showed little voltage-dependence.

5 The unitary conductance was 12 pS in the presence of 50 mM Ba^{2+}. Within a burst, the distribution of opening times was a single exponential with a mean open time of 10.4 ms.

6 The channel described here represents either a new class of voltage-independent Ca channel or an entirely modified form of voltage-gated Ca channel. This channel may account for the mechanism of enhanced Ca^{2+} influx through the cell membrane induced by MTX, and presumably regulates some ionic movements in myocardial cells.

Introduction

In research aimed at characterizing and purifying ion channels, one of the essential aspects has been the discovery and development of chemical tools that selectively modulate the channel function (Hille, 1984). From this viewpoint, a variety of natural toxins such as tetrodotoxin, saxitoxin, veratridine, batrachotoxin and sea anemone toxins have been extensively studied and have attracted the attention of pharmacologists, physiologists and biochemists because they interact with specific channels on excitable membranes (Albuquerque *et al.*, 1971; Narahashi, 1974; Catterall, 1980; Honerjäger, 1982). Using these toxins as specific ligands, biochemical studies concerning molecular properties of ion channels have developed dramatically (Catterall, 1986). Maitotoxin (MTX), the most potent marine toxin known, has been isolated from the toxic dinoflagellate *Gambierdiscus toxicus* as the water soluble toxin of ciguatera seafood poisoning in tropical regions (Yasumoto *et al.*, 1979). The chemical structure of MTX has been only partially determined. It is considered to be a non-peptidic substance having a large molecular weight (Yasumoto *et al.*, 1979). We have shown that MXT produces Ca^{2+}-dependent excitatory effects on various tissues such as cardiac muscle (Kobayashi *et al.*, 1985a, b; 1986), smooth muscle (Ohizumi & Yasumoto, 1983; Ohizumi *et al.*, 1983), skeletal muscle (Miyamoto *et al.*, 1984) and neuronal cells (Takahashi *et al.*, 1982; 1983). Since Ca^{2+} plays a central role in the regulation of many cellular functions, MTX has been widely used as a valuable tool by numerous investigators (Freedman *et al.*, 1984; Gomi *et al.*, 1984; Schettini *et al.*, 1984; Kim *et al.*, 1985; Login *et al.*, 1985; Ueda *et al.*, 1986; Niki *et al.*, 1986). However, the mechanism of the Ca^{2+}-dependent excitatory actions of MTX has not yet been clarified. Here we describe the first direct evidence based on patch-clamp experiments that MTX activates voltage-independent Ca channels in cardiac myocytes, an action which is responsible for the highly enhanced Ca^{2+} influx through the cell membrane. These channels may play a role in the regulation of ionic movements.

[1]Author for correspondence.

Methods

Preparation of single myocytes

Single rod-shaped cells were isolated from ventricular muscle of male guinea-pigs (250–300 g) by enzymatic digestion according to the method of Cavalié *et al.* (1983). Animals were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}) and the ascending aorta was cannulated *in situ* under artificial respiration. The heart was attached to the base of a 60-cm high Langendorff column and perfused with normal Tyrode solution containing (mM); NaCl 135, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 5 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5 (pH 7.4) followed by a nominally Ca^{2+}-free solution. For enzymatic digestion, 40 mg collagenase (Worthington) and 40 μM $CaCl_2$ were added to Ca-free Tyrode solution, which was then recirculated for 30 min. All perfusates were oxygenated with O_2 and prewarmed to 37°C. The enzyme solution was washed out with a high-K^+, low-Cl^- solution (KB medium) containing (mM): K glutamate 70, KCl 25, KH_2PO_4 10, taurine 10, oxalic acid 10, EGTA 0.5, glucose 10 and HEPES 5 (pH 7.4), and the dissociated cells were stored at 4°C in the same solution.

