Activation of rabbit platelets by Ca^{2+} influx and thromboxane A_2 release in an external Ca^{2+} -dependent manner by zooxanthellatoxin-A, a novel polyol

Mun-Chual Rho, Norimichi Nakahata, *Hideshi Nakamura, *Akio Murai & ¹Yasushi Ohizumi

Department of Pharmaceutical Molecular Biology, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980, Japan and *Department of Chemistry, Faculty of Sciences, Hokkaido University, Sapporo 060, Japan

1 Zooxanthellatoxin-A (ZT-A), a novel polyhydroxylated long chain compound, isolated from a symbiotic marine alga *Symbiodinium* sp., caused aggregation in rabbit washed platelets in a concentration-dependent manner (1–4 μM), accompanied by an increase in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$).

2 ZT-A did not cause platelet aggregation or increase $[\text{Ca}^{2+}]_i$ in a Ca^{2+} -free solution, and Cd^{2+} (0.1–1 mM), Co^{2+} (1–10 mM) and Mn^{2+} (1–10 mM) inhibited ZT-A-induced aggregation. SK&F96365 (1–100 μM), a receptor operated Ca^{2+} channel antagonist, and mefenamic acid (0.1–10 μM), a non-specific divalent cation channel antagonist, inhibited platelet aggregation and the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A.

3 Indomethacin (0.1–10 μM), a cyclo-oxygenase inhibitor, and SQ-29548 (0.1–10 μM), a thromboxane A_2 (TXA_2) receptor antagonist, inhibited platelet aggregation and the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A.

4 Methysergide (0.01–1 μM), a 5-HT₂ receptor antagonist, inhibited ZT-A-induced platelet aggregation but did not affect the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A.

5 Tetrodotoxin (1 μM), a Na^+ channel blocker and chlorpheniramine (1 μM), a H₁-histamine receptor antagonist, neither affected ZT-A-induced platelet aggregation nor the increase in $[\text{Ca}^{2+}]_i$ induced by ZT-A.

6 Genistein (1–100 μM), a protein tyrosine kinase inhibitor, and staurosporine (0.01–1 μM), a protein kinase C inhibitor, also inhibited ZT-A-induced platelet aggregation.

7 The present results suggest that ZT-A elicits Ca^{2+} -influx from platelet plasma membranes. The resulting increase in $[\text{Ca}^{2+}]_i$ subsequently stimulates the secondary release of TXA_2 from platelets. Furthermore, the response to ZT-A may be associated with tyrosine phosphorylation.

Keywords: Aggregation; platelets; Ca^{2+} ; zooxanthellatoxin-A; cytosolic free Ca^{2+} ; thromboxane A_2

Introduction

Platelets which play a role in thrombus formation, haemostasis and regeneration of vessels respond to a wide variety of stimuli. These small disc-shaped cells recognize even minor damages on the surface of the endothelial cells lining blood vessels and promptly respond to the physical or chemical stimuli by undergoing adhesion, shape change, secretion and aggregation. Such a series of cell responses ends in the formation of a haemostatic plug or thrombus (Nozawa *et al.*, 1991). Platelets have frequently been used as a potentially useful model for studying transmembrane signalling since they are highly responsive to various agents and also influence active biochemical and morphological events.

Recently, a number of natural products have been isolated from marine organisms and have been extensively studied by numerous investigators. Marine products such as palytoxin (Ishida *et al.*, 1985; Hirata *et al.*, 1988) and maitotoxin (MTX) (Kobayashi *et al.*, 1987; Gusovsky & Daly, 1990; Watanabe *et al.*, 1993) have proved to be useful chemical tools for helping to resolve the mechanism of cellular functions.

Zoosanthellatoxin-A (ZT-A) and -B, novel polyhydroxylated long chain compounds, were recently isolated from a symbiotic marine alga *Symbiodinium* sp. as potent vasoconstrictor substances (Nakamura *et al.*, 1993a,b; 1995). They were characterized as relatively large molecules (molecular weight 2,872 Da) containing a large number of oxygen

atoms and olefinic carbons. These chemical characters are different from those of other marine toxins such as palytoxin and MTX which exhibit potent vasoconstrictor activities and contain a large number of olefins and a small number of ethereal rings, respectively (Asari *et al.*, 1993; Nakamura *et al.*, 1993a).

Although ZT-A is known to contract vascular smooth muscle, it is unknown whether it activates platelets. Thus, we have investigated the effects of ZT-A on aggregation and Ca^{2+} metabolism in rabbit platelets. This is the first report concerning the pharmacological properties of ZT-A in rabbit washed platelets.

