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Ca^{2+}-dependent aggregation of rabbit platelets induced by maitotoxin, a potent marine toxin, isolated from a dinoflagellate

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1 Administration of maitotoxin (MTX), a dinoflagellate toxin, caused aggregation of rabbit washed platelets. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), measured by fura-2 fluorescence technique, was also increased by the presence of MTX. Rates of aggregation response and $[Ca^{2+}]_i$-increase were dependent on tested concentrations (3–100 ng ml^{-1}) of the toxin.

2 The MTX-induced platelet aggregation and $[Ca^{2+}]_i$-increase were totally abolished in a Ca^{2+}-free solution. The successive administration of Ca^{2+} in the presence of MTX elicited the aggregation and increase in $[Ca^{2+}]_i$.

3 Ba^{2+} was capable of substituting for Ca^{2+} in the MTX-induced platelet aggregation. In the presence of external Ca^{2+}, transition metals, Co^{2+}, Cd^{2+} and Ni^{2+}, inhibited the aggregation response to MTX.

4 Organic calcium antagonists (verapamil and nifedipine) as well as a cyclo-oxygenase-inhibitor (aspirin) did not apparently inhibit the aggregation response to MTX, except for a high concentration (10^{-5} M) of verapamil, while procaine (10 mM) reduced the rate of platelet aggregation.

5 MTX also elicited a release of ATP from platelets, which was abolished in the absence of external Ca^{2+}.

6 In contrast, thrombin 0.5 unit ml^{-1} could elicit platelet shape change, $[Ca^{2+}]_i$-increase and ATP-release in the absence of external Ca^{2+}.

7 These results suggest that the MTX-induced platelet activation is caused by an enhanced Ca^{2+}-influx presumably through voltage-independent Ca^{2+} channels on the plasma membrane.

Keywords: Maitotoxin; aggregation; rabbit platelets; cytosolic Ca^{2+} concentration; divalent cation

Introduction

Maitotoxin (MTX) is a water-soluble toxin isolated from the dinoflagellate *Gambierdiscus toxicus* which causes a seafood poisoning in tropical regions (Yasumoto *et al.*, 1979). The minimum lethal dose of the toxin is 0.13 μg kg^{-1} i.p. in mice, being approximately 50 times more potent than tetrodotoxin. The chemical structure of MTX has partly been elucidated with approximate Mr 3500 by mass spectroscopy (Yokoyama *et al.*, 1988): the molecule characteristically contains sulphate esters besides many hydroxy groups and ester rings along carbonyl or acetal/ketal structure.

Since we reported for the first time that MTX causes a Ca^{2+}-dependent release of noradrenaline from rat phaeochromocytoma cells (Takahashi *et al.*, 1982; 1983) and a Ca^{2+}-dependent contraction of smooth muscles (Ohizumi *et al.*, 1983; Ohizumi & Yasumoto, 1983a,b), various stimulatory effects of MTX have been revealed to be highly dependent on the external Ca^{2+} in smooth muscles (Berta *et al.*, 1988; Sladeczek *et al.*, 1988), insect skeletal muscle (Gomi *et al.*, 1984; Miyamoto *et al.*, 1984), neuroblastoma cells (Freedman *et al.*, 1984; Yoshii *et al.*, 1987), anterior pituitary cells (Login *et al.*, 1987), synaptosomes (Ueda *et al.*, 1986a,b), pancreatic islets (Niki *et al.*, 1986) and mussel spermatozoa (Nishiyama *et al.*, 1986). Most MTX-induced responses described above were sensitive to organic calcium antagonists and divalent cations such as verapamil and Co^{2+}. Thus, it has been suggested that MTX activates some Ca^{2+} channels on the plasma membrane. Recently, MTX has been reported to stimulate phosphoinositide breakdown in many cell types (Gusovsky & Daly, 1990). The MTX-induced stimulation of phospholipid metabolism was abolished in the absence of external Ca^{2+} (Sladeczek *et al.*, 1988; Gusovsky *et al.*, 1989).

In cardiac muscle of the rat ventricle, MTX has been reported to prolong the action potential plateau (Legrand & Bagnis, 1984). Further, MTX elicited arrythmogenic effects as well as positive inotropic effects in cardiac tissues and isolated cells (Kobayashi *et al.*, 1985a,b). The arrythmogenic changes were associated with a morphological damage of cardiac cells (Kobayashi *et al.*, 1986). Applying patch-clump techniques to the guinea-pig myocytes, we have demonstrated that the Co^{2+}-sensitive Ca^{2+}-channels activated by MTX are quite different from normal voltage-gated Ca^{2+}-channels with respect to the current-voltage relationship, unitary conductance and mean open time of channels (Kobayashi *et al.*, 1987a,b). Recently, in rat ventricular myocytes, MTX has also been reported to elicit a transient activation of voltage-gated Ca^{2+} current and an increase in voltage-independent current carried by Ca^{2+} and Na^{+} (Faivre *et al.*, 1990).