Patch-clamp experiments

Guinea-pig myocardial cells were superfused at 37°C with normal Tyrode solution. Voltage-clamp experiments were performed by use of the patch-clamp method in the whole-cell recording configuration (Hamill *et al.*, 1981; Marty & Neher, 1983). Gigaohm seals were obtained with fire-polished patch-clamp pipettes having a resistance of 1–3 MΩ. Addition of EGTA kept the free Ca^{2+} concentration in the internal dialysate at a low level. On rupturing the membrane patch, the approximate resting potential ranged beween −70 and −80 mV. The holding potential was set at −80 mV and the cell was allowed to stand for several minutes until the cell interior had equilibrated with the pipette solution. Then the superfusion medium was changed by changing from normal Tyrode solution to Na-free, Cs, Ba-Tyrode solution. Under these conditions, currents flowing through Na and K channels were minimal, and only inward Ba currents through Ca channels were observed in response to depolarizing pulses. For single-channel current measurements, standard patch-clamp recording techniques (Hamill *et al.*, 1981; Marty & Neher, 1983) were used to form cell-attached patches of cardiac cell membrane using pipettes with the resistance of 2–5 MΩ. Experiments were repeated in cells from different hearts. The current was measured by means of a patch-clamp amplifier (L/M-EPC 7, List Medical, Darmstadt, West Germany), stored on tape through a digital PCM data recording system (Model RP-880, NF Electronic Instruments, Yokohama, Japan) and analysed later on a NEC PC-9801 Computer at appropriate sampling rates.

Solutions for patch-clamp experiments

The composition of the solutions used in patch-clamp experiments is shown in Table 1. Other superfusion media were made by mixing these solutions. The pipettes for whole-cell recordings were filled with a solution containing (mM); Cs aspartate 110, CsCl 20, EGTA 5, ATP-Tris 5 and HEPES-Tris 5 (pH 7.3). The pipettes for single-channel current measurements were filled with a solution containing (mM): $BaCl_2$ 50, choline Cl 75, tetrodotoxin 0.03 and HEPES-Tris 5 (pH 7.4).

Table 1 Composition of solutions (mM)[a]

	NaCl	KCl	$CaCl_2$	$MgCl_2$	Choline Cl	CsCl	$BaCl_2$
Normal Tyrode	135	5.4	1.8	1	—	—	—
Cs-Tyrode	135	—	1.8	1	—	5.4	—
Ca-free, Cs-Tyrode	135	—	—	1	—	5.4	—
Na-free, Cs-Tyrode	—	—	1.8	1	135	5.4	—
Na-free, Cs, Ba-Tyrode	—	—	—	1	135	5.4	1.8

[a]All solutions also contained (mM): glucose 5 and HEPES 5 and were adjusted to pH 7.4.

Results

Whole cell current recordings

MTX produced a sustained inward current in ventricular cells isolated from adult guinea-pig heart. The whole-cell patch-clamp technique was applied to the cells superfused with Ba^{2+} (1.8 mM)-containing Na-free, Cs, Ba-Tyrode solution. After external application of MTX (0.3 ng ml^{-1}), the holding current at −80 mV shifted gradually in the inward direction (Figure 1A). This inward current was enhanced by