Methods

Isolation of ZT-A

ZT-A was isolated as described previously (Nakamura *et al.*, 1993b). The cultured dinoflagellate (*Symbiodinium* sp.) was extracted three times by suspension with 70% ethanol and the ethanol extract (22.9 g) was subsequently dissolved in water and extracted with ethyl acetate, followed by n-butanol. The n-butanol extracts (5.8 g) were fractionated on a polystyrene column (MCI gel CHP-20P, 75–150 μm , Mitsubishi Chemical Industries Ltd., Tokyo, Japan). The 40% ethanol eluate was chromatographed on a diethyl amino ethyl Sepadex A-25 (1/30 M phosphate buffer, pH 6.9). The eluate was applied to a polystyrene column, eluted with 80% ethanol, and then sepa-

¹ Author for correspondence.

rated by a reversed phase high performance liquid chromatography column (YMC Pack D-ODS-5, 2 cm $\times$ 20 cm, YMC Ltd., Kyoto, Japan) using 4:1 methanol-water containing 0.2 M NaCl (9 ml min⁻¹), to give two active substances named ZT-A and -B, after desalting with a polystyrene column. The negative fast atom bombardment mass spectrum of ZT-A exhibited a predominant ion peak at m/z 2872. Figure 1 shows the full structure of ZT-A. This compound has characteristic functions such as a diepoxide, two conjugated dienes, macrolactone ring, a sulphate ester and an exomethylene (Nakamura *et al.*, 1995).

Preparation of washed platelets

Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2–3 kg), collected into plastic tubes containing acid citrate dextrose solution (1/6 volume of blood) composed of citric acid (65 mM), trisodium citrate (85 mM), and dextrose (2%) at pH 4.5, subsequently centrifuged at 250 *g* for 10 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 650 *g* for 10 min at room temperature (20–25°C). The pellet was washed twice with Tyrode/4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) solution (pH 6.35). The resultant pellet was resuspended in the second Tyrode/HEPES solution (pH 7.35) with a final density of approximately 5×10^8 cells ml⁻¹. The Tyrode/HEPES solution was composed of (mM): NaCl 138.3, KCl 2.68, MgCl₂ 6H₂O 1.0, NaHCO₃ 4.0, HEPES 10, glucose 0.1% (w/v) and albumin 0.35% (w/v) at pH 6.35 or 7.35.

Determination of platelet aggregation

Platelet aggregation was determined by a standard turbidometric method (Born, 1962) in an aggregometer (PAM-6C, Merbanix, Tokyo, Japan). Platelet aggregation was expressed as an increase in light transmission. The levels of light transmission were calibrated as 0% for a platelet suspension and 100% for the Tyrode/HEPES solution. Platelet suspension (0.3 ml) in the aggregometer cuvette was preincubated for 5 min at 37°C under continuous stirring at 1000 r.p.m. and

then CaCl₂ or ethylene glycol *bis*(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was added at the final concentration of 1 mM. After 5 min, ZT-A was added and platelet aggregation was monitored for 10 min. Divalent cation and various blockers were preincubated for 5 min before the addition of ZT-A.

Measurement of intracellular Ca²⁺ concentration

For loading fura-2 into platelets, the platelet pellets prepared as described above were resuspended in the Tyrode/HEPES solution (pH 7.35) containing 1 μ M fura-2/AM and incubated for 15 min at 37°C. Platelets were subsequently washed with the Tyrode/HEPES solution (pH 7.35) to remove extracellular fura-2/AM. After the number of platelets was adjusted to 1×10^8 ml⁻¹, the fura-2-Ca²⁺ signal was measured with a fluorescence spectrophotometer (F-2000, Hitachi). Platelets were exposed at excitation light with wavelengths at 340/380 nm and the light emission was measured at 510 nm. The platelet suspension (1.5 ml) in a quartz cuvette was preincubated for 2 min at 37°C in the spectrophotometer. Two min after the addition of CaCl₂ at a final concentration of 1 mM, ZT-A was added and the responses were monitored for a maximum of 10 min. Various blockers were preincubated for 1 min before the addition of ZT-A.

Radioimmunoassay of thromboxane B₂

The release of thromboxane B₂ (TXB₂) from rabbit platelets was determined by radioimmunoassay (Matsuoka *et al.*, 1989). In brief, platelet aggregation was terminated by addition of an equal volume of ice-cold 50 μ M indomethacin/50 mM ethylenediaminetetraacetic acid solution and the sample solution was centrifuged at 1700 *g* for 10 min at 4°C. The assay mixture of 0.1 ml sample (1700 *g* supernatant) or standard (0.1–3000 ng ml⁻¹), 0.1 ml antiserum against TXB₂ (20,000 times dilution) and 0.1 ml [³H]-TXB₂ (10 nCi) was incubated overnight at 4°C. [³H]-TXB₂ bound to antiserum was assayed in a liquid scintillation spectrophotometer after the sedimentation of free [³H]-TXB₂ with dextran-coated charcoal.

Figure 1 Chemical structure of zooxanthellatoxin-A (ZT-A).