Platelets are activated by many substances like thrombin, noradrenaline, etc (for a review, see Siess, 1989). However, the type and nature of Ca^{2+} channels in platelets have not been well clarified. Doyle & Rüegg (1985) reported that depolarization of platelets with 50 mM KCl did not induce Ca^{2+} entry into platelets and that a Ca^{2+} channel agonist Bay K 8644 did not affect the Ca^{2+} uptake or the cytosolic Ca^{2+} concentration of platelets. Further, a binding assay using [^{3}H]-nimodipine did not show any specific site for binding of the Ca^{2+}-channel antagonist (Grossmann *et al.*, 1992). These studies suggest that activation of platelets occurs through the operation of voltage-independent Ca^{2+} channels as well as the Ca^{2+} liberation from the cellular store. Thus, we have investigated how MTX affects Ca^{2+}

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metabolism of platelets by measuring fura-2-fluorescence and aggregation of the rabbit platelets. We show here that MTX-induced activation of rabbit washed platelets is strictly dependent on the presence of external Ca^{2+} and that the toxin-induced activation is insensitive to organic calcium antagonists at their specific concentrations for the inhibition of voltage-gated Ca^{2+} channels. Part of this work was presented at the 64th Annual Meeting of the Japanese Pharmaceutical Society at Kobe, Japan (Watanabe *et al.*, 1991).

Methods

Preparation of platelets

Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2–3 kg), collected into 77 mM EDTA, pH 7.4, and subsequently centrifuged at 950 *g* for 5 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 1800 *g* for 5 min. The pellet was washed twice by suspension and centrifugation (1800 *g*, 5 min). The resultant pellet was resuspended in the Ca^{2+}-free Tyrode-HEPES solution with a final density of approximately 2×10^5 cells μl^{-1}. The number of platelets was counted by use of a fluorescence microscope and haemocytometer. The first suspension solution for washing platelets contained: NaCl 140, 4-morpholinepropanesulphonic acid (MOPS) 12 and EDTA 1.2 (mM) at pH 7.4. The second solution did not contain EDTA. The Tyrode-HEPES solution was composed of NaCl 136.9, KCl 5.4, $MgCl_2$ 0.53, $NaHCO_3$ 11.9, NaH_2PO_4 0.33, glucose 11.0 and HEPES 5.0 (mM), pH 7.4.

Measurements of aggregation and cytosolic Ca^{2+} concentration of platelets

For loading fura-2 to platelets, the platelet pellet prepared as described above was resuspended in the Tyrode-HEPES solution containing 0.5 μM fura-2/AM and incubation for 10 min at 37°C and was subsequently washed with the Tyrode-HEPES solution to remove extracellular fura-2/AM. When platelets were exposed to fura-2/AM for more than 10 min, the rate of aggregation induced by agents was much smaller compared to those of non-loaded platelets, presumably due to the known buffering effect of fura-2 on $[Ca^{2+}]_i$ (Pollock & Rink, 1986). Fura-2-Ca^{2+} signal and aggregation were measured with a spectrofluorometer (CAF-100, Japan Spectroscopic, Tokyo, Japan), specially designed for the simultaneous measurement of fluorescence and light transmission. Platelets were exposed to excitation light having wavelengths of 340 and 380 nm and emission was measured at 500 nm. The platelet suspension (0.3 ml) in a siliconized cuvette was prewarmed at least for 1 min at 37°C in the spectrofluorometer. Then, Ca^{2+} at the final concentration of 1 mM was added. Five min after the Ca^{2+}-addition, MTX or thrombin was administered and platelet responses were monitored for a maximum of 30 min. Effects of Ca^{2+}-removal on platelet responses were examined without the addition of Ca^{2+}. At the end of experiments, the maximum and minimum fluorescence levels were determined with 0.2% Triton X-100 and 3 mM ethyleneglycol*bis*(β-aminoethyl)-N',N'-tetraacetic acid (EGTA) at pH 8.2, respectively, and the $[Ca^{2+}]_i$ was calculated by use of the fura-2-Ca^{2+} dissociation constant of 224 nM as reported by Pollock *et al.* (1986). Aggregation response was determined by a standard turbidimetric method and was expressed as % of light transmission. Attained levels and rates of aggregation and $[Ca^{2+}]_i$-increase were taken as parameters of the platelet responses. The rate of aggregation represents the maximum slope of the response determined with a ruler by eye and is expressed as % min^{-1}. The rate of $[Ca^{2+}]_i$-increase is also estimated by the maximum slope of the response and is expressed as the change in the fluorescence ratio excited by 340 to 380 nm per min. In some instances, the rate of $[Ca^{2+}]_i$-increase was expressed by $[Ca^{2+}]_i$-change min^{-1}.

In some experiments, the effect of divalent cations or various pharmacological blockers on platelet aggregation without loading fura-2 was examined by the turbidimetric technique using an aggregometer (PAM-6C, Mebanix). Various cations and blockers were added 4 min before administration of MTX. Ba^{2+}-substitution was performed by the addition of 1 mM Ba^{2+} instead of Ca^{2+} after 1 min equilibration of the platelet suspension.

Determination of ATP released from platelets

Purified luciferin-luciferase was added to a tube containing 0.3 ml washed platelet solution. The tube was placed in a dark chamber of a photon counter (Biocounter 2000, Lumac B.V./3M, The Netherlands). After equilibration for 10 min at 37°C, MTX was added and the yielded light was determined in the presence and absence of 1 mM Ca^{2+}. For standardization, known amounts of ATP (10 nmol–10 μmol) were added to the 0.3 ml medium with luciferin-luciferase. When luciferase was exposed to ATP, light intensity reached a peak in 1 s and it decayed only less than 10% of the peak intensity during 10 min observation time. The light intensity was linearly correlated to the amounts of ATP.