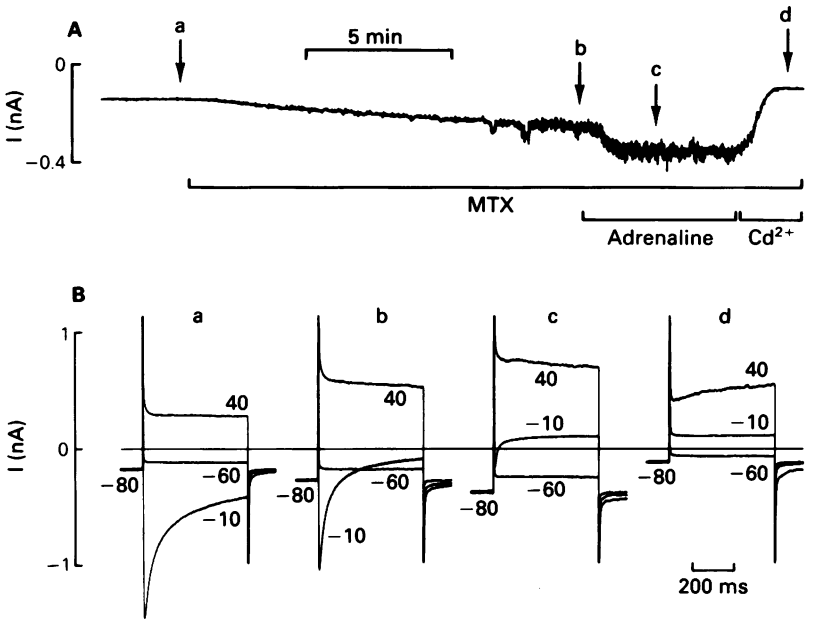

Figure 1 (A) Current changes induced by treatment with maitotoxin (MTX) in a whole-cell clamped ventricular cell superfused with Na-free, Cs, Ba-Tyrode solution. The holding potential was set at −80 mV. The lower bars indicate the range of exposure to MTX (0.3 ng ml^{-1}), adrenaline (2 μM) or Cd^{2+} (1 mM). At (a)–(d), the current records shown in (B) were obtained. The superfusion medium was altered by changing from one solution to another containing MTX, adrenaline and/or Cd^{2+} as indicated. (B) Superimposed current records taken at each of the positions shown by (a)–(d) in (A): (a) control, (b) 0.3 ng ml^{-1} MTX, (c) 0.3 ng ml^{-1} MTX and 2 μM adrenaline and (d) 0.3 ng ml^{-1} MTX and 1 mM Cd^{2+}. Transient currents were elicited by 500 ms command pulses from a holding potential (−80 mV) to three different potentials (−60, −10 and +40 mV). The pulse potential used is indicated for each record. Almost the same results were obtained reproducibly from three different cells.

adrenaline (2 μM) and abolished by Cd^{2+} (1 mM). These results suggested the possible opening of voltage-gated Ca channels by MTX. However, as shown in Figure 1B, MTX (0.3 ng ml^{-1}) did not increase the transient Ca-channel currents. Instead, MTX produced changes in the steady-state current; inward shifts at −60 and −80 mV and outward shifts at −10 and +40 mV (Figure 1B, a,b). This indicates that the reversal potential of this steady current is between −60 and −10 mV, quite different from that of voltage-gated Ca-channel current (+50 to +70 mV) (Reuter & Scholz, 1977; Lee & Tsien, 1982). Adrenaline (2 μM) enhanced both the inward and outward currents, while Cd^{2+} (1 mM) suppressed them (Figure 1B c,d). The effect of MTX on the steady current was poorly reversed by washing out with MTX-free medium.

In the Ca^{2+} (1.8 mM)-containing Na-free, Cs-Tyrode solution, exposure to MTX (1 ng ml^{-1}) also caused a similar shift in the holding current, indicating that Ca^{2+} can carry this inward current. The detailed voltage-dependence of the current activated by MTX is illustrated in Figure 2. The I–V relations in Na-free, Cs-Tyrode solution with or without MTX (1 ng ml^{-1}) are shown in Figure 2a. The data points in Figure 2b show the difference current amplitudes. The I–V relation of the MTX-activated current was almost linear and the reversal potential was about −23 mV. The current amplitudes at −80 and +30 mV were −0.44 ± 0.02 and +0.62 ± 0.06 nA ($n = 4$) per cell, respectively. The reversal potential ranged between −20 and −30 mV.

In the Cs-Tyrode solution containing 135 mM Na^+ and 0.9 mM Ca^{2+}, the reversal potential of MTX (1 ng ml^{-1})-activated current was about −30 mV. When the Na^+ concentration was reduced to 13.5 mM, the reversal potential shifted by about 20 mV in the negative direction. Furthermore, when the Ca^{2+} concentration in the Cs-Tyrode solution with 135 mM Na^+ was raised from 0.45 to 5.4 mM, the reversal potential shifted by about 28 mV in the positive direction. By means of the constant field equation (Reuter & Scholz, 1977), the relative permeability ratio P_{Ca}/P_{Na} for the MTX-activated current

