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The mechanism of action of maitotoxin in relation to Ca^{2+} movements in guinea-pig and rat cardiac muscles

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1 Maitotoxin (MTX), the most potent marine toxin known, produced a dose-dependent positive inotropic effect on guinea-pig isolated left atria and rat ventricle strips at concentrations of 0.1 ng to 4 ng ml^{-1}. MTX (4 ng ml^{-1}) also exhibited a positive chronotropic effect on guinea-pig right atria.

2 The MTX-induced inotropic effect was almost abolished by Co^{2+} or verapamil, but was little affected by propranolol, reserpine or tetrodotoxin.

3 The tissue Ca content of guinea-pig left atria was increased by MTX (2–30 ng ml^{-1}) in a dose-dependent manner, and this increase was markedly inhibited by Co^{2+} or verapamil.

4 Furthermore, on the rat isolated cardiac myocytes MTX (0.01–10 ng ml^{-1}) caused an arrhythmogenic effect which was followed by their transformation into irreversibly rounded cells. The effects of MTX on the isolated cells were inhibited by verapamil or Ca^{2+}-free solution.

5 These results suggest that the excitatory effects of MTX on heart muscle are caused by a direct action on the cardiac muscle membrane mainly due to an increase in Ca^{2+} permeability.

Introduction

In studies aimed at elucidating the function of ion channels, one of the essential aspects has been the discovery and development of chemical tools that selectively modulate the channel function. From this viewpoint, marine toxins such as tetrodotoxin (TTX) (Narahashi, 1974), saxitoxin (Catterall, 1980), palytoxin (Ohizumi & Shibata, 1980), and sea anemone toxins (Romey *et al.*, 1976; Norton *et al.*, 1981; Ohizumi & Shibata, 1981; 1982) have been extensively studied and have drawn the attention of pharmacologists and physiologists because of their action on specific channel sites on the cell membrane. Recently, MTX isolated from toxic dinoflagellates and poisonous fishes inhabiting tropical seas has been revealed to be the most potent marine toxin known, with the minimum lethal dose of 0.17 $\mu g\,kg^{-1}$, i.p. in mice (Yasumoto *et al.*, 1979; Yasumoto, 1980). Its chemical structure has only been partially determined. More recently, we have shown that MTX produces a Ca^{2+}-dependent release of noradrenaline from a rat phaeochromocytoma cell line, PC12h (Takahashi *et al.*, 1982; 1983) and Ca^{2+}-dependent contraction of smooth muscle (Ohizumi & Yasumoto, 1983a, b; Ohizumi *et al.*, 1983) and skeletal muscle (Gomi *et al.*, 1984). It has also been reported that MTX may have excitatory effects on voltage-dependent Ca channels of insect skeletal muscle (Miyamoto *et al.*, 1984) and cultured neuronal cells (Freedman *et al.*, 1984) However, the physiological and pharmacological properties of MTX on the cardiac muscle have not yet been described apart from a few preliminary studies (Miyahara *et al.*, 1979; Legrand & Bagnis, 1984a, b). The present study was undertaken to clarify the mechanism of Ca^{2+}-dependent excitatory effects of MTX on guinea-pig atria, rat ventricle and rat isolated cardiac myocytes.

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Methods

Mechanical response

Male guinea-pigs (250–350 g) and male Wistar rats (280–300 g) were killed by a blow to the head. The guinea-pig left and right atria or rat ventricle strips were excised and mounted vertically in a 20 ml organ bath containing a Krebs-Ringer bicarbonate solution (30°C) of the following composition (mM): NaCl 120,

KCl 4.8, $CaCl_2$ 1.2, $MgSO_4$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose 5.8 at pH 7.4 and were aerated with 95% O_2 and 5% CO_2. One end of the tissue was connected to a force-displacement transducer by a silk ligature and the other end was secured to the plastic tissue holder with the platinum stimulating electrodes. Apart from right atria, the tissue preparations were electrically stimulated at a frequency of 2 Hz by rectangular pulses of 5 ms duration and intensity of 5 V. Resting tensions of 1 g and 0.2 g were applied to the atria and the ventricular strips, respectively. The tissues were equilibrated for at least 1 h before starting the experiments. In determining inotropic effects of MTX, only the response to the first administration of MTX to each preparation was used because the effects of MTX gradually decreased during repeated applications. Contractile force was recorded isometrically with a force-displacement transducer and was displayed on a chart recorder.