Drugs

The following drugs were used: verapamil hydrochloride (Sigma, St Louis, MO, U.S.A.), nifedipine (Sigma), procaine hydrochloride (Daiichi Seiyaku, Tokyo, Japan), fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), luciferin-luciferase reagent (Lumac B.V./3M). MTX was a gift of Professor T. Yasumoto, Tohoku University.

Statistical comparison

Results of the experiments are expressed as mean ± s.e.mean. Student's *t* test and paired *t* test were used for stastistical analysis of the results.

Results

Effects of maitotoxin and thrombin on aggregation and $[Ca^{2+}]_i$

Aggregation responses and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) changes of the fura-2-loaded washed platelets of the rabbit were monitored simultaneously. Administration of MTX (3–100 ng ml^{-1}) elicited aggregation of the platelets after a lag time (Figures 1 and 2a,b). During the lag time, light transmission often decreased transiently, presumably reflecting a shape change of platelets (Milton & Frojmovic, 1983). The lag time was dependent on MTX-concentrations tested. MTX, 100 ng ml^{-1}, elicited aggregation with a similar time course to 30 ng ml^{-1} of the toxin (data not shown). MTX at 1 ng ml^{-1} failed to cause aggregation during 30 min observation time. MTX at concentrations of 10–100 ng ml^{-1} elicited nearly maximum aggregation. The aggregation response to 3 ng ml^{-1} seemed to be submaximal in Figure 2a, but this might be due to the limitation of reaction time for 30 min because of the variance of turbidimetric response in time. At the termination of the reaction the aggregation response to 3 ng ml^{-1} MTX continued to increase as shown in Figure 1. Thus, it is suggested that, once MTX triggers aggregation, platelets aggregate completely. Compared to the finally attained level of aggregation, the rate of aggregation was dependent on the concentration of MTX.

Figure 1 (lower panel) shows that MTX (3–30 ng ml^{-1}) elicited a gradual increase in $[Ca^{2+}]_i$ of the fura-2-loaded platelets. The onset of $[Ca^{2+}]_i$-increase was prompt after administration of the toxin. The rate of $[Ca^{2+}]_i$-increase was

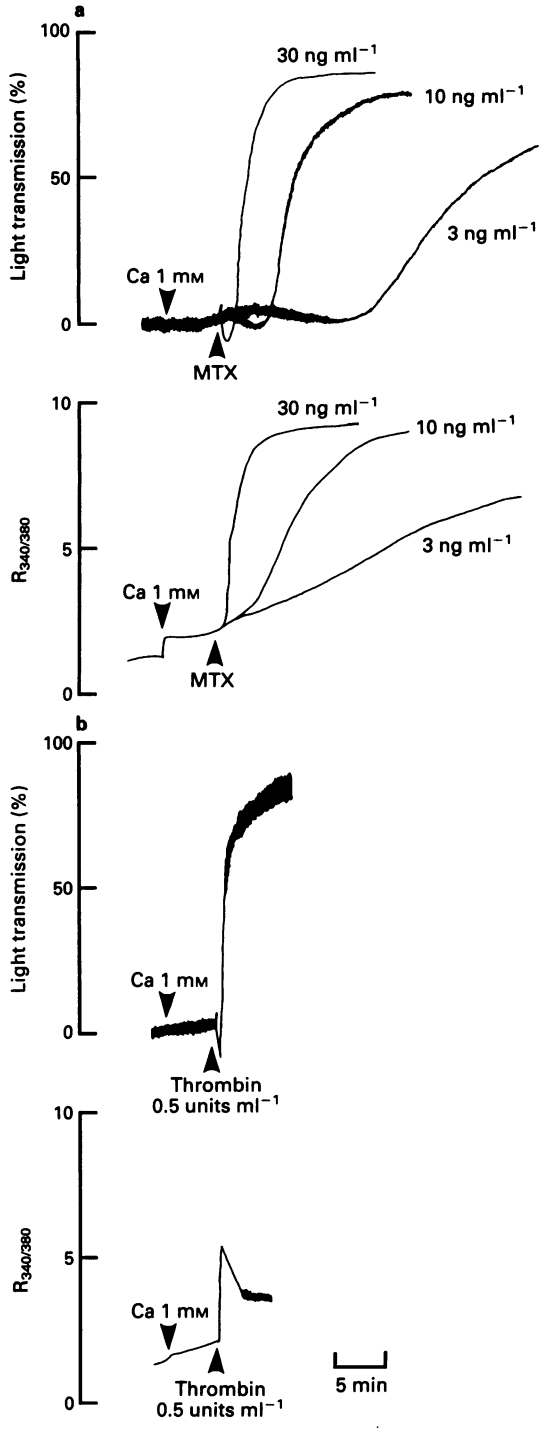

Figure 1 Simultaneous recordings of aggregation (upper panel) and fura-2 fluorescence (lower panel) of the rabbit washed platelets in response to maitotoxin (MTX) and thrombin. MTX 3–30 ng ml^{-1} (a) and thrombin 0.5 units ml^{-1} (b) were applied 5 min after addition of 1 mM $CaCl_2$ to the nominally Ca^{2+}-free solution. R340/380 in the ordinate of the lower panel represents the ratio of fluorescence intensity excited by 340 nm to 380 nm. Maximum and minimum values for R340/380 when platelets were exposed to Triton X-100 and EGTA were as follows: 9.33 and 0.66 in (a), and 11.78 and 0.80 in (b), respectively.