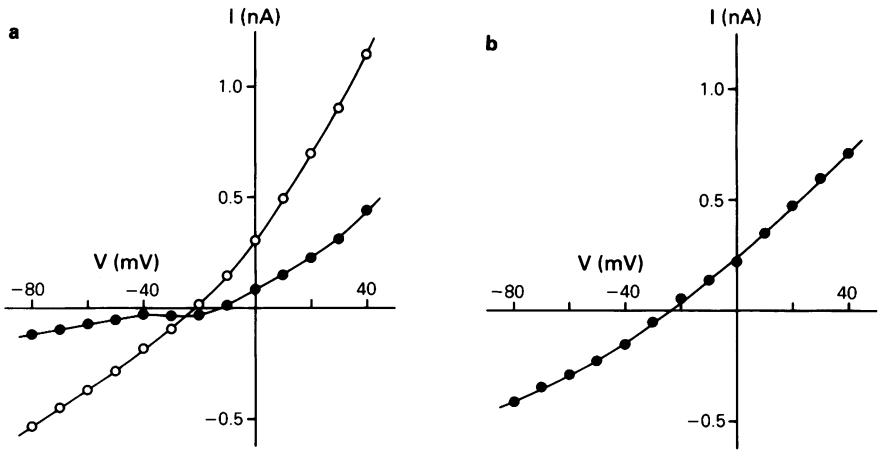

Figure 2 (a) Effect of maitotoxin (MTX) on the current-voltage relationships before (●) and 4 min after (○) treatment with MTX 1 ng ml^{-1}. The cells were superfused with Na-free, Cs-Tyrode solution. Currents were elicited by 200 ms depolarizing pulses from a holding potential (−80 mV) to various potentials. Ordinate scale, steady-state current levels measured at the end of each pulse. Abscissa scale, membrane potential during the pulse. Almost the same results were obtained reproducibly from three different cells. (b) Steady-state levels of difference currents derived by subtracting currents recorded in the absence of MTX (1 ng ml^{-1}) from those recorded, at the same voltage, in its presence. Data are from the experiment described in (A). Ordinate scale, amplitudes measured as in (A); abscissa scale, membrane potential.

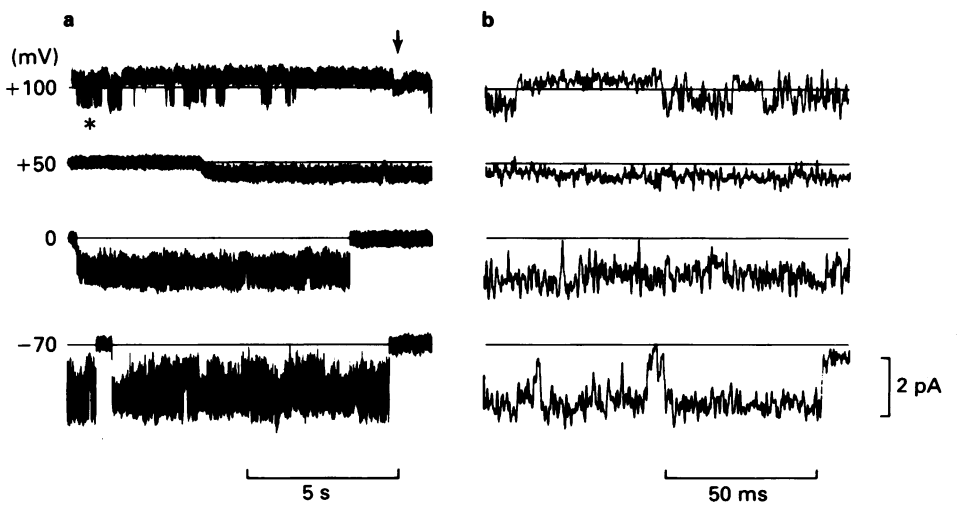

Figure 3 Maitotoxin (MTX)-activated channel currents from cell-attached patches of single ventricular cells. Recordings of single-channel currents displayed at slower (a) and faster (b) speed. The pipette facing the external side of the membrane patch contained 50 mM $BaCl_2$ saline with 100 ng ml^{-1} MTX. The cell was superfused with normal Tyrode solution. Similar channel currents to those shown here were observed in 12 different myocytes. Deviation of the membrane potential (Δ V) from the resting potential is indicated on the left of each trace. Currents flowing from the external (pipette) to the internal (cell interior) side are shown as downward deflections. The solid line indicates the zero current level at each holding potential. Arrow, closing state of all channels including MTX-activated channel; asterisk, short bursts of single voltage-gated Ca-channel current. The traces were displayed through a low-pass filter with a cut-off frequency of 1 kHz (a) or 1.5 kHz (b).

was calculated to be approximately 1/0.02. Assuming that Ca^{2+}, Na^+ and Cs^+ are the only charge carriers of the MTX-activated current in Cs-Tyrode solution, the permeability ratio P_{Ca}/P_{Cs} for this current was estimated to be about 1/0.07. These results suggest that the dominant charge carrier of this current is Ca^{2+} in Cs-Tyrode solution. Actually, the MTX-activated current increased as the Ca^{2+} concentration in the Cs-Tyrode solution was raised from 0.45 to 5.4 mM.