Materials

The following drugs were used; verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan), mefenamic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan), tetrodotoxin (Sankyo Co. Ltd., Tokyo, Japan), indomethacin (Merck Company Inc., Rahway, NJ, U.S.A.), [1S-[1 α ,2 β (5Z),3 β ,4 α]-7-[3-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2,1]hepta-2-yl]-5-heptenoic acid (SQ-29548, Squibb Japan Inc., Tokyo, Japan), methysergide (Sandoz Pharmaceuticals, Hanover, NJ, U.S.A.), genistein (Wako Pure Chemical Industries Ltd., Osaka, Japan), staurosporine (Wako Pure Chemical Industries Ltd., Osaka, Japan), chlorpheniramine maleate (Sankyo Co. Ltd., Tokyo, Japan), maitotoxin (Wako Pure Chemical Industries Ltd., Osaka, Japan), bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.). [3 H]-thromboxane B₂ (110.6 Ci mmol⁻¹) was obtained from DuPont/NEN (Boston, MA, U.S.A.). Anti-thromboxane B₂ serum was given to us by ONO Pharmaceutical (Osaka, Japan). 1-{ β -[3-4-methoxyphenyl]propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride (SK&F96365) was provided by Smith Kline Beecham (London, England). All other chemicals were of analytical grade.

Data analysis

The data are presented as mean $\pm$ s.e.mean. Statistical comparisons were made by Student's *t* test.

Results

Effects of ZT-A on platelet aggregation and [Ca²⁺]_i

ZT-A elicited platelet aggregation after a lag time of 10–90 s (Figure 2a). During the lag time, light transmission often decreased transiently, presumably reflecting a platelet shape change (Milton & Frojmovic 1983). The lag time was dependent on the concentration of ZT-A tested (Figure 2a). ZT-A-induced aggregation was concentration-dependent with an EC₅₀ value of 1.85 μ M (Figure 3). On the other hand, ZT-A (0.5–4 μ M) elicited an increase in [Ca²⁺]_i of the fura-2-loaded platelets (Figure 2b). The increase in [Ca²⁺]_i was dependent on concentrations of ZT-A with the EC₅₀ of 1.7 μ M, which was similar to that for platelet aggregation (Figure 3). While ZT-A at a concentration of 1 μ M failed to cause platelet aggregation, it significantly increased [Ca²⁺]_i, suggesting that a slight increase in [Ca²⁺]_i was not enough to cause aggregation.

Effects of external Ca²⁺ concentrations and Ca²⁺ channel blockers on ZT-A-induced aggregation and increase in [Ca²⁺]_i

ZT-A could not induce platelet aggregation nor increase [Ca²⁺]_i in a Ca²⁺-free solution (Figure 4). ZT-A caused aggregation to a small extent below 0.3 mM external Ca²⁺ (Figure 4a) and the ZT-A-induced increase in [Ca²⁺]_i was dependent on external Ca²⁺ concentrations from 0.3 mM to 3 mM (Figure 4b). Figure 5 shows the external Ca²⁺-dependency of ZT-A (2 μ M)-induced platelet aggregation and increase in [Ca²⁺]_i. Since Co²⁺, Cd²⁺ and Mn²⁺ are known to interfere with Ca²⁺ influx through Ca²⁺ channels at the plasma membrane (Evans, 1988), we investigated the effects of these divalent cations on the platelet aggregation induced by ZT-A (2 μ M) in the presence of 1 mM Ca²⁺ (Figure 6). Cd²⁺ (0.1–1 mM), Co²⁺ (1–10 mM) and Mn²⁺ (1–10 mM) inhibited platelet aggregation induced by ZT-A (2 μ M) (Figure 6). We further investigated the effects of verapamil, an organic Ca²⁺ antagonist, mefenamic acid, a non selective Ca²⁺ channel blocker, SK&F96365, a receptor-operated Ca²⁺ entry blocker and tetrodotoxin, a Na⁺ channel blocker (Figure 7). The platelet aggregation induced by ZT-A was slightly inhibited by verapamil (10 μ M). SK&F96365 (100 μ M) and mefenamic acid

(10 μ M) inhibited the aggregation induced by ZT-A by approximately 70–90% of control (Figure 7a), suggesting that platelet aggregation in response to ZT-A requires an increase

Figure 2 Typical recordings of aggregation (a) and increase in [Ca²⁺]_i (b) of rabbit washed platelets in response to zooxanthellatoxin-A (ZT-A). ZT-A (0.1–4 μ M) was applied 5 (a) or 2 min (b) after addition of 1 mM CaCl₂ to the nominally Ca²⁺-free solution. Aggregation response was measured by a turbidometric method. R340/380 on the ordinate scale of the lower panel represents the ratio of fluorescence intensity at 510 nm excited by 340 nm to 380 nm.

Figure 3 The concentration-response relationships for zooxanthellatoxin-A (ZT-A, 1–10 μ M) on aggregation level (□) and increase in [Ca²⁺]_i (○). Platelet aggregation or [Ca²⁺]_i change in response to ZT-A (4 μ M) in the presence of 1 mM Ca²⁺ was taken as 100%. The level of light transmission induced by 4 μ M ZT-A was 75.9% $\pm$ 1.55 (*n* = 4). The ratio of [Ca²⁺]_i change after addition of 4 μ M ZT-A was 2.14 $\pm$ 0.161 (*n* = 4). Values are given as mean $\pm$ s.e.mean (*n* = 4).