dependent on the MTX concentration rather than the finally raised level of $[Ca^{2+}]_i$ and it seemed to be associated with the rate of platelet aggregation rather than the lag period (Figure 2c vs 2b). As in the aggregation response, 100 ng ml^{-1} MTX elicited the $[Ca^{2+}]_i$-increase with a similar time course to that induced by 30 ng ml^{-1}, and 1 ng ml^{-1} failed to raised $[Ca^{2+}]_i$ (data not shown). The cytosolic Ca^{2+} concentrations before

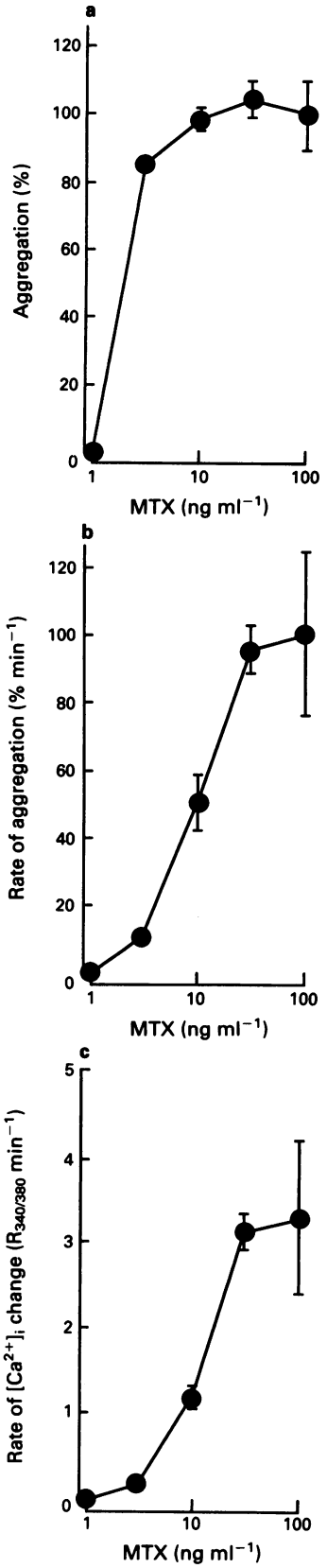

Figure 2 The concentration-response relationships for maitotoxin (MTX) on aggregation level (a), aggregation rate (b) and rate of fura-2-signal increase (c) in the rabbit platelets. Aggregation response was measured by turbidimetric method. In (a) and (b), the responses to 100 ng ml^{-1} MTX were taken as 100%. The maximum level of light transmission in the presence of 30 ng ml^{-1} MTX was 83.3 ± 2.64% (n = 4) and the rate of light transmission change after the administration of 30 ng ml^{-1} MTX was 40.5 ± 4.85% min^{-1} (n = 4). In (c), the rate of $[Ca^{2+}]_i$ change was given as the change of fura-2 fluorescence ratio (R340/380) per min. The rate of $[Ca^{2+}]_i$ change after administration of 30 ng ml^{-1} MTX was 272.6 ± 80.1 nM min^{-1} (n = 4). Values are mean with s.e.mean (n = 3–5).

