Involvement of Phospholipase C-γ2 in Activation of Mitogen-Activated Protein Kinase and Phospholipase A2 by Zooxanthellatoxin-A in Rabbit Platelets

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ABSTRACT

Zooxanthellatoxin-A (ZT-A), a polyhydroxypolyene isolated from a symbiotic dinoflagellate Symbiodinium sp., caused thromboxane A2 (TXA2) dependent and genistein-sensitive aggregation in rabbit platelets. Our study was performed to clarify the mechanism of the action of ZT-A. ZT-A caused an increase in tyrosine phosphorylation of 42-kDa protein, which is defined as p42 mitogen-activated protein kinase (MAPK) by immunoprecipitation. Although indomethacin (10 μM) completely inhibited ZT-A-induced TXB2 release, it partially inhibited the MAPK activation. The remained MAPK activation was completely inhibited by genistein (50 μM). Genistein (50 μM), by itself, abolished TXB2 release induced by ZT-A. ZT-A (2 μM) stimulated liberation of arachidonic acid and the subsequent metabolites such as TXB2 and 12-hydroperoxyeicosatetraenoic acid. However, ZT-A-stimulated phosphoinositide hydrolysis which was due to an increase in tyrosine phosphorylation of phospholipase C-(PLC)γ2. The phosphorylation of PLC-γ2 and the phosphoinositide hydrolysis were also partially inhibited by indomethacin (10 μM), and were abolished by a combined treatment of indomethacin (10 μM) and genistein (50 μM). ZT-A (2 μM) induced MAPK activation in the presence of indomethacin (10 μM) which was concentration-dependently inhibited by staurosporine and calphostin C, protein kinase C inhibitors. PD98059 (50 μM), a MAPK kinase inhibitor, also inhibited ZT-A-induced TXB2 release. Depletion of external Ca2+ abolished ZT-A (2 μM) induced MAPK activation, phosphoinositide hydrolysis, arachidonic acid liberation and TXB2 release. These results suggest that ZT-A stimulates a protein tyrosine kinase in the presence of external Ca2+, resulting in the activation of MAPK probably via PLC-γ2 and protein kinase C. The MAPK stimulated a liberation of arachidonic acid that is rapidly converted to TXA2. The released TXA2 causes aggregation accompanied with second stimulation of MAPK cascade.

Many platelet proteins become phosphorylated at tyrosine residues during agonist stimulation (Ferrel and Martin, 1988; Golden and Brugge, 1989; Nakamura and Yamamura, 1989; Oda et al., 1992; Bachelot et al., 1992), although the functional significance of this is not clear. Tyrosine phosphorylation might play a pivotal role in platelet signal transduction (Pumiglia et al., 1992) where signaling enzymes, such as PLC-γ, phosphatidylinositol-3 kinase and nonreceptor tyrosine kinase including the src gene products, may cluster with interactions between these molecules stabilized by their SH2 domains (Sadowski et al., 1986; Ullrich and Schlessinger, 1990; Koch et al., 1991). Tyrosine phosphorylation may also be induced by [Ca2+]i elevation or by Ca2+ depletion in intracellular Ca2+ storage site (Vostal et al., 1991).

MAPK is a family of serine/threonine kinase that appears to be a component common to signaling pathway initiated by a wide range of factors including hormones, differentiation factors and mitogens (Tomas, 1992; Nishida and Gotoh, 1993; Davis, 1993). Phosphorylation of tyrosine and threonine residues is required for the activation of MAPK (Chatani et al., 1992), which is mediated by a dual phosphorylating specificity of MAPK kinase (Nishida and Gotoh, 1993; Davis, 1993).