Figure 4 Typical recordings of the concentration-response relationships for external Ca^{2+} on zooxanthellatoxin-A (ZT-A)-induced aggregation (a) and increase in $[Ca^{2+}]_i$ (b). ZT-A ($2 \mu M$) was applied to 5 (a) or 2 min (b) after addition of $CaCl_2$ (0.3–3.0 mM) to the nominally Ca^{2+} -free solution. EGTA (1 mM) was added instead of $CaCl_2$. R340/380 on the ordinate scale of (b) represents the ratio of fluorescence intensity at 510 nm excited by 340 nm to 380 nm.

Figure 5 Effects of various external Ca^{2+} concentrations ($[Ca^{2+}]_o$) on platelet aggregation ($\square$) and increase in $[Ca^{2+}]_i$ ($\circ$) induced by zooxanthellatoxin-A (ZT-A). ZT-A ($2 \mu M$) was applied 5 ($\square$) or 2 ($\circ$) min after addition of $CaCl_2$ (0.1–10.0 mM) to the nominally Ca^{2+} -free solution. EGTA (1 mM) was added instead of $CaCl_2$. Platelet aggregation or $[Ca^{2+}]_i$ change induced by ZT-A ($2 \mu M$) in the presence of 3 mM Ca^{2+} was taken as 100%. The maximum level of light transmission in the presence of 3 mM $CaCl_2$ was 74.7 ± 2.33 ($n=4$). Values are given as mean $\pm$ s.e.mean ($n=4$).

in $[Ca^{2+}]_i$ and therefore implying that ZT-A stimulates Ca^{2+} influx. The inhibition of ZT-A-induced platelet aggregation by the above drugs was concentration-dependent, and the IC_{50} values of the drugs are shown in Table 1. ZT-A-induced increase in $[Ca^{2+}]_i$ was also inhibited by SK&F96365 ($100 \mu M$)

Figure 6 Effects of transition metals on zooxanthellatoxin-A (ZT-A)-induced platelet aggregation. Cd^{2+} ($\square$), Co^{2+} ($\circ$) or Mn^{2+} ($\blacksquare$) was added 5 min before addition of ZT-A in the presence of 1 mM Ca^{2+} . ZT-A ($2 \mu M$)-induced aggregation in the presence of 1 mM Ca^{2+} was taken as 100% (control). Values are given as mean $\pm$ s.e.mean ($n=4$).

Figure 7 Effects of Ca^{2+} channel blockers on zooxanthellatoxin-A (ZT-A, $2 \mu M$)-induced platelet aggregation (a) and increase in $[Ca^{2+}]_i$ (b). Aggregation or $[Ca^{2+}]_i$ change induced by ZT-A ($2 \mu M$) in the presence of 1 mM Ca^{2+} was taken as 100% (control). The following drugs were applied 5 (a) or 2 min (b) before addition of ZT-A in the presence of 1 mM Ca^{2+} : verapamil (Ver, $10 \mu M$), SK&F96365 (SK, $100 \mu M$), mefenamic acid (MEA, $10 \mu M$), tetrodotoxin (TTX, $1 \mu M$). Values are given as mean $\pm$ s.e.mean ($n=4-6$). Significant difference ($*P<0.05$) by paired t test when compared to control.

and mefenamic acid ($10 \mu M$), but not verapamil ($10 \mu M$) (Figure 7b). Tetrodotoxin ($1 \mu M$) did not affect either ZT-A-

induced platelet aggregation or increase in $[Ca^{2+}]_i$, suggesting no involvement of Na^+ -influx in the action of ZT-A (Figure 7).

Table 1 IC_{50} values of various drugs in zooxanthellatoxin-A (ZT-A)-induced aggregation

Drug	IC_{50} (μM)
Verapamil	10.9 ± 3.20
SK&F96365	28.0 ± 11.7
Mefenamic acid	6.60 ± 1.40
Indomethacin	5.12 ± 1.52
SQ-29548	0.57 ± 0.25
Methysergide	0.39 ± 0.14
Genistein	20.1 ± 7.31
Staurosporine	0.20 ± 0.07

Drugs were preincubated with rabbit platelet suspensions 5 min before the addition of ZT-A in the presence of 1 mM Ca^{2+} . ZT-A ($2 \mu M$)-induced aggregation in the presence of 1 mM Ca^{2+} was taken as 100% (control). Each IC_{50} was obtained from 3–4 separate concentrations of the drugs. Values are given as mean $\pm$ s.e.mean ($n=3-5$).

Figure 8 Effects of receptor antagonists and protein kinase inhibitors on zooxanthellatoxin-A (ZT-A, $2 \mu M$)-induced aggregation (a) and increase in $[Ca^{2+}]_i$ (b). Aggregation or $[Ca^{2+}]_i$ change induced by ZT-A ($2 \mu M$) in the presence of 1 mM Ca^{2+} was taken as 100% (control). The following drugs were applied 5 (a) or 2 min (b) before addition of ZT-A in the presence of 1 mM Ca^{2+} : indomethacin (Ind, $10 \mu M$), SQ-29548 (SQ, $10 \mu M$), methysergide (Met, $1 \mu M$), genistein (Gen, $100 \mu M$), staurosporine (Sta, $0.1 \mu M$), chlorpheniramine (Chl, $1 \mu M$). Values are given as mean $\pm$ s.e.mean ($n=4-6$). Significant difference ($*P<0.05$) by paired t test when compared to control.