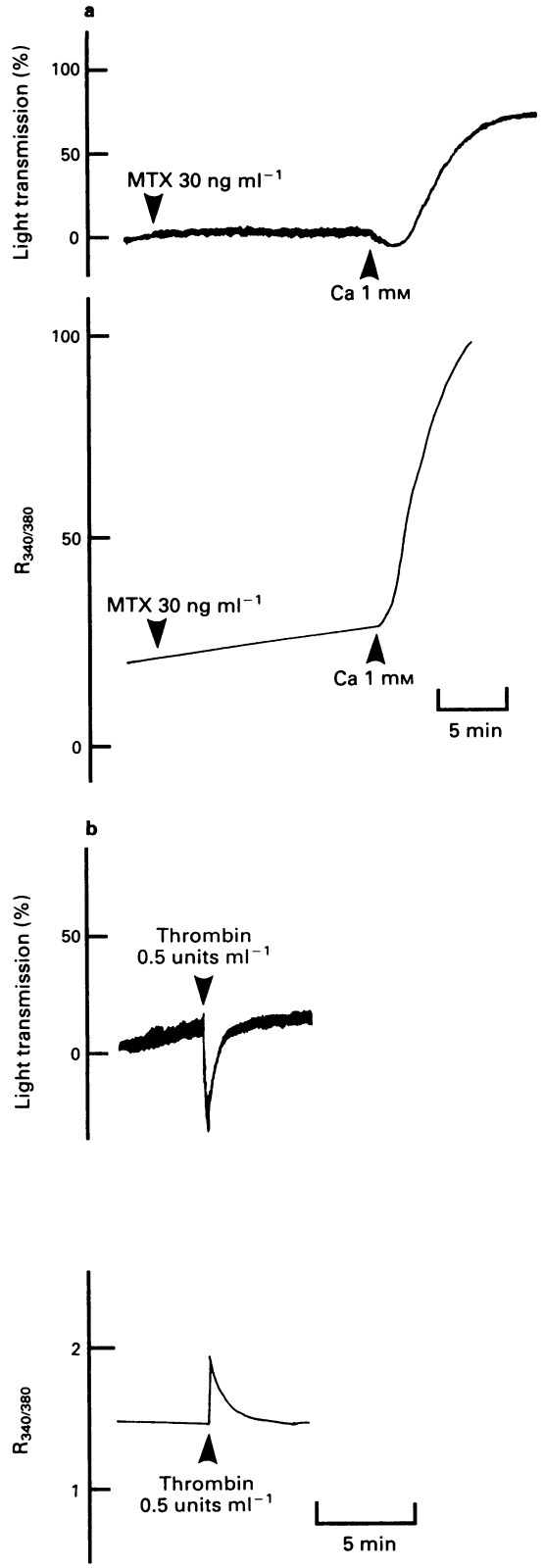

Figure 3 Effects of maitotoxin (MTX) 30 ng ml^{-1} (a) and thrombin 0.5 units ml^{-1} (b) on aggregation (upper panel) and fura-2-signal (lower panel) of the platelets in the absence of external Ca^{2+}. In (a) $CaCl_2$ (1 mM) was added 15 min after administration of 30 ng ml^{-1} MTX. Maximum and minimum values for R340/380 were as follows: 10.50 and 0.56 in (a), and 5.56 and 0.57 in (b), respectively.

stimulation and at 90% of the maximum level in the presence of 10–100 ng ml^{-1} MTX were 77.9 ± 11.6 (n = 12) and 2713.5 ± 130.5 nM (n = 12), respectively. The final level of $[Ca^{2+}]_i$ in the presence of MTX could not be estimated since the Ca^{2+}-fura-2 fluorescence signal in the presence of MTX was equivalent to that after application of Triton X-100.

For comparison of MTX-induced response with receptor-mediated response, we also investigated the effects of thrombin on aggregation and $[Ca^{2+}]_i$ of the rabbit washed platelets (Figure 1b). Thrombin 0.5 unit ml^{-1} elicited a transient decrease followed by a rapid increase in light transmission, indicating the shape change followed by aggregation of platelets. On the other hand, 0.5 unit ml^{-1} thrombin raised $[Ca^{2+}]_i$ with a rapid transient phase followed by a relatively small plateau phase: $[Ca^{2+}]_i$ was 298.0 ± 124.6 μM (n = 4) for the transient phase or 217.8 ± 124.6 μM (n = 4) for the plateau phase. These results indicate that MTX is much more effective in raising $[Ca^{2+}]_i$ of platelets than thrombin.

Effects of varying external Ca^2 concentration and Ba^{2+}-substitution for Ca^{2+} on platelet responses

When fura-2-load platelets were exposed to Ca^{2+}-free solution, administration of 30 ng ml^{-1} MTX elicited neither aggregation nor $[Ca^{2+}]_i$-increase (Figure 3). The subsequent addition of 1 mM Ca^{2+} elicited a transient decrease followed by a rapid increase in light transmission, suggesting the shape change followed by the aggregation of platelets (Figure 3). Fura-2-fluorescence signal showed that $[Ca^{2+}]_i$ of platelets started to increase promptly after the Ca^{2+} addition in the presence of MTX (Figure 3).

When external Ca^{2+} was replaced by Ba^{2+} (1 mM), administration of MTX 30 ng ml^{-1} elicited aggregation of the platelet. The rate of MTX-induced aggregation in the presence of Ba^{2+} was approximately 45% lower than in the presence of Ca^{2+}, while aggregation was nearly maximal (Table 1). This suggests that, like many other Ca^{2+}-dependent responses of a number of cells (review: Evans, 1988), Ba^{2+} is capable of substituting for Ca^{2+} in the MTX-induced aggregation response of the platelet.

Figure 4 shows concentration-response relationships for external Ca^{2+} in the MTX (30 ng ml^{-1})-induced aggregation and $[Ca^{2+}]_i$-increase of the fura-2-loaded platelets. Rates of aggregation were small at external Ca^{2+} concentrations less than 0.3 mM and dose-dependently increased at 1 and 3 mM Ca^{2+}. Similarly, the rates of $[Ca^{2+}]_i$-increase were small at external Ca^{2+} concentrations less than 0.3 mM and markedly increased at 1 and 3 mM Ca^{2+}. On the other hand, attained levels of aggregation seemed to be dose-dependent on Ca^{2+} concentrations less than 0.1 mM and reached nearly maximum at higher concentrations between 0.1 and 3 mM. Apparently, this Ca^{2+}-concentration-aggregation relationship is affected by the limited reaction time of 30 min, as in the case of MTX-induced response in normal media. From these results it appears that the platelet aggregation response to MTX is markedly accelerated by the presence of external Ca^{2+} concentrations over 0.3 mM.