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ABBREVIATIONS: PLC, phospholipase C; SH, src homology; [Ca2+]i, intracellular Ca2+ concentration; MAPK, mitogen-activated protein kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; IP3, inositol-1,4,5-trisphosphate; PKC, protein kinase C; PDGF, platelet-derived growth factor; cPLA2, cytosolic phospholipase A2; ZT-A, zooxanthellatoxin-A; TX, thromboxane; SQ-29548, 1S-[1α,2β(5Z,8Z,4α)]-7-[3-(2-phenylamino)carbonyl]hydrazino)methyl]7-oxaebicyclo[2,2,1][hept-2-yl]-5-heptenoic acid; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; PAG, polycyclic amide gel electrophoresis; TBS, tris-buffered saline; TCA, trichloroacetic acid; IP3, inositol phosphates; PI, phosphoinositide; MEK1, mitogen-activated protein kinase kinase 1; 12-HETE, 12-hydroperoxyeicosatetraenoic acid; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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The MAPK pathway is not a single pathway, but represents a common mechanism of signal transduction that has been adapted to couple different stimuli to distinct physiological responses. Ligand binding to a receptor protein tyrosine kinase triggers the activation and autophosphorylation of the kinase, which leads to activation of ras, a small GTP-binding protein. Activated ras then initiates a cascade of sequential MAPK (Avruch et al., 1994; Cobb et al., 1994). MAPK is well characterized as one of intracellular signaling enzyme involved in proliferation of cells. Because platelets are nonproliferative cells, the signal transduction pathway, including MAPK, cannot lead to a mitogenic signal and instead may regulate cytoskeletal or secretory changes during platelet activation (Papkoff et al., 1994).

Upon stimulation, PIP$_2$ is cleaved by PLC to form IP$_3$ and diacylglycerol. IP$_3$ releases Ca$^{2+}$ from the intracellular stores and rises the cytosolic-free Ca$^{2+}$ level (O'Rourke et al., 1985), although diacylglycerol activates PKC, leading to protein phosphorylation, granule secretion and fibrinogen-receptor expression (Rünk et al., 1983). PLC-$γ$2 is immunologically distinct from PLC-$γ$1, but does contain the SH2 regions (Kumjan et al., 1991). When PLC-$γ$2 is transfected and overexpressed in rat-2 fibroblasts, PLC-$γ$2 is transiently tyrosine-phosphorylated by PDGF, with a similar kinetics to those for PLC-$γ$1 (Sultzman et al., 1991). However, several isoforms of PLC, e.g., PLC-$β$, -$γ$, -$δ$ and -$ε$, exist in platelets (Banno et al., 1992) and are capable of contributing to increase in diacylglycerol and IP$_3$. However, the physiological function and control of each isoform in platelets is still relatively undefined.

Recent studies have revealed that the regulation of cPLA$_2$ is complex and involves both changes in [Ca$^{2+}$], mediated by PLC activation and phosphorylation of cPLA$_2$ (Clark et al., 1991; Lin et al., 1992; Nemenoff et al., 1993). It has also been shown that the p42 MAPK phosphorylates cPLA$_2$ that results in a 3- to 4-fold increase in specific activity in the presence of a high Ca$^{2+}$ concentration (Nemenoff et al., 1993; Lin et al., 1993), and that arachidonic acid liberation may be regulated by activated MAPK and increase in [Ca$^{2+}$], in human platelets (Nakashima et al., 1994).

ZT-A was recently isolated from a symbiotic marine alga Symbiodinium sp. as potent vasoconstrictor compound (Nakamura et al., 1993, 1995), and characterized as large molecules (2872 Da) containing a large number of oxygen and olefinic carbons. The chemical characters are different from those of other marine toxins such as palytoxin and maito-olefinic carbons. The chemical characters are different from those of other marine toxins such as palytoxin and maito-

**Methods**

**Materials.** ZT-A was isolated as described previously (Nakamura et al., 1993). Indomethacin was obtained from Merck Company Inc. (Rahway, NJ). Ionomycin, genistein, staurosporine and calphostin C were obtained from Wako Pure Chemical Industries (Osaka, Japan). BSA were from Sigma Chemical CO. (St. Louis, MO). [$^{3}H$]TXB$_2$ (110.6 Ci/mmol), [$^{3}H$]Arachidonic acid (50 mCi/mmol) and [$^{3}H$]myo-inositol (23.4 Ci/mmol) were obtained from Du Pont/NEC (Boston, MA). [γ-32P]ATP was from Amersham International plc. (Bucking-