Effects of receptor antagonists, a cyclo-oxygenase inhibitor and protein kinase inhibitors on ZT-A-induced aggregation and increase in $[Ca^{2+}]_i$

We next investigated several receptor antagonists on ZT-A ($2 \mu M$)-induced aggregation (Figure 8). ZT-A-induced platelet aggregation was inhibited by SQ-29548 ($10 \mu M$), a TXA_2 receptor antagonist, and methysergide ($1 \mu M$), a $5-HT_2$ receptor antagonist, suggesting the involvement of TXA_2 and $5-HT$ in ZT-A-induced aggregation (Figure 8a). Indomethacin, a cyclo-oxygenase inhibitor also inhibited ZT-A-induced aggregation, supporting the results obtained with SQ-29548 (Figure 8a). SQ-29548 and indomethacin also significantly reduced ZT-A-induced increase in $[Ca^{2+}]_i$ (Figure 8b). However, methysergide did not affect ZT-A-induced increase in $[Ca^{2+}]_i$ (Figure 8b). Chlorpheniramine ($1 \mu M$), a H_1 -histamine receptor antagonist, neither affected ZT-A-induced platelet aggregation nor the increase in $[Ca^{2+}]_i$ (Figure 8b).

Furthermore, we investigated the effects of protein kinase inhibitors on ZT-A ($2 \mu M$)-induced platelet aggregation (Figure 8a) and increase in $[Ca^{2+}]_i$ (Figure 8b) in the presence of external Ca^{2+} . ZT-A-induced aggregation and increase in $[Ca^{2+}]_i$ were inhibited by genistein ($100 \mu M$), suggesting the involvement of tyrosine kinase in ZT-A action. Staurosporine, a protein kinase C (PKC) inhibitor, inhibited ZT-A-induced aggregation (Figure 8a), but not the increase in $[Ca^{2+}]_i$ (Figure 8b). Inhibitory effects of indomethacin, SQ-29548, methysergide, genistein and staurosporine on ZT-A-induced aggregation were concentration-dependent, and the IC_{50} values of these agents are shown in Table 1.

Effects of ZT-A on TXB_2 release from rabbit platelets

Since SQ-29548 and indomethacin inhibited ZT-A-induced platelet aggregation, TXA_2 would be involved in ZT-A action. Thus, the effect of ZT-A ($2 \mu M$) on the release of TXB_2 , a stable metabolite of TXA_2 , was investigated in the presence or absence of external Ca^{2+} . ZT-A stimulated TXB_2 release from platelets in the presence of 1 mM Ca^{2+} (Figure 9). However, ZT-A failed to release TXB_2 in a Ca^{2+} -free solution (Figure 9).

Figure 9 Effect of zooxanthellatoxin-A (ZT-A, $2 \mu M$) on thromboxane B_2 (TXB_2) release from rabbit platelets determined with radioimmunoassay. ZT-A was added 5 min after addition of 1 mM $CaCl_2$ to the nominally Ca^{2+} -free solution. EGTA (1 mM) was added instead of $CaCl_2$. Reaction was terminated 2 min after addition of ZT-A by ice-cold 50 μM indomethacin/50 mM ethylenediaminetetraacetic acid solution. Values are given as mean $\pm$ s.e.mean ($n=3$). Significant difference ($**P<0.01$) by paired t test when compared to control.

Figure 10 Inhibitory effect of indomethacin on zooxanthellatoxin-A (ZT-A)- or maitotoxin (MTX)-induced platelet aggregation. Aggregation response was measured by turbidometric method. Indomethacin ($10 \mu\text{M}$) was added 5 min before addition of ZT-A ($2 \mu\text{M}$) or MTX (30 ng ml^{-1}) in the presence of 1 mM Ca^{2+} . Data are representative of three separate experiments.

Effect of a cyclo-oxygenase inhibitor on ZT-A- or MTX-induced platelet aggregation

ZT-A-induced platelet aggregation is similar to MTX-induced platelet aggregation in the dependency on external Ca^{2+} . We compared the effects of indomethacin ($10 \mu\text{M}$), a cyclo-oxygenase inhibitor on MTX (30 ng ml^{-1})-induced platelet aggregation and ZT-A ($2 \mu\text{M}$)-induced platelet aggregation (Figure 10). ZT-A-induced platelet aggregation was significantly inhibited by indomethacin ($10 \mu\text{M}$) as already shown in Figure 8 and Table 1, but MTX-induced platelet aggregation was scarcely inhibited by indomethacin, showing the different contribution of cyclo-oxygenase metabolites to ZT-A- and MTX-induced platelet aggregation.