In contrast to MTX, administration of thrombin 0.5 units ml^{-1} to the Ca^{2+}-free solution elicited a transient increase in $[Ca^{2+}]_i$ associated with a transient decrease in light transmis-

Table 1 Maitotoxin (MTX, 30 ng ml^{-1})-induced aggregation of the rabbit washed platelet in the presence of Ca^{2+} (1 mM) or Ba^{2+} (1 mM)

	Aggregation (%)	*Rate of aggregation* (%)
Ca^{2+}	100	100
Ba^{2+}	88.1 ± 0.4*	54.9 ± 8.7**

Platelet aggregation was measured by the turbidimetric method. To examine the effects of Ba^{2+} on platelet aggregation, Ba^{2+} was added to the medium instead of Ca^{2+}. Responses in the presence of Ca^{2+} were taken as 100% and the responses in the presence of Ba^{2+} were compared by paired experiments. Values are given as mean ± s.e.mean (n = 3 or 4).
Significantly different value from control (Ca^{2+}) by paired t test at *P < 0.01 and **P < 0.05, respectively.

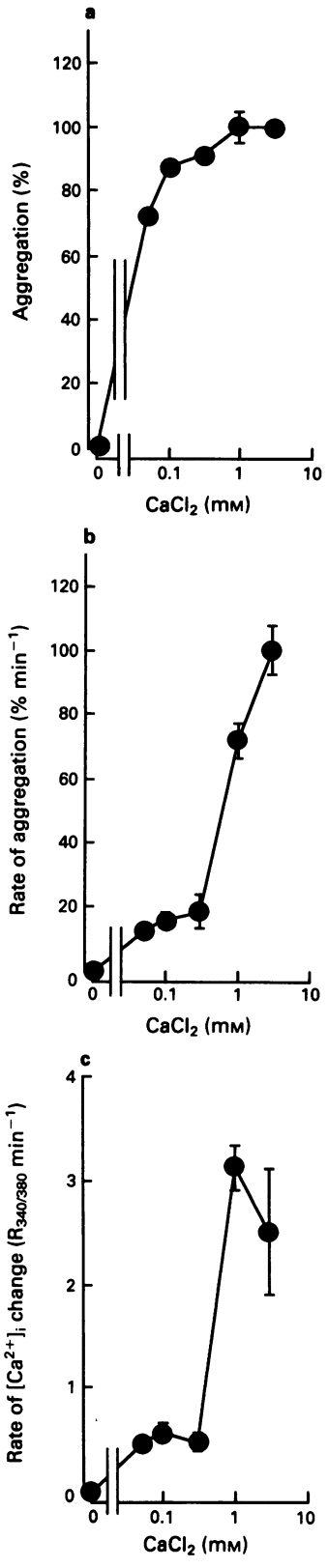

Figure 4 Effects of various external Ca^{2+} concentrations on aggregation level (a), aggregation rate (b) and rate of fura-2-signal increase (c) of the platelets in the presence of maitotoxin (MTX) 30 ng ml^{-1}. In (a) and (b), the aggregation responses to 3 mM Ca^{2+} were taken as 100%. Values are mean with s.e.mean. ($n = 3-6$). The maximum level of light transmission in the presence of 3 mM Ca^{2+} was 85.1 ± 1.79% ($n = 4$) and the rate of light transmission change after addition of 3 mM Ca^{2+} was 66.0 ± 5.00% min^{-1} ($n = 4$). In (c), the rate of $[Ca^{2+}]_i$ change was given as the change of fura-2 fluorescence ratio (R340/380) per min. The rate of $[Ca^{2+}]_i$ change after addition of 3 mM Ca^{2+} MTX was 536.4 ± 108.3 nM min^{-1} ($n = 4$).

sion (Figure 3). Similarly, Pollock & Rink (1986) reported the thrombin-induced responses of aggregation and $[Ca^{2+}]_i$ in a Ca^{2+}-free solution. They observed a small aggregation response in a Ca^{2+}-free solution. This difference may be due to the difference in cellular fura-2-concentrations or may be due to species differences, rabbit washed platelets in our studies and human platelets in theirs. These results suggest that thrombin, but not MTX, could activate the platelet aggregation process through the release of Ca^{2+} sequestered at some cellular sites.

Inhibitory effects of divalent ions and various blockers on platelet aggregation

Since Co^{2+}, Cd^{2+} and Ni^{2+} are known to interfere with a Ca^{2+}-influx through Ca^{2+} channels at the plasma membrane (Evans, 1988), we investigated effects of these divalent cations on MTX (30 ng ml^{-1})-induced platelet aggregation in the presence of 1 mM Ca^{2+} (Table 2). Co^{2+} and Cd^{2+} (both 0.3 mM) significantly inhibited the platelet aggregation by approximately 20–30% of control. The rate of aggregation was also inhibited by Co^{2+} and Cd^{2+}. In the presence of Ni^{2+} (1 mM), MTX apparently elicited a smaller aggregation at a slower rate than control. However, we could not examine effects of higher concentrations of Co^{2+} and Cd^{2+}, since their presence at 1 mM elicited aggregation by themselves (data not shown). These results suggest that the transition metals tested could interfere with the platelet aggregating response to MTX.