**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. Platelet-rich plasma was centrifuged at 650 × g for 10 min at room temperature (20–25°C). The pellet was washed twice with Tyrode/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$^{11}$ platelets/ml (Ardlie et al., 1970; Vickers et al., 1982). The Tyrode/HEPES solution was composed of NaCl 138.3 mM, KCl 2.68 mM, MgCl$_2$ 2.68 mM, CaCl$_2$ 1.25 mM, MgCl$_2$ · 6H$_2$O 1.0 mM, NaHCO$_3$ 4.0 mM, HEPES 10 mM, 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) using 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 % for a platelet suspension and 100% for the Tyrode/HEPES solution. Platelet suspension (0.3 ml) in the aggregometer cuvette was preincubated for 2 min at 37°C under continuous stirring at 1000 r.p.m. and then CaCl$_2$ or EGTA was added at the final concentration of 1 mM. After 2 min, ZT-A was added and platelet aggregation was monitored for 10 min. Various blockers were preincubated for 5 min before the addition of ZT-A.

**Immunoblotting.** For analysis of total platelet proteins, the reaction was terminated by addition of Laemmli sample buffer, and the mixture was then boiled for 5 min and analyzed on an 8% SDS-PAGE. Immunoblot assays were performed as described by Papkoff et al. (1994), with slight modifications. For immunoblotting, proteins were electrophoretically transferred to the polyvinylidene difluoride membrane for 80 min at 120 mA. Blots were incubated for 2 hr with 1% (w/v) BSA in TBS to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved by using a specific antiphosphotyrosine monoclonal murine antibody (4G10, 2 μg/ml) in TBS containing 1% BSA for 2 hr. MAPK was detected by rabbit anti-MAPK (ERK2, C-14) antibody (1 μg/ml) in TBS containing 1% BSA for 2 hr. PLC-$γ$2 was detected by rabbit anti-PLC-$γ$2 antibody (Q-20, 1 μg/ml) in TBS containing 1% BSA for 2 hr. The primary antibody was removed and blots were washed in TBS with 0.05% Tween-20 five times. To detect the primary antibody, blots were incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibody (Bio-Rad, Hercules, CA) diluted 1:3000 in TBS containing 1% BSA for 2 hr, and then washed five times in TBS with 0.05% Tween-20. After the blots were exposed to enhanced chemiluminescence reagents (Bio-Rad for 30 min), they were then exposed to Hyper film-enhanced chemiluminescence (Amersham) for 10 to 30 min.
Immunoprecipitation. For immunoprecipitation of MAPK, platelet suspensions (0.3 ml) were harvested by addition of 20 μl denaturing buffer (10% SDS, 10 mM dithiothreitol, 20 mM HEPES, pH 7.4) (Papkoff et al., 1994). The sample was heated at 95°C for 5 min and diluted with 0.8 ml of immunoprecipitation buffer (150 mM NaCl, 10% glycerol, 1% triton X-100, 1.5 mM MgCl2, 2 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate and 50 mM HEPES, pH 7.4). The samples were centrifuged at 15,000 × g × 20 min. The supernatants (0.8 ml) were then incubated for 3 hr at 4°C with 1 μg/ml of the anti-MAPK (ERK2, C-14) antibody, and were further incubated for 1 hr at 4°C after addition of protein A-Sepharose (20 μg). Immune complexes were collected by centrifugation in a microcentrifuge and then washed three times with washing buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate and 50 mM HEPES, pH 7.4). The precipitate was solubilized with Laemmli sample buffer, and the mixture was then boiled for 5 min and applied to 10% SDS-PAGE and immunoblots described above. For immunoprecipitation of PLC-γ2, platelet suspensions (0.3 ml) were harvested by the addition of an equal volume of ice-cold Nonidet P-40 lysis buffer containing 2% (w/v) Nonidet P-40, 100 mM Tris-acetate (pH 8.0), 2.0 mM EDTA, 2.0 mM EGTA, 100 mM NaF, 200 mM NaCl, 6.0 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 2.0 mM phenylmethylsulfonyl fluoride, 0.4 mM leupeptin and 20 μg/ml apro tinin (Tate and Rittenhouse, 1993). The samples were then incubated for 50 min on ice and spun at 15,000 × g × 10 min. Each sample (1 ml) was incubated with 1 μg/ml of the anti-PLC-γ2 antibody (Q-20) for 2 hr, followed by an 1-hr incubation with protein A-Sepharose (20 μg) at 4°C. Immune complexes were collected by centrifugation in a microcentrifuge, and then washed once with lysis buffer, twice with phosphate-buffered saline containing 2% (w/v) Nonidet P-40, 0.1% SDS, 1% aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate and 50 mM HEPES, pH 7.4). The precipitate was solubilized by Laemmli sample buffer, and the mixture was then boiled for 5 min and applied to 10% SDS-PAGE and immunoblots described above, after boiling for 10 min.