Single-channel current measurements

Single-channel currents were recorded from cell-attached patches of myocardial cell membrane exposed to a pipette solution with or without MTX (1–100 ng ml^{-1}). Channel currents of the type shown in Figure 3 were seen in most cell-attached patches (12 among 20) which had the extracellular face exposed to MTX (100 ng ml^{-1}). These channel currents usually developed slowly within 2 to 10 min after establishment of the patches. Also in patches exposed to the low concentration of MTX (1 ng ml^{-1}), similar single-channel currents were seen although the frequency was low. When gigaohm seals were obtained by use of pipettes filled with MTX-free solution, such channel currents were observed only rarely (1 among 10), suggesting that these currents are activated by MTX and responsible for the MTX-induced current shift in whole-cell clamped myocytes. As shown in Figure 3a, channel opening events occurred as long bursts, and each burst of channel openings and closings lasted between 1 s and 1 min. Within such a burst, there were numerous open-close events with long open and short closed times (Figure 3b). The gating showed little voltage-dependence. Bursts were sometimes separated by prolonged closings lasting some minutes. This behaviour indicates that the gating is more complicated than a single first-order transition between an open and a closed state. At 100 mV positive to the resting potential ($\Delta V = +100$ mV), the MTX-activated current was outward-going, and the voltage-gated Ca-channel current appeared simultaneously as inward short bursts (Figure 3a, asterisk).

Figure 4 shows the unitary current-potential deviation (ΔV) relation of MTX-activated channels, the slope of which is approximately linear in the ΔV range between -70 and $+30$ mV and gives a conductance of 12 pS. The reversal potential of the unitary current was estimated to be about 80 mV positive to the resting potential. Furthermore, the open-time histograms of the inward currents occurring inside of each burst recorded at the resting potential (Figure 5a)

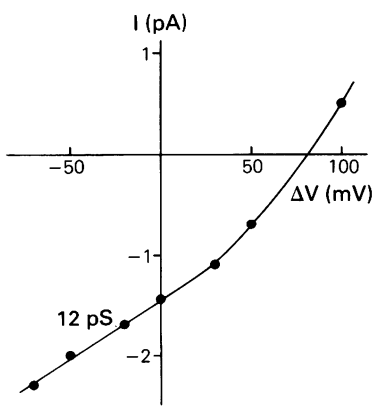

Figure 4 Voltage-dependence of single-channel current activated by maitotoxin (MTX, 1 ng ml^{-1}). Ordinate scale, unit amplitude of the current; abscissa scale, deviation of the membrane potential (ΔV) from the resting potential. Each value is the mean of data from four different experiments.

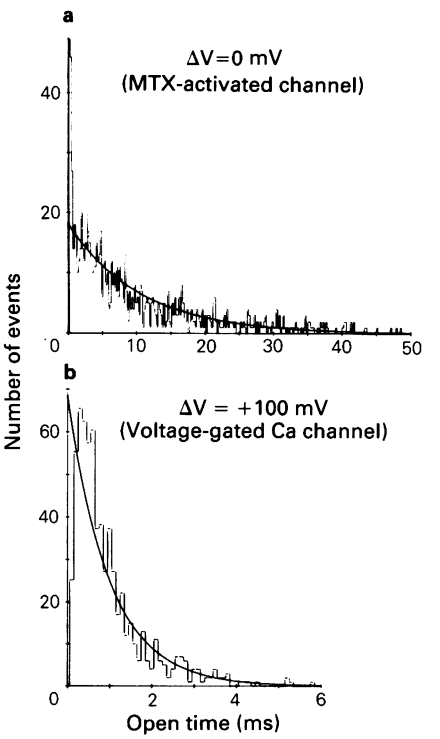

Figure 5 Open-time histogram of maitotoxin (MTX)-activated channels at the resting potential (a) and that of voltage-gated Ca channels at $\Delta V = +100$ mV (b). Data were filtered at 1.5 kHz and digitized at 5 kHz (a) or 10 kHz (b). The histogram was fitted with the theoretical single exponential distribution for a mean open time of 10.4 ms (a) or 1.0 ms (b). Note that the abscissa scale in (b) is expanded in comparison with that in (a). Almost the same results were reproducibly observed in three different myocytes.

and at $\Delta V = +100$ mV (Figure 5b) are illustrated. The distributions of opening times were essentially single exponential for both channels, though there was an excess of short open times (Figure 5a, first 0.6 ms). The mean open time of MTX-activated channels (10.4 ms) was much longer than that of voltage-gated Ca channels (1.0 ms). The gating property of MTX-activated channels showed little voltage-dependence and their long-opening behaviour was observed in the ΔV range between -70 and $+100$ mV.