We further investigated effects of organic Ca^{2+} antagonists (verapamil and nifedipine) and a non-specific channel blocker (procaine) (review: Godfraind *et al.*, 1988) on MTX-induced platelet aggregation (Table 3). Pretreatments with verapamil and nifedipine (both 10^{-6} M) had no significant effects on the extent or rate of the aggregation response to 30 ng ml^{-1} MTX. However, 10^{-5} M verapamil inhibited the rate of ag-

Table 2 Effects of transition metals, Cd^{2+}, Co^{2+} and Ni^{2+}, on maitotoxin (MTX, 30 ng ml^{-1})-induced platelet aggregation

	Aggregation (%)	*Rate of aggregation* (%)
Control	100	100
Cd^{2+} (0.3 mM)	71.7 ± 3.0***	62.4 ± 13.3*
Co^{2+} (0.3 mM)	77.5 ± 3.4***	69.5 ± 20.5*
Ni^{2+} (1.0 mM)	62.4 ± 7.9***	22.2 ± 7.1***

Aggregation responses to MTX in the presence of 1 mM Ca^{2+} were taken as 100% (control). Transition metals were added in the presence of Ca^{2+} before administration of MTX. Values are given as mean ± s.e.mean ($n = 6$ or 8). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, by paired t test when compared to control.

Table 3 Effects of verapamil (10^{-6} and 10^{-5} M), nifedipine (10^{-6} M), procaine (10 mM) and aspirin (10^{-5} M) on maitotoxin (MTX, 30 ng ml^{-1})-induced platelet aggregation

	Aggregation (%)	*Rate of aggregation* (%)
Control	100	100
Verapamil (10^{-6} M)	96.2 ± 2.9	80.6 ± 7.6
Verapamil (10^{-5} M)	95.2 ± 5.7	44.1 ± 3.7*
Nifedipine (10^{-6} M)	100.3 ± 2.5	101.4 ± 7.5
Procaine (10 mM)	99.4 ± 2.7	19.8 ± 1.8*
Aspirin (10^{-5} M)	100.0 ± 2.5	91.6 ± 5.8

Tested agents were added in the presence of 1 mM Ca^{2+} before administration of MTX. Control responses in the medium without agents are expressed as 100%. Values are given as mean ± s.e.mean ($n = 6$ or 8).
*$P < 0.001$, by paired t test when compared to control.

gregation to approximately 45% of control, but did not significantly affect the maximum aggregation response to MTX. This verapamil-induced inhibition may be due to a non-specific action rather than the inhibition of voltage-gated Ca^{2+} channels, since its concentration exceeds the specific range (Godfraind *et al.*, 1986). Similarly, the rate of aggregation, but not the maximum aggregation, was markedly inhibited by the presence of 10 mM procaine.

We also investiated the effects of aspirin, a cyclo-oxygenase inhibitor (Smith & Willis, 1971; Hamberg *et al.*, 1974). However, pretreatment with 10^{-5} M aspirin did not affect the platelet aggregation responses to 30 ng ml^{-1} MTX (Table 3).

Effects of maitotoxin and thrombin on ATP release from platelets

When platelets are activated, several substances including ATP are released from dense granules (Holmsen *et al.*, 1969). Thus, effects of MTX on ATP release were investigated in comparison to the thrombin-induced response (Figure 5). MTX 3–100 ng ml^{-1} as well as thrombin 0.5 units ml^{-1} elicited a transient increase in external ATP concentration measured with luciferase, indicating that both agents release ATP from platelets. The rate of increase and the peak of released ATP concentration were dependent on MTX concentrations in a similar range to those causing aggregation and $[Ca^{2+}]_i$-increase of the platelets. In the Ca^{2+}-free solution, MTX failed to release ATP, whereas thrombin still released ATP (Figure 5) as reported by Grette (1962). These results suggest that both aggregation and secretion following MTX activation of platelets is highly dependent on the presence of external Ca^{2+}.

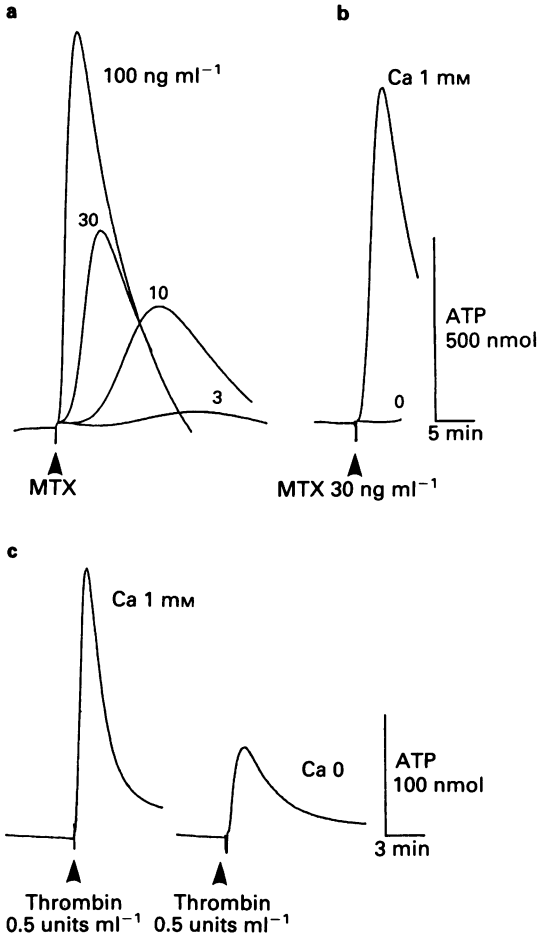

Figure 5 Effects of maitotoxin (MTX) (a and b) and thrombin (c) on ATP secretion of the rabbit platelets determined with luciferin-luciferase. In (a), various concentrations (3–100 ng ml^{-1}) of MTX were administered in the presence of 1 mM Ca^{2+}. (b) and (c) show a different set of experiments representing the effects of 30 ng ml^{-1} MTX and 0.5 units ml^{-1} thrombin on ATP secretion in the presence and absence of 1 mM Ca^{2+}, respectively.