MAPK assay. Platelets were lysed by sonication in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, pH 7.4 measured at 4°C and centrifuged at 15,000 × g × 20 min. Supernatants (15 μl) were then incubated for 30 min at 30°C with 10 μl of the substrate buffer containing 6 mM substrate peptide (NH2- KRELVEPLTPAGEAPNQALL-39 R-OH), 75 mM HEPES, 300 mM NaCl, 0.05% sodium azide, pH 7.4, and 5 μl of ATP buffer containing 0.3 mM [γ-32P]ATP (0.67 Ci/mmol) and 90 mM MgCl2. After incubation, 10 μl of 300 mM orthophosphoric acid were added to terminate the reaction. After 30 μl of each were washed onto phosphocellulose discs, the discs were washed twice for 2 min in 1% acetic acid, and then washed twice for 2 min in distilled water. The radioactivity on each disc was determined by scintillation counting. To examine the effect of indomethacin and genistein, platelets were preincubated with the drugs for 5 min at 37°C before addition of ZT-A.

Radioimmunoassay of TXB2. The release of TXB2 from rabbit platelets was determined by a radioimmunoassay (Matsuoka et al., 1989). In brief, the reaction of platelets was terminated by addition of equi-volume of ice-cold 50 μM indomethacin/50 mM EDTA solution. After centrifugation, the supernatant was adjusted pH to 3.0 with 1 N HCl, and the released [3H]arachidonic acid metabolites were extracted with ethyl acetate. The ethyl acetate solubles were dried by stream of nitrogen gas and applied to thin-layer chromatography plate (LKSD, Whatman, Clifton, NJ). The developer used was benzene/isooctane/acetic acid (60:30:3, v/v). [3H]Arachidonic acid metabolites were visualized in radioluminogram with a molecular imager (GS363, Bio-Rad, Hercules, CA).

Determination of arachidonic acid liberation. For the determination of arachidonic acid liberation, platelets were labeled with [3H]arachidonic acid (10 μCi/ml) at 37°C for 1 hr. Then, platelets were washed twice with Tyrode/HEPES-albumin solution (pH 7.35). Platelets were incubated with drugs in aggregometer cuvettes with constant stirring at 37°C. The reaction was terminated by addition of equi-volume of ice-cold 50 μM indomethacin/50 mM EDTA solution (Matsuoka et al., 1989). After centrifugation, the released [3H]radioactivity in the supernatant was estimated as arachidonic acid liberation.