Modified medium

Low-Ca^{2+} (0.3 mM) or high-Ca^{2+} (3.0 mM) solutions were prepared by reducing or increasing $CaCl_2$ in the normal Krebs-Ringer bicarbonate solution. A Na^+-free, low-Ca^{2+} medium had the following composition (mM): sucrose 280, KCl 4.8, $CaCl_2$ 0.3, $MgSO_4$ 1.3, KH_2PO_4 1.2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Sigma) 5 and glucose 5.8 at pH 7.4, was bubbled with O_2. For the experiments in a modified medium, the tissue preparations were incubated for 30 min before the application of MTX.

Reserpine pretreatment

Reserpine (2 mg kg^{-1}, i.p.) was twice administered to guinea-pigs 48 and 24 h before the experiment.

Tissue Ca content

The left atria were excised from male guinea-pigs (300–350 g) and were cut in half, and then suspended in a Krebs-Ringer solution aerated with 95% O_2 and 5% CO_2 at 30°C. A tension of 0.3 g was applied; the atria were stimulated electrically (2 Hz, 5 ms) and allowed to equilibrate for 1 h. After a 30 min incubation under control or test conditions, the tissues were blotted, weighed and then ashed with 0.1 ml of 60% perchloric acid in a quartz tube at 500°C for 7 h. The ashed samples were dissolved in 2 ml of the solution containing 0.4% EDTA and 0.2% $SrCl_2$. The amount of Ca was determined with an atomic absorption spectrophotometer (Varian, AA-175).

Rat isolated cardiac myocytes

Cells were isolated from ventricular muscle of adult male Wistar rats weighing 250 to 300 g by digestion with collagenase (Worthington) according to the method of Miyakoda & Nakamura (1982). The perfusion medium aerated with O_2 contained (mM): NaCl 120, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 5, glucose 10 and HEPES 20; pH 7.4. A Ca^{2+}-free medium was prepared by omitting $CaCl_2$ from the normal perfusion medium. The beating activity of isolated myocytes was observed under a phase contrast microscope equipped with a thermostatically controlled heated stage. In the presence or absence of MTX, cardiac cells were placed in the chamber which was perfused at 37°C with the medium (flow rate, 0.2 ml min^{-1}). Myocytes were driven by a stimulator through a pair of platinum wire electrodes with 3 ms rectangular pulses of 15 V cm^{-1} at a frequency of 2 Hz. The image of the cells was recorded with a video recording system (Victor) for counting the myocytes.

Na^+,K^+-ATPase assay

Na^+,K^+-ATPase was prepared from the cardiac muscle of guinea-pig by the method of Pitts & Schwartz (1975). After 5 min pretreatment with the drug, the enzyme reaction was carried out at 37°C for 15 min in 0.5 ml reaction mixture containing (mM): NaCl 100, KCl 20, $MgCl_2$ 5, ATP 3 and Tris-HCl 50; pH 7.4. The reaction procedure was the same as described by Ohizumi & Yasumoto (1983a).

Statistical analysis of the data

The data on mechanical response, chemical assay of Ca and enzyme activity are expressed as the mean ± s.e.mean. Statistical comparisons were made using Student's *t* tests.

Purification of maitotoxin

The dinoflagellate (*Gambierdiscus toxicus*) was extracted with hot methanol and the methanol extract was chromatographed on silicic acid and ODS columns to give highly purified MTX having the minimum lethal dose in mice of 0.2 μg kg^{-1}, i.p. The details of purification of MTX have been published (Yasumoto *et al.*, 1979; Yasumoto, 1980). In the present paper, the concentration of MTX was expressed as g ml^{-1} because its molecular weight has not been determined. MTX was dissolved in distilled water at concentrations of 1–100 μg ml^{-1} and was kept frozen as stock solutions.