Discussion

The present experiments demonstrate that ZT-A is capable of eliciting an initial shape change followed by a prolonged aggregation accompanied by an increase in $[\text{Ca}^{2+}]_i$ in rabbit washed platelets. These stimulatory effects of ZT-A are specifically characterized by the very strict dependency on the presence of external Ca^{2+} and by the involvement of TXA_2 . The Ca^{2+} influx across the plasma membrane may be solely responsible for ZT-A-induced aggregation of platelets. Thus, ZT-A-induced platelet aggregation was inhibited by inorganic Ca^{2+} channel antagonists such as Cd^{2+} , Co^{2+} and Mn^{2+} (Evans, 1988). Doyle & Regg (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+} influx or $[\text{Ca}^{2+}]_i$ in platelets, showing that platelets may lack voltage-gated Ca^{2+} channels (Siess, 1989;

Rink & Sage, 1990). Although verapamil inhibited ZT-A-induced aggregation, the 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). In addition, mefenamic acid, a non selective Ca^{2+} channel blocker, and SK&F96365, a receptor-operated Ca^{2+} entry blocker also inhibited aggregation and Ca^{2+} entry induced by ZT-A (Figure 7a). These results suggest that ZT-A-induced aggregation occurs through the operation of voltage-independent Ca^{2+} channels in rabbit platelets. Alternatively, ZT-A may activate a new voltage-independent Ca^{2+} channel.

Platelet stimulating agents such as the Ca^{2+} ionophore A23187, collagen and U46619 induce the liberation of arachidonic acid (AA) from membrane phospholipids (Rittenhouse, 1984; Hanasaki & Arita, 1988). Once released, AA is rapidly converted to biologically active metabolites by the actions of cyclo-oxygenase and lipoxygenase enzymes (Siess *et al.*, 1983). In platelets, AA may be converted to prostaglandin endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2 by cyclo-oxygenase, then to TXA_2 by TXA_2 synthase (Hamberg *et al.*, 1975). TXA_2 , a predominant metabolite of AA in platelets, is a very potent inducer of platelet aggregation (Ogletree, 1987). TXA_2 activates phospholipase C via a G protein of $\text{G}_{q/11}$ family (Baldassare *et al.*, 1993), resulting in generation of the two second messengers, inositol-1,4,5-trisphosphate, a Ca^{2+} mobilizer, and diacylglycerol, a PKC activator (Wilson *et al.*, 1985; Priess *et al.*, 1986). Our results showing that indomethacin, a cyclo-oxygenase inhibitor and SQ-29548, a TXA_2 receptor antagonist inhibited the platelet aggregation induced by ZT-A indicate that ZT-A also activates TXA_2 generation after Ca^{2+} entry and that the released TXA_2 activates platelets to induce aggregation. In fact, ZT-A released TXA_2 from platelets (Figure 9). Methysergide, a 5-HT₂ receptor antagonist, potentially inhibited the aggregation induced by ZT-A, showing a possible involvement of 5-HT in ZT-A-induced platelet aggregation. However, methysergide did not affect ZT-A-induced increase in $[\text{Ca}^{2+}]_i$. The reason for this discrepancy remains unknown.

Platelets possess a high level of tyrosine kinase activity, most of which is attributed to pp 60^{src} (Golden *et al.*, 1986; Feder & Bishop, 1990). Activation of platelets by several agonists increases the level of tyrosine phosphorylation resulting in the appearance of a new set of tyrosine-phosphorylated proteins (Ferrel & Martin, 1988; Golden & Bruggs, 1989; Nakamura & Yamamura, 1989; Bachelot *et al.*, 1992). Fibrinogen binding to the glycoprotein IIb/IIIa which plays a critical role in platelet aggregation appears to regulate protein tyrosine phosphorylation (Ferrel & Martin, 1989; Golden *et al.*, 1990). Tyrosine phosphorylation may also be induced by the elevation of $[\text{Ca}^{2+}]_i$ or by Ca^{2+} depletion in intracellular Ca^{2+} storage sites (Vostal *et al.*, 1991; Paul *et al.*, 1993). These findings suggest the possible involvement of tyrosine kinase in the signal transduction system in platelets, although the precise role of tyrosine phosphorylation in platelet activation remains elusive. Genistein inhibited the increase in $[\text{Ca}^{2+}]_i$ in response to ZT-A, as well as platelet aggregation, indicating the importance of tyrosine kinase in Ca^{2+} entry.

Staurosporine was originally considered as a protein kinase C (PKC) inhibitor (Tamaoki *et al.*, 1986). Recent studies indicate staurosporine inhibits a variety of tyrosine kinase (Daniel *et al.*, 1994) as well as other serine-threonine kinases including protein kinase A (PKA) (Spacey *et al.*, 1990; Tamaoki, 1991; Rasouly & Lazarovici, 1994). If staurosporine inhibits PKA activity, ZT-A-induced platelet aggregation may be enhanced. However, staurosporine inhibited ZT-A-induced aggregation, but it did not affect Ca^{2+} mobilization (Figure 9). Therefore, PKA might not be involved in the action of staurosporine in the ZT-A-induced platelet activation and PKC might be involved in secondary activation after Ca^{2+} entry by ZT-A.