Measurement of inositol phosphates. Washed platelets suspended in albumin-free Tyrode/HEPES solution (pH 7.35) were labeled with 25 μCi/ml [3H]myo-inositol at 37°C for 1 hr. Platelets were washed with Tyrode/HEPES-albumin solution (pH 7.35), and resuspended at 5 × 108 platelets/ml. After platelets were preincubated for 2 min, they were incubated with drugs in the presence or absence of 10 μM LiCl for 5 min. Reaction was terminated by addition of equal volume of ice-cold 10% TCA. The TCA extracts were washed three times with diethyl ether to remove TCA. Diethyl ether was removed by keeping the samples at 47°C for 30 min. [3H]H1P2 or total [3H]H1Ps were separated by anion exchange column (Bio-Rad AG IX-8, 100-200 mesh, formate form) previously described (Nakahata et al., 1989). The elute was counted with a liquid scintillation spectrophotometer.

Data analysis. The results obtained were expressed as mean ± S.E. and the statistical difference was determined with unpaired Student’s t test.

Results

Effects of indomethacin and genistein on ZT-A- and ionomycin-induced platelet aggregation. ZT-A and ionomycin caused aggregation in rabbit platelets (fig. 1). Though ZT-A (2 μM) induced platelet aggregation was inhibited by indomethacin (10 μM) or genistein (50 μM), ionomycin (5 μM) induced aggregation persisted after the treatment of platelets with above drugs (fig. 1). These results suggest that ZT-A activates platelets through cyclooxygenase products and tyrosine phosphorylation, not due to Ca2+ ionophore-like action.

Effects of ZT-A on tyrosine phosphorylation. The stimulation of platelets by ZT-A (2 μM) resulted in an increase in the amount of tyrosine phosphorylation of 135-, 140-, and 130-kDa proteins (fig. 2A). To determine whether 42-kDa protein is MAPK or not, platelet lysates were immunoprecipitated with anti-MAPK (ERK2, C-14) antibody and MAPK was analyzed by using of antiphosphotyrosine antibody or anti-MAPK (ERK2, C-14) antibody (fig. 2B and C). ZT-A (2 μM) increased phosphorylation of the tyrosine residue of p42 MAPK but not p44 MAPK (fig. 2B). In these analyses, the amount of immunoprecipitated p42 MAPK was unchanged by ZT-A (fig. 2C) and p44 MAPK was not immunoprecipitated with anti-MAPK (ERK2, C-14) antibody.
Elevation of MAPK activity during ZT-A-induced platelet activation. MAPK activity was measured by incorporation of \( ^{32} \text{P} \) to the specific substrate for MAPK. ZT-A increased MAPK activity in a concentration-dependent manner with a maximum concentration of 2 \( \mu \text{M} \) (fig. 3A). Time course analysis revealed that ZT-A activated MAPK by two phases. Within 60 sec, ZT-A increased MAPK activity 2-fold, and then it increased 10-fold at 5 min after addition of ZT-A (fig. 3B).

The effect of indomethacin (10 \( \mu \text{M} \)) on ZT-A (2 \( \mu \text{M} \)) induced MAPK activation was examined. Although the TXB\(_2\) release was completely inhibited by pretreatment of platelets with indomethacin (fig. 4A), the MAPK activation was partially (60–70\%) inhibited by indomethacin (fig. 4B). Genistein (50 \( \mu \text{M} \)), by itself, also inhibited the TXB\(_2\) release induced by ZT-A (fig. 4A). Because genistein is known to acts as a TXA\(_2\) antagonist in addition to tyrosine kinase inhibitor (Nakashima et al., 1991), we next investigated the effect of genistein (50 \( \mu \text{M} \)) on ZT-A-induced MAPK activation in the presence of indomethacin (10 \( \mu \text{M} \)). Genistein completely reduced ZT-A (2 \( \mu \text{M} \)) induced MAPK activation in the presence of indomethacin (fig. 4B), showing that ZT-A activates protein tyrosine kinase upstream of MAPK activation without contribution of TXA\(_2\).

Effects of PD98059 on ZT-A-induced TXB\(_2\) release. We further investigated the effects of PD98059, a MAPK kinase 1 (MEK1) inhibitor, on ZT-A-induced TXB\(_2\) release, because cPLA\(_2\) can be activated by MAPK (Qui et al., 1993). PD98059 (50 \( \mu \text{M} \)) potently inhibited TXB\(_2\) release in response to ZT-A (2 \( \mu \text{M} \)), indicating that MAPK is responsible for TXB\(_2\) release probably via cPLA\(_2\) (table 1).