Drugs

The following drugs were used: TTX (Sankyo Company, Ltd., Tokyo, Japan), verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan), propranolol

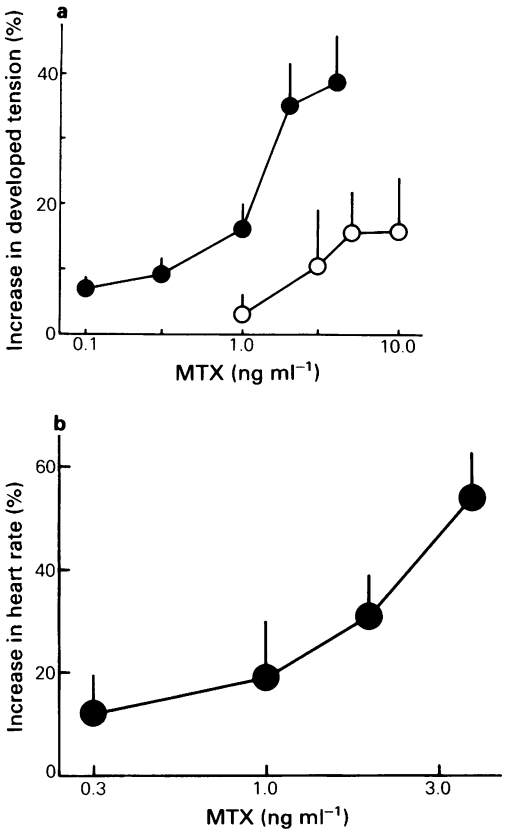

Figure 1 The log concentration-contractile response curves for maitotoxin (MTX). (a) Effect on the developed tension of the guinea-pig left atria (●) and the rat ventricle strips (○). (b) Effect on the heart rate of the spontaneously beating guinea-pig right atria. Vertical lines indicate s.e.mean ($n = 6$).

(Sumitomo Chemical Co. Ltd., Osaka, Japan) and reserpine (apoplone; Daiichi-Seiyaku Company, Ltd., Tokyo, Japan).

Results

Mechanical response

MTX produced a positive inotropic effect on guinea-pig isolated left atria. As shown in Figure 1a, the contractile force of the atria increased with MTX concentrations in the range of 0.1 to 4 ng ml^{-1} in a dose-dependent manner. In this concentration range, the effect of MTX continued for over 1 h without an increase in diastolic tension of the atria. In the rat ventricle strips, MTX also caused a dose-dependent increase in the developed tension at concentrations of 1–10 ng ml^{-1}. The 50% effective doses of MTX were 1 ng ml^{-1} for the left atria and 2 ng ml^{-1} for the ventricles (Figure 1a). These results indicate the approximate equipotent effects of MTX on both tissues. The maximum increase in developed tension of the left atria was 39%, but that of the ventricle strips was only 16%. As shown in Figure 2, MTX (2 ng ml^{-1}) increased the developed tension by 166%, 34% and 14% in the low-Ca^{2+} (0.3 mM), normal (1.2 mM) and high-Ca^{2+} (3.0 mM) solutions, respectively. The MTX (2 ng ml^{-1})-induced increase in the contractile force was almost abolished by treatment with Co^{2+} (2 mM) or verapamil (3×10^{-7} M) in the low-Ca^{2+} and normal solutions. However, the elevation of the external Ca^{2+} concentration to 30 mMM abolished the inhibitory effect of Co^{2+} (2mM) or verapamil (3×10^{-7} M). In addition, after treatment