Although ZT-A activates platelets in a similar manner to a Ca^{2+} ionophore, ZT-A is not a Ca^{2+} ionophore. Ionomycin, a

Ca^{2+} ionophore elicits an increase in $[\text{Ca}^{2+}]_i$ of platelets in the absence of external Ca^{2+} (Rink *et al.*, 1982), but ZT-A could not increase $[\text{Ca}^{2+}]_i$ in the absence of external Ca^{2+} . ZT-A-induced platelet aggregation is similar to MTX-induced platelet aggregation, but ZT-A has different characteristics from MTX. MTX-induced platelet aggregation was inhibited by the presence of Co^{2+} (0.3 mM) or Cd^{2+} (0.3 mM) (Watanabe *et al.*, 1993). Although ZT-A-induced platelet aggregation was inhibited by Cd^{2+} , Co^{2+} and Mn^{2+} , effective concentration of Co^{2+} was different from the effective concentration on MTX-induced aggregation (Figure 6). Furthermore, ZT-A-induced platelet aggregation was inhibited by treatment of a cyclooxygenase inhibitor as described above, but MTX-induced platelet aggregation was not affected (Figure 10). Thus, ZT-A activates platelets in a different manner from a Ca^{2+} ionophore or MTX.

In conclusion, ZT-A elicits Ca^{2+} -influx in platelet plasma membranes. The resulting increase in cytosolic Ca^{2+} appears to stimulate the release of TXA_2 , which thereafter activates platelets to cause aggregation. The Ca^{2+} -influx may be associated with tyrosine phosphorylation.

We are very grateful to Squibb Japan for providing us with SQ-29548, to Smith Kline Beecham for providing us with SK&F96365 and to ONO Pharmaceutical for providing us with anti-TXB₂ serum. This work was supported by a Grant-in-Aid from Scientific Research (No. 04304048, 05271102 and 05671805 to N.N. and No. 05152011, 05256203, 05454567 and 05557103 to Y.O.) from the Ministry of Education, Science and Culture of Japan, and by a Grant-in-Aid from the Marine Biotechnology Institute and Sapporo Bioscience Foundation.

References

- ASARI, T., NAKAMURA, H., MURAI, A. & KAN, Y. (1993). Structures of periodate oxidation products with a conjugated diene or an exomethylene from zooxanthellatoxin-A. *Tetrahedron Lett.*, **34**, 4059–4062.
- BACHELOT, C., CANO, E., GRELLAC, F., SALEUM, S., DRUKER, B.J., LEVY-TOLEDANO, S., FISCHER, S. & RENDU, F. (1992). Functional implications of tyrosine protein phosphorylation in platelet. *Biochem. J.*, **284**, 923–928.
- BALDASSARE, J.J., TARVER, A.P., HANDERSON, P.A., MACKIN, W.M., SAHAGAN, B. & FISHER, G.J. (1993). Reconstitution of thromboxane A₂ receptor-stimulated phosphoinositide hydrolysis in isolated platelet membranes: involvement of phosphoinositide-specific phospholipase C- β and GTP-binding protein G_q. *Biochem. J.*, **291**, 235–240.
- BORN, G.V.R. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, **194**, 927–929.
- DANIEL, J.D., DANGELMAIER, C. & SMITH, J.B. (1994). Evidence for a role for tyrosine phosphorylation of phospholipase C γ 2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem. J.*, **302**, 617–622.
- DOYLE, V.M. & RÜEGG, U.T. (1985). Lack of evidence for voltage dependent calcium channels on platelets. *Biochem. Biophys. Res. Commun.*, **127**, 161–167.
- EVANS, C.H. (1988). Alkaline earths, transition metals, and lanthanides. In *Calcium in Drug Action*. ed. Backer, P.F. pp. 527–546. Berlin: Springer-Verlag.
- FEDER, D. & BISHOP, J.M. (1990). Purification and enzymatic characterization of pp60^{c-src} from human platelets. *J. Biol. Chem.*, **265**, 8205–8211.
- FERREL, J.E. JR & MARTIN, G.S. (1988). Platelet tyrosine-specific protein phosphorylation is regulated by thrombin. *Mol. Cell. Biol.*, **8**, 3603–3610.
- FERREL, J.E. JR & MARTIN, G.S. (1989). Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb–IIIa in platelets. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2234–2238.
- GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.*, **38**, 321–416.
- GOLDEN, A. & BRUGGS, J.S. (1989). Thrombin treatment induces rapid changes in tyrosine phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 901–905.
- GOLDEN, A., BRUGGS, J.S. & SHATTIL, S.J. (1990). Role of platelet membrane glycoprotein IIb–IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J. Cell. Biol.*, **111**, 3117–3127.
- GOLDEN, A., NEMETH, S.P. & BRUGGE, J.S. (1986). Blood platelets express high levels of the pp60^{c-src} specific tyrosine kinase activity. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 852–856.
- GUSOVSKY, F. & DALY, W. (1990). Maitotoxin: a unique pharmacological tool for research on calcium-dependent mechanisms. *Biochem. Pharmacol.*, **39**, 1633–1639.
- HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2994–2998.
- HANASAKI, K. & ARITA, H. (1988). Characterization of a new compound, S-145, as a specific TXA₂ receptor antagonist in platelets. *Thrombosis Res.*, **50**, 365–376.
- HIRATA, Y., UEMURA, D. & OHIZUMI, Y. (1988). Chemistry and pharmacology of palytoxin. In *Handbook of Natural Toxins*. ed. Tu, A.T. pp. 241–258. New York: Marcel Dekker, Inc.
- ISHIDA, Y., SATAKE, N., HABON, J., KITANO, H. & SHIBATA, S. (1985). Inhibitory effect of ouabain on the palytoxin-induced contraction of human umbilical artery. *J. Pharmacol. Exp. Ther.*, **232**, 557–560.
- KOBAYASHI, M., OCHI, R. & OHIZUMI, Y. (1987). Maitotoxin-activated single calcium channels in guinea-pig cardiac cells. *Br. J. Pharmacol.*, **92**, 665–671.
- MATSUOKA, I., NAKAHATA, N. & NAKANISHI, H. (1989). Inhibitory effect of 8-bromo cyclic GMP on an extracellular Ca^{2+} -dependent arachidonic acid liberation in collagen-stimulated rabbit platelets. *Biochem. Pharmacol.*, **38**, 1841–1847.
- MILTON, J.G. & FROJMOVIC, M.M. (1983). Turbidometric evaluations of platelet aggregation: relative contributions of measured shape change, volume, and early aggregation. *J. Pharmacol. Methods*, **9**, 101–115.
- NAKAMURA, H., ASARI, T. & MURAI, A. (1993a). Structure of periodate oxidation products with characteristic partial structures of zooxanthellatoxin-A, a potent vasoconstrictive polyol from a symbiotic dinoflagellate. *J. Org. Chem.*, **58**, 313–314.
- NAKAMURA, H., ASARI, T. & MURAI, A. (1995). Zooxanthellatoxin-A, a potent vasoconstrictive 62-membered lactone from a symbiotic dinoflagellate. *J. Am. Chem. Soc.*, **117**, 550–551.
- NAKAMURA, H., ASARI, T., OHIZUMI, Y., KOBAYASHI, J., YAMASU, T. & MURAI, A. (1993b). Isolation of zooxanthellatoxins, novel vasoconstrictive substances from the zooxanthella *Symbiodinium* sp. *Toxicon*, **31**, 371–376.
- NAKAMURA, S. & YAMAMURA, H. (1989). Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets. *J. Biol. Chem.*, **264**, 7089–7091.
- NOZAWA, Y., NAKASHIMA, S. & NAGATA, K. (1991). Phospholipid-mediated signaling in receptor activation of human platelets. *Biochim. Biophys. Acta*, **1082**, 219–238.
- OGLETREE, M.L. (1987). Overview of physiological and pathophysiological effects of thromboxane A₂. *Fed. Proc.*, **46**, 133–138.
- PAUL, S., FARNDAL, R.W. & SAGE, S.O. (1993). The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets. *FEBS Lett.*, **315**, 242–246.
- PRIESS, J., LOOMIS, C.R., BISHOP, W.R., STEIN, R., NIEDEL, J.E. & BELL, R.M. (1986). Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. *J. Biol. Chem.*, **261**, 8597–8600.
- RASOULY, D. & LAZAROVICI, P. (1994). Staurosporine induces tyrosine phosphorylation of a 145 kDa protein but does not activate gp140^{trk} in PC12 cells. *Eur. J. Pharmacol.*, **269**, 255–264.
- RINK, T.J. & SAGE, S.O. (1990). Calcium signaling in human platelets. *Annu. Rev. Physiol.*, **52**, 431–449.