Effect of ZT-A on arachidonic acid metabolites liberation. To confirm PLA\(_2\) activation induced by ZT-A (2 \( \mu \text{M} \)), we examined the liberation of arachidonic acid and its subsequent metabolites. ZT-A (2 \( \mu \text{M} \)) stimulated liberation of arachidonic acid from membrane phospholipids, which was converted to 12-hydroperoxyeicosatetraenoic acid (12-HETE) and TXB\(_2\) in platelets (fig. 5).

Effect of ZT-A on phosphoinositide hydrolysis. ZT-A stimulated PI hydrolysis in a concentration-dependent manner with a maximum at 2 \( \mu \text{M} \) in the presence of external Ca\(^{2+}\) (fig. 6A). Figure 6B shows the time course of ZT-A-induced accumulation of IP\(_3\) in the absence of LiCl. The peak of IP\(_3\) accumulation was 30 sec after the addition of ZT-A. The time course analysis showed that ZT-A activated PLC before MAPK.
Although ZT-A- (2 μM) induced TXB₂ release was completely inhibited by indomethacin (fig. 4A), ZT-A- (2 μM) induced accumulation of IPs was partially inhibited by indomethacin (fig. 7A). The results suggest that released TXA₂ by ZT-A partly contributes to accumulation of IPs, but the remained ZT-A-induced accumulation of IPs was independent of TXA₂. However, ZT-A- (2 μM) induced accumulation of IPs in the presence of 10 μM indomethacin was concentration-dependently inhibited by genistein, a protein tyrosine kinase inhibitor (fig. 7B).

**PLC-γ2 activation upon ZT-A stimulation.** The stimulation of platelets by ZT-A (2 μM) resulted in an increase in protein tyrosine phosphorylation of PLC-γ2, determined by immunoprecipitation (fig. 8). Protein tyrosine phosphorylation of PLC-γ2 was partially inhibited by indomethacin (10 μM), and completely inhibited by genistein (50 μM) plus indomethacin (10 μM) (fig. 8A). In these analyses, the amount of immunoprecipitated PLC-γ2 was not affected by indomethacin, genistein or ZT-A (fig. 8B).

**Effects of staurosporine and calphostin C on ZT-A-induced MAPK activation.** Because PLC activation was responsible for activation of PKC, leading to MAPK activation (Rink et al., 1983; Qiu and Leslie, 1994), we investigated the effects of staurosporine and calphostin C, PKC inhibitors, on MAPK activation induced by ZT-A in the presence of indomethacin (10 μM). ZT-A- (2 μM) induced MAPK activation was inhibited by above drugs in a concentration-depen-

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**Fig. 3.** Effect of ZT-A on MAPK activity. Platelets were incubated with the indicated concentration of ZT-A for 5 min in the presence of 1 mM CaCl₂ (A) and were incubated with ZT-A (2 μM) in the presence of 1 mM CaCl₂ for the indicated time (B). MAPK activity (A, 566.0 ± 26.4 cpm/tube, B, 588.5 ± 54.8 cpm/tube) that was not treated with ZT-A in the presence of 1 mM CaCl₂ was taken as 1 (control). Data are expressed as fold of control. Values represent the mean ± S.E. (n = 3) and are representative of three independent experiments.

**Fig. 4.** Effects of indomethacin and genistein on ZT-A-induced TXB₂ release (A) and MAPK activation (B). Platelets were pretreated with indomethacin (10 μM) or genistein (50 μM) for 5 min, and then incubated with ZT-A (2 μM) for 5 min in the presence of 1 mM CaCl₂ (A). After preincubation of indomethacin (10 μM) for 5 min, genistein (50 μM) was applied 5 min before addition of ZT-A (2 μM) (B). TXB₂ release (0.8 ± 0.1 ng/5 × 10⁸ platelets) and MAPK activity (674.0 ± 22.6 cpm/tube) that were not treated with ZT-A in the presence of 1 mM CaCl₂ was taken as 1 (control). Values represent the mean ± S.E. (n = 4) and are representative of three independent experiments. Significant difference (*P < .05) by unpaired t test when compared to control.
dent manner (fig. 9). Both staurosporine and calphostin C had no effect on the phosphorylation of PLC-\(\gamma\)2 (data not shown).