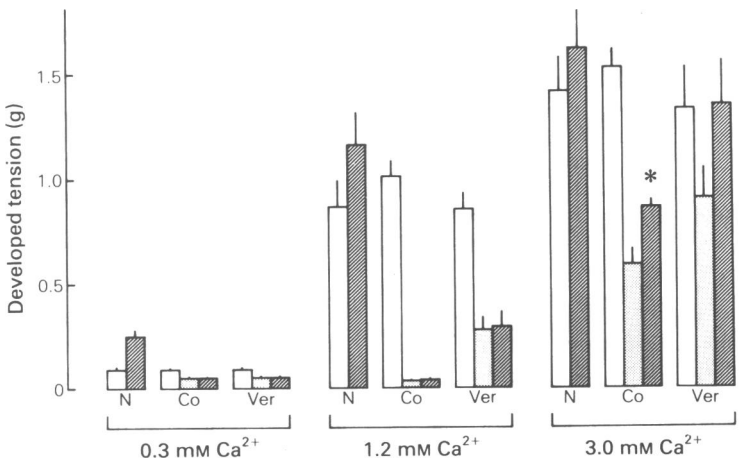

Figure 2 Effect of maitotoxin (MTX, 2 ng ml^{-1}) on the developed tension of the guinea-pig left atria in the presence or absence of Co^{2+} or verapamil. Open columns, control; stippled columns, after drug pretreatment; hatched columns, after MTX treatment. Pretreatment (15 min): N = none, Co = Co^{2+} (2 mM), Ver = verapamil (3×10^{-7} M). Vertical lines indicate s.e.mean ($n = 4$). *Significantly higher than the values before the application of MTX, $P < 0.05$.

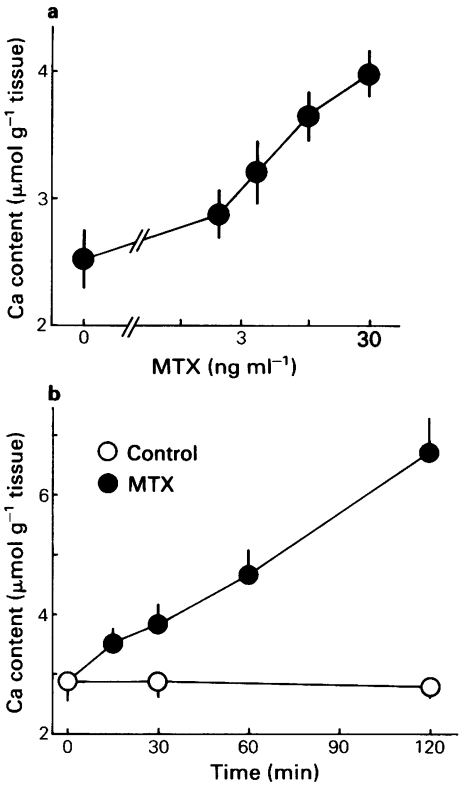

Figure 3 Effect of maitotoxin (MTX) on the tissue Ca content of the guinea-pig left atria. (a) The log dose-response curve for MTX treatment (30 min). (b) Time course of an increase in the Ca content induced by MTX (4 ng ml^{-1}) Vertical lines indicate s.e.mean ($n = 6$).

with TTX (5×10^{-7} M), propranolol (10^{-6} M) and reserpine (2 mg kg^{-1}, i.p.), MTX still increased the developed tension by 64, 42 and 96%, respectively. As shown in Figure 1b, MTX (0.3–4 ng ml^{-1}) caused a dose-dependent increase in the heart rate of the spontaneously beating right atria of the guinea-pig. In the right atria of reserpinized guinea-pigs, MTX (4 ng ml^{-1}) also caused a positive chronotropic effect (from 123.9 ± 8.2 (control) to 164.1 ± 5.9 beats min^{-1}).