Discussion

The present experiments demonstrate that MTX is capable of eliciting a shape change followed by aggregation as well as an ATP-secretion in the rabbit washed platelet. These stimulatory effects of MTX are specifically characterized by the very strict dependency on the presence of external Ca^{2+}. In the absence of external Ca^{2+}, MTX failed to cause any changes in the platelet function described above. While, many other stimulants such as thrombin, ADP, etc. could activate platelets through the release of intracellular Ca^{2+} (for reviews: see Siess, 1989; Rink & Sage, 1990). Ionophores like ionomycin and A23187 elicit an increase in $[Ca^{2+}]_i$ of platelets in the absence of external Ca^{2+} (Rink *et al.*, 1982; Thompson & Scrutton, 1985). However, MTX was reported to have no ionophore-like action on mitochondrial and liposomal membranes (Takahashi *et al.*, 1983). Thus, it is suggested that MTX activates platelets at the level of plasma membrane in a different manner from ionophore-induced activation. Further, Ca^{2+}-influx across the plasma membrane may be solely responsible for the MTX-induced activation of platelets.

By use of fura-2, MTX was shown to elicit a marked increase in $[Ca^{2+}]_i$ which was totally abolished in the absence of external Ca^{2+}. MTX at effective concentrations of 3–100 ng ml^{-1} raised the fluorescence signal induced by Ca^{2+}-fura-2 complex nearly to the maximum, suggesting that $[Ca^{2+}]_i$ reaches more than 3×10^{-6} M in the presence of MTX. For comparison, thrombin at the concentration which elicited the maximum aggregation raised the fluorescence signal to 50% of the maximum at the most effective case, i.e. $[Ca]_i < 10^{-6}$ M. Further, lower concentrations of MTX elicited slower increases in $[Ca^{2+}]_i$ and aggregation, but activated both to the maximum. These results indicate that once MTX activates platelets, the activation does not cease, resulting in the massive increase in $[Ca^{2+}]_i$ even at the lower concentrations. Presumably, such a long-lasting activation by MTX is consistent with the facts that MTX elicits the increase in Ca^{2+} content of smooth muscles (Ohizumi & Yasumoto, 1983a,b) and the marked prolongation of mean open time of Ca^{2+} channels in cardiac myocytes (Kobayashi *et al.*, 1987a).

The MTX-induced aggregation was inhibited by the presence of transition metals (Co^{2+}, Cd^{2+} and Ni^{2+}). When external Ca^{2+} was replaced by Ba^{2+}, MTX could cause the maximum aggregation. On the other hand, organic Ca^{2+} antagonists did not inhibit aggregation except at the highest concentration of verapamil. These results are comparable to the reported aggregation responses of platelets to agonists such as thrombin and ADP with respect to the transition metal-sensitivity, Ba^{2+}-substitution, and organic Ca^{2+} antagonist-ineffectiveness (Hallam & Rink, 1985; Blache *et al.*, 1985; Jy & Haynes, 1987; Sage & Rink, 1987; Avdonin *et al.*, 1988; Zschauer *et al.*, 1988). The fact that procaine significantly slowed the platelet aggregation response to MTX may be explained by its non-specific inhibition of Ca^{2+} influx through the plasma membrane (Godfraind *et al.*, 1986). Also, considering the previously reported facts that MTX could stimulate voltage-independent Ca^{2+} channels in cardiac muscle (Kobayashi *et al.*, 1987a,b; Faivre *et al.*, 1990) and that platelets may lack voltage-gated Ca^{2+} channels (reviews: Siess, 1989; Rink & Sage, 1990), it is suggested that MTX, like receptor agonists, is capable of activating voltage-independent Ca^{2+} channels on the plasma membrane of platelets.

In contrast, MTX-induced aggregation was not affected by the presence of aspirin. The irreversible platelet aggregation due to the stimulation of receptors by high concentrations of agonists is known to be amplified by a reaction of cyclo-oxygenase (Hamberg *et al.*, 1974; 1975), which is effectively blocked by the presence of aspirin (Smith & Willis, 1971; Hamberg *et al.*, 1974). These results suggest that if identical Ca^{2+} channels are responsible for both MTX and agonists, MTX acts more directly on the Ca^{2+} channels than the agonists do. Alternatively, MTX may activate other voltage-independent Ca^{2+} channels than those for agonists. Although the identification of specific Ca^{2+} channels for MTX awaits a molecular study, MTX may be a good tool for analysing the voltage-independent Ca^{2+} channels especially in platelets, since MTX did not affect the process of Ca^{2+} release from cellular stores and its effective concentration is very low (1–10 nM) based on the recently estimated molecular weight of 3500 (Yokoyama *et al.*, 1988).

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