Effects of depletion of external Ca\(^{++}\) on ZT-A-induced platelet activation. Because ZT-A-induced platelet aggregation was dependent on the presence of external Ca\(^{++}\) (Rho et al., 1995), we investigated the effect of depletion of external Ca\(^{++}\) on ZT-A-induced MAPK activation, TXB\(_2\) release, arachidonic acid liberation and PI hydrolysis. These ZT-A-induced activations were also strictly dependent on the presence of external Ca\(^{++}\) (table 2).

Discussion

Platelet activation is accompanied by the phosphorylation of numerous proteins at tyrosine residue (Findik and Presec, 1988; Golden et al., 1990; Bading and Greenberg, 1991). In an effort to clarify the roles of tyrosine phosphorylation in platelet signal transduction, we have investigated the platelet activation induced by ZT-A, which is genistein sensitive (Rho et al., 1995). ZT-A increased the tyrosine phosphorylation of p42 MAPK in rabbit platelets. In human platelets, thrombin has been shown to stimulate the activation of p42 MAPK but not p44 MAPK (Papkoff et al., 1994). PMA and platelet-activating factor also activated p42 MAPK, but not p44 MAPK in sheep platelets (Samiei et al., 1993). In agreement with other reports, we also observed that ZT-A increased protein tyrosine phosphorylation of p42 MAPK but not p44 MAPK in rabbit platelets. ZT-A also increased the tyrosine phosphorylation of 110- and 105-kDa proteins. The contribution of these proteins to p42 MAPK activity and platelet aggregation in response to ZT-A is left to be studied.

Although indomethacin completely inhibited TXB\(_2\) release and aggregation induced by ZT-A, it partially inhibited the MAPK activation induced by ZT-A. Therefore, TXA\(_2\) released by ZT-A is able to activate MAPK and cPLA\(_2\) in rabbit plate-

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<th>TABLE 1 Effects of PD98059 on ZT-A-induced TXB(_2) release</th>
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<tr>
<td>TXB(_2) (ng/5 \times 10^8 Platelets)</td>
</tr>
<tr>
<td>No addition</td>
</tr>
<tr>
<td>ZT-A (2 \muM)</td>
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<tr>
<td>PD98059 (50 \muM)</td>
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<td>PD98059 (50 \muM) + PD98059 (50 \muM)</td>
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PD98059 (50 \muM) was applied 5 min before addition of ZT-A (2 \muM). TXB\(_2\) release (0.4 ± 0.15 ng/5 \times 10^8 platelets) that was not treated with ZT-A in the presence of 1 mM CaCl\(_2\) (control). Values represent the mean ± S.E. (n = 4) and are representative of three independent experiments.

* Significant difference (P < .05) by unpaired t test when compared to control.

Fig. 5. Effect of ZT-A on liberation of arachidonic acid and its metabolites. Platelets were labeled with 0.8 \muCi/ml \[^{14}\text{C}\]arachidonic acid for 1 hr at 37°C. Platelets were incubated with ZT-A (2 \muM) for 5 min in the presence of 1 mM CaCl\(_2\). \[^{14}\text{C}\]arachidonic acid metabolites were visualized in radioluminogram with a molecular imager (GS363, Bio-Rad). The results are representative in three similar experiments.