Tissue Ca content

The effects of various concentrations of MTX on tissue Ca content of the guinea-pig left atria were examined after a 30 min application. As shown in Figure 3a, MTX (2–30 ng ml^{-1}) caused a dose-dependent increase in the tissue Ca content of the atria. After treatment with MTX (30 ng ml^{-1}), the Ca content was increased from 2.53 ± 0.24 (control) to 3.98 ± 0.18 μmol g^{-1} wet weight or 57% increase. Figure 3b shows the time course of an increase in the

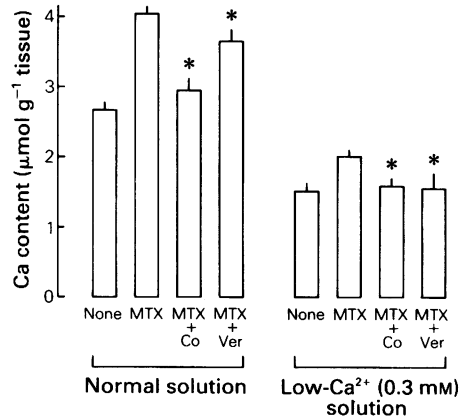

Figure 4 Effect of maitotoxin (MTX, 4 ng ml^{-1}) on the tissue Ca content of the guinea-pig left atria in the presence or absence of Co^{2+} or verapamil. Pretreatment (15 min): Co = Co^{2+} (2 mM), Ver = verapamil (1×10^{-5} M). Vertical lines indicate s.e.mean ($n = 6$). *Significantly different from MTX alone in each solution, $P < 0.05$.

tissue Ca content of the left atria after exposure to MTX (4 ng ml^{-1}). The tissue Ca content continued to increase linearly during 120 min after the application of MTX. As shown in Figure 4, in the normal solution MTX (4 ng ml^{-1}) increased the tissue Ca content by approximately 49%. The MTX (4 ng ml^{-1})-induced increase in the Ca content was inhibited approximately 78% and 25% by treatment with Co^{2+} (2 mM) and verapamil (1×10^{-5} M), respectively. When the concentration of external Ca^{2+} was decreased to 0.3 mM, MTX (4 ng ml^{-1}) elevated the Ca content 32% and this effect of MTX was suppressed about 86% and 93% by the administration of Co^{2+} (2 mM) and verapamil (1×10^{-5} M), respectively. In addition, in Na^+-free medium containing 0.3 mM $CaCl_2$ the Ca content was increased by MTX (4 ng ml^{-1}) from 1.651 ± 0.054 ($n = 10$) to 1.804 ± 0.036 ($n = 10$) μmol g^{-1} wet weight, i.e. a 9% increase.

Rat isolated cardiac myocytes

The myocardial cells isolated from adult rats were stimulated in a perfusion medium, and the time course change in the movement of the cells was observed by video recording. In the medium containing 2 mM Ca^{2+}, almost all the rod-shaped cells continued beating regularly in response to the electrical stimulation. On exposure to the medium containing concentrations of Ca^{2+} above 3 mM, most of the normally beating cells gradually started moving arrhythmically followed by irreversible contracture giving round cells. In the 2 mM-Ca^{2+} medium, the effects of MTX on the isolated cardiac myocytes were examined. As shown in Figure 5, at 10 min after the addition of MTX

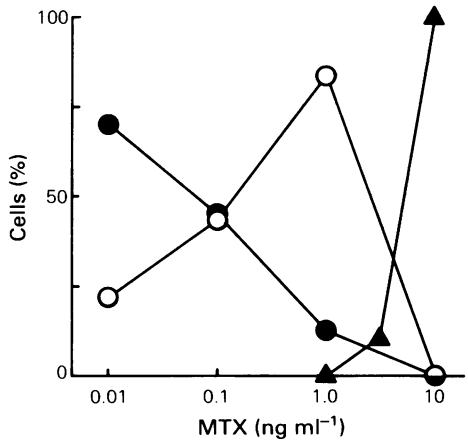

Figure 5 The log concentration-response curves for maitotoxin (MTX) on the percentages of regularly beating cells (●), arrhythmically moving cells (○) and irreversibly shortened cells (▲) among total rod-shaped myocardial cells ($n = 27-60$) isolated from adult rats. Each value is the mean of data from three different experiments.