- RINK, T.J., SMITH, S.W. & TSIEN, R.Y. (1982). Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca^{2+} -independent activation for shape-change and secretion. *FEBS Lett.*, **148**, 21–26.
- RITTENHOUSE, S.E. (1984). Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclo-oxygenase products and ADP. *Biochem. J.*, **222**, 103–110.
- SIESS, W. (1989). Molecular mechanisms of platelet activation. *Physiol. Rev.*, **69**, 58–178.
- SIESS, W., SIEGEL, F.L. & LAPETINA, E.G. (1983). Arachidonic acid stimulates the formation of 1,2-diacylglycerol and phosphatidic acid in human platelets. *J. Biol. Chem.*, **258**, 11236–11242.
- SPACEY, G.D., BONSER, R.W., RANDALL, R.W. & GARLAND, L.G. (1990). Selectivity of protein kinase inhibitors in human intact platelets. *Cell Signal.*, **2**, 329–338.
- TAMAOKI, T. (1991). Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods Enzymol.*, **201**, 340–347.
- TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.
- VOSTAL, J.G., JACKSON, W.L. & RAPHAEL-SHULMAN, N. (1991). Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *J. Biol. Chem.*, **266**, 16911–16916.
- WATANABE, A., ISHIDA, Y., HONDA, H., KOBAYASHI, M. & OHIZUMI, Y. (1993). Ca^{2+} -dependent aggregation of rabbit platelets induced by maitotoxin, a potent marine toxin, isolated from a dinoflagellate. *Br. J. Pharmacol.*, **109**, 29–36.
- WILSON, D.B., NEUFELD, E.J. & MAJERUS, P.W. (1985). Phosphoinositide interconversion in thrombin-stimulated human platelets. *J. Biol. Chem.*, **260**, 1046–1051.

(Received December 5, 1994

Revised February 6, 1995

Accepted February 22, 1995)