Fig. 6. Effect of ZT-A on PI hydrolysis. Platelets were labeled with 25 \muCi/ml \[^{3}\text{H}\]myo-inositol for 1 hr at 37°C. Platelets were incubated with various concentrations of ZT-A for 5 min (A) and incubated with ZT-A (2 \muM) for the indicated time (B) in the presence of 1 mM CaCl\(_2\). The reaction was carried out in the presence (A) or absence (B) of 10 mM LiCl, and was terminated by the addition of 0.3 ml of 10% TCA. Total \[^{3}\text{H}\]IPs and \[^{3}\text{H}\]IP3 were determined as described in “Materials and Methods.” Values represent the mean ± S.E. (n = 4) and are representative of three independent experiments.
lets, i.e., ZT-A activates cPLA₂ and releases TXA₂, which secondarily enhances cPLA₂ activation via a TXA₂ receptor. In fact, TXA₂ has been shown to activate MAPK and arachidonic acid liberation in rabbit platelets (Ohkubo et al., 1996b). Furthermore, it is assumed that the remained ZT-A-induced MAPK activation in the presence of indomethacin is independent of TXA₂. MAPK can be activated by extracellular stimuli via tyrosine kinase receptors (McCormic, 1994) or G protein-coupled receptors (Lange-Carter et al., 1993; Crespo et al., 1994). Because the remained ZT-A-induced MAPK activation in the presence of indomethacin was completely inhibited by genistein, a protein tyrosine kinase is responsible for ZT-A-induced MAPK activation independently of TXA₂. The fact that MAPK activity was increased 2-fold within 60 sec by ZT-A supports the idea that there is a primary MAPK activation in ZT-A action. In addition, ZT-A-induced platelet aggregation and p42 MAPK activation were also inhibited by tyrphostin 23, another tyrosine kinase inhibitor (Rho et al., 1997).

PKC has been shown to play a role in cPLA₂ activation (Qiu et al., 1993; Wijkander and Sundler, 1992). Diacylglycerol stimulates PKC synergistically with Ca²⁺, leading to activation of MAPK cascade in several cell types (Qiu and Leslie, 1994). To elucidate the primary signaling pathway of ZT-A to activate MAPK, the involvement of PLC and PKC was investigated in the presence of indomethacin. ZT-A caused genistein-sensitive PI hydrolysis in the presence of indomethacin, suggesting that tyrosine phosphorylation was
involved in PLC activation. The involved PLC isoform is defined as PLC-γ2, based on the observation that ZT-A-induced tyrosine phosphorylation of PLC-γ2 in the presence of indomethacin was completely inhibited by genistein. The result is consistent with the case of thrombin or collagen in tyrosine phosphorylation of PLC-γ2 in human platelets (Tate and Rittenhouse, 1993; Daniel et al., 1994). However, ZT-A-induced MAPK activation was concentration-dependently inhibited by staurosporine and calphostin C, PKC inhibitors, in the presence of indomethacin. These results imply that ZT-A primarily causes PLC-γ2 activation, leading to PKC and MAPK activation. In agreement with the results, ZT-A-induced platelet aggregation was also inhibited by staurosporine (Rho et al., 1995) and calphostin C (M.-C. Rho, N. Nakahata, H. Nakamura, A. Murai and Y. Ohizumi, unpublished observation).

ZT-A-induced platelet activation was strictly dependent on the presence of external Ca**++. A possibility raised that ZT-A acts as a Ca**+ ionophore. However, ZT-A- (2 μM) induced aggregation was quite different from ionomycin- (5 μM) induced one, i.e., indomethacin (10 μM) and genistein (100 μM) potently inhibited ZT-A but not ionomycin-induced aggregation. Therefore, ZT-A does not act as a Ca**+ ionophore. Inflused Ca**+ by ZT-A may participate in its pharmacological action cooperatively with its activation of tyrosine kinase signaling pathway.

In conclusion, ZT-A may primarily activates a protein tyrosine kinase in the presence of external Ca**+. The activated protein tyrosine kinase subsequently stimulates the activation of MAPK probably via PLC-γ2 and PKC. The activated MAPK in turn stimulates cPLA2 and liberates arachidonic acid that is rapidly converted to TXA2. Released TXA2 may enhance platelet function including MAPK activity.

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**References**


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