(10 pg–10 ng ml^{-1}) the percentage of normally beating cells among total rod cells was decreased in a dose-dependent manner. The proportion of myocytes showing arrhythmic motions such as arbitrary beating and fibrillatory movement increased from 22 to 84% by increasing the MTX concentration from 10 pg to 1 ng ml^{-1}. After treatment with MTX (10 ng ml^{-1}), all the cells were irreversibly shortened (Figure 5). As shown in Table 1, when the rod cells were pretreated with verapamil (1×10^{-6} M) 10 min before the application of MTX (1 ng ml^{-1}), a delay was observed in the change of normally beating myocytes into arrhythmically moving cells and then into rounded cells. At 6 min after the addition of MTX (1 ng ml^{-1}), 84% of regularly beating cells turned into arrhythmically moving cells. However, in the verapamil-treated group, 40% of the cells remained beating normally (Table 1). Furthermore, in the absence of Ca^{2+} no apparent change was observed for 15 min following treatment with MTX (10 ng ml^{-1}). After addition of Ca^{2+} (2 mM) to the Ca^{2+}-free medium, almost all the myocytes transformed into irreversibly contracted cells via the arrhythmically moving state within 5 min.

Table 2 Effect of maitotoxin (MTX) or ouabain on Na^+,K^+-ATPase activity of guinea-pig heart

Treatment	*Inhibition*[a] (%)
MTX (1 ng ml^{-1})	7.6 ± 2.7
MTX (10 ng ml^{-1})	4.6 ± 2.0
MTX (100 ng ml^{-1})	4.9 ± 2.2
Ouabain (1×10^{-5} M)	79.8 ± 1.3

[a]Mean ± s.e.mean ($n = 3$)

Na^+,K^+-ATPase

The effect of MTX (1 ng to 100 ng ml^{-1}) and ouabain on the Na^+,K^+-ATPase activity using purified enzyme preparations isolated from guinea-pig heart muscle were examined. Ouabain (1×10^{-5} M) inhibited the Na^+,K^+-ATPase activity by approximately 80%, whereas MTX had little effect on the activity at concentrations up to 100 ng ml^{-1} (Table 2).

Discussion

MTX (0.1 to 4 ng ml^{-1}) caused a positive inotropic effect on the cardiac muscle of the guinea-pig isolated left atria and the rat ventricle strips. The effect of MTX was little affected by a β-adrenoceptor blocking drug (propranolol), a catecholamine depleting drug (reserpine) or a Na channel blocker (TTX). In addition, MTX produced a positive chronotropic effect on the isolated right atria, and a marked increase in the heart rate caused by MTX was also observed in reserpine-pretreated guinea-pigs. Furthermore, in rat isolated cardiac cells that are free from neural influence, MTX was still able to produce an increase in the degree and the rate of the contraction which were measured by a high-speed cine camera system (unpublished observations). These results would eliminate the possible

Table 1 Effect of maitotoxin (MTX) on rat isolated cardiac myocytes in the presence or absence of verapamil

Treatment	*% of Cells*[a] *Beating normally* (%)	*Moving arrhythmically* (%)
None	100	0
MTX (1 ng ml^{-1})	16.0 ± 2.2	84.0 ± 2.2
MTX (1 ng ml^{-1}) + verapamil (1×10^{-6} M)	40.0 ± 1.8	60.0 ± 1.8

[a]Mean ± s.e.mean (data from three different experiments).

involvement of an indirect action elicited by the release of catecholamines and direct activation of the β-adrenoceptor or the Na channel as a major mechanism of action of MTX.