Discussion

During recent decades there has been a dramatically growing awareness that the Ca movements in the cell can play an essential role in many cellular events. From this point of view, substances that influence cellular Ca movements provide valuable probes for the elucidation of various cellular functions. Recently, we have shown that the cardiotonic effects of MTX on guinea-pig atria are not affected by a β-adrenoceptor blocker, a catecholamine depleting drug, a Na channel blocker or histamine receptor antagonists (Kobayashi *et al.*, 1985b) and that these effects of MTX are strongly dependent on the Ca^{2+} concentration (Kobayashi *et al.*, 1986). It has also been shown by pharmacological and biochemical experiments that MTX exerts Ca^{2+}-dependent excitatory actions on smooth muscle (Ohizumi & Yasumoto, 1983; Ohizumi *et al.*, 1983), skeletal muscle (Miyamoto *et al.*, 1984; Gomi *et al.*, 1984), neuronal cells (Takahashi *et al.*, 1982; 1983; Freedman *et al.*, 1984; Kim *et al.*, 1985; Ueda *et al.*, 1986), anterior pituitary cells (Schettini *et al.*, 1984; Login *et al.*, 1985) and pancreatic islets (Niki *et al.*, 1986). All these actions of MTX are inhibited or abolished by Ca channel blockers or a Ca^{2+}-free solution. A possible explanation of these results has been that MTX may activate voltage-gated Ca channels. In our electrophysiological experiments reported here, MTX-induced current was enhanced by adrenaline and abolished by Cd^{2+}, a result that is compatible with this interpretation. However, a quite important observation is that the current-voltage relationship and unitary conductance of MTX-activated channels described here are clearly different from those of normal voltage-gated Ca channels. Also important is the finding that MTX-activated channels have the mean open time which is ten times longer than that of normal voltage-gated Ca channels. An attractive interpretation of all these observations is that MTX activates a new class of voltage-independent Ca channels to increase the Ca^{2+} permeability through the cardiac cell membrane, which may account for the mechanism of Ca^{2+}-dependent excitatory actions of MTX. If this is the case, the appearance of MTX-induced steady-state current may be independent of a loss of transient Ca channel current. Alternatively, another possible explanation is that normal voltage-gated Ca channels are modified with MTX and transformed into Ca channels that look like a new type, just as voltage-gated Na channels have been reported to be modified with veratridine (Sutro, 1986; Leibowitz *et al.*, 1986).

It is well-known that K^+ and Cs^+ can move through voltage-gated Ca channels in the outward direction, causing a shift of the measured reversal potential by 50–70 mV negative with respect to the equilibrium potential of Ca^{2+}, above 120 mV (Reuter & Scholtz, 1977; Lee & Tsien, 1982). In the whole-cell clamped myocyte superfused with Cs-Tyrode solution, the reversal potential of the MTX-induced steady current ranges between -20 and -30 mV, a value which is more negative than that of voltage-gated Ca-channel current. A probable interpretation of the difference between the two reversal potentials is that MTX-activated Ca channels are more permeable to Cs^+ than usual voltage-gated Ca channels. Actually, the permeability ratio P_{Cs}/P_{Ca} for the MTX-induced current is estimated to be larger than the reported value ($P_{Cs}/P_{Ca} < P_K/P_{Ca} = 0.01/1$) for usual voltage-gated Ca channels in the cardiac muscle membrane (Reuter & Scholtz, 1977; Lee & Tsien, 1982).

Non-selective cation channels have recently been shown to be activated by intracellular Ca^{2+} in cardiac myocytes (Colquhoun *et al.*, 1981), neuroblastoma cells (Yellen, 1982), pancreatic acinar cells (Maruyama & Petersen, 1982) and crustacean nerve terminals (Lemos *et al.*, 1986). These channels are characterized by their poor voltage-dependence and complex kinetics. The MTX-activated channels resemble the non-selective cation channels in these properties. The crucial difference is, however, that the MTX-activated channels are dominantly permeable to Ca^{2+}. In the pipette solution with 5 mM EGTA, the cytoplasmic free Ca^{2+} concentration is quite low, at least before the initial activation of these Ca channels and subsequent Ca^{2+} influx through them, suggesting that these channels can be activated by MTX even in the absence of intracellular Ca^{2+}.

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