It is generally accepted that organic Ca antagonists (such as verapamil and methoxyverapamil (D600)) as well as polyvalent cations (such as Co^{2+}, Mn^{2+}, Mg^{2+} and La^{3+}) selectively inhibit Ca channel function in the cardiac muscle (Fleckenstein, 1977). In the present experiments, the positive inotropic effect of MTX on the atria was abolished by Ca channel blockers, such as Co^{2+} and verapamil. In the left atria, MTX profoundly increased the tissue Ca content in the presence or absence of external Na^+, and this action of MTX was markedly inhibited by Co^{2+} or verapamil. Similarly, the ^{45}Ca uptake of the left atria was strikingly increased by MTX and this increase was abolished by treatment with Co^{2+} (unpublished observations). Furthermore, in the present experiments MTX caused an arrhythmogenic effect on the isolated cardiac myocytes, followed by their transformation into irreversibly shortened cells. The effects of MTX on the myocardial cells were inhibited or abolished by verapamil or the Ca^{2+}-free solution. In the rat isolated heart, it has been shown that MTX lengthens the action potential plateau and that the effect of MTX is abolished by Ca channel blockers (verapamil and Mn^{2+}) and low-Ca^{2+} solution. The increase in the duration of plateau by MTX, however, was not affected by a Na channel blocker (TTX) or a potassium conductance inhibitor (4-aminopyridine). These findings suggest that the MTX-induced lengthening of the action potential is due to an increase in Ca conductance of the cell membrane of cardiac muscles (Legrand & Bagnis, 1984b). In addition, it has been reported that MTX also causes Ca-dependent excitatory effects on smooth muscle (Ohizumi & Yasumoto, 1983a, b; Ohizumi *et al.*, 1983), skeletal muscle (Miyamoto *et al.*, 1984; Gomi *et al.*, 1984), cultured neuronal cells (Takahashi *et al.*, 1982; 1983; Freedman *et al.*, 1984) and hormone-releasing anterior pituitary cells (Schettini *et al.*, 1984). All these actions of MTX were inhibited or abolished by Ca channel blockers or a Ca^{2+}-free solution, suggesting the possible involvement of the activation of Ca channels in the mechanism of MTX-induced effects. Based on these observations, it is suggested that the excitatory effects of MTX on heart muscle are caused by a direct action on the cardiac muscle membrane mainly due to increasing Ca^{2+} permeability which occurs possibly through Ca channels.

It is well-known that, on exposure to high-Ca^{2+} medium, the intact cardiac myocytes begin to beat abnormally and then transform into irreversibly-contracted form resulting from an increase in the intracellular Ca^{2+} concentration (Dow *et al.*, 1981; Williamson *et al.*, 1983). In the present experiments, MTX caused a closely similar arrhythmogenic effect on the myocyte to that induced by a high-Ca^{2+} solution, and the effects of MTX were markedly suppressed by Ca channel blockers. These observations suggest that in the normal solution, MTX may produce such a rise in intracellular Ca^{2+} concentrations as occurs in the myocytes incubated in the high-Ca^{2+} medium without MTX.

It has been reported that A23187, a Ca ionophore, transports Ca across numerous membranes (Reed & Lardy, 1972; Caswell & Pressman, 1972; Garcia *et al.*, 1975; Cochrane *et al.*, 1975), and that the ionophore produces the positive inotropic effect on the isolated heart that appears to be mediated via increasing concentrations of Ca^{2+} (Holland *et al.*, 1975). However, MTX did not exhibit any ionophoretic activities on rat liver mitochondria and liposomes (Takahashi *et al.*, 1983), and the excitatory effect of MTX on the aorta and PC12h cells was competitively antagonized by verapamil, whereas that caused by A23187 was not affected by verapamil, (Ohizumi & Yasumoto, 1983a; Takahashi *et al.*, 1982). These observations suggest that the mechanism of action of MTX is quite different from that of the ionophores. On the other hand, ouabain, a Na^+,K^+-ATPase inhibitor, has been reported to increase the cardiac contractility which is accompanied by an increase in Ca^{2+} influx through the Na^+-Ca^{2+} exchange system (Langer & Serena, 1970; Langer, 1971; Akera & Brody, 1978). In the present experiments, MTX has little effect on the Na^+,K^+-ATPase activity from guinea-pig heart muscle. These observations suggest that the mode of positive inotropic action of MTX cannot be explained by the inhibition of the Na^+,K^+-pump.

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