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Antiplatelet mechanisms of TA-993 and its metabolite MB3 in ADP-induced platelet aggregation

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Abstract

We investigated the antiplatelet mechanisms of TA-993 [(−)-*cis*-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-8-methyl-2-(4-methylphenyl)-1,5-benzothiazepin-4(5*H*)-one maleate] and its metabolite MB3 (deacetyl and *N*-monomethyl TA-993) in human platelets stimulated by ADP in vitro. TA-993 and MB3 concentration-dependently inhibited fibrinogen binding to the ADP-stimulated platelets as well as inhibiting platelet aggregation. The antiplatelet effect of MB3 was about 300 times more potent than those of TA-993 and a glycoprotein IIb/IIIa receptor antagonist, Arg-Gly-Asp-Ser (RGDS). Aggregation of ADP-treated fixed platelets caused by the addition of fibrinogen was inhibited by RGDS but not by TA-993 and MB3. TA-993 and MB3 inhibited ADP-induced polymerization of actin filaments. Neither TA-993 nor MB3 affected cyclic AMP and cyclic GMP levels in resting platelets, and nor suppressed the increase in intracellular Ca^{2+} concentration induced by ADP. These results suggest that the antiplatelet mechanisms of TA-993 and MB3 may involve inactivation of glycoprotein IIb/IIIa receptors via inhibition of the polymerization of actin.

Keywords: TA-993; Platelet; Glycoprotein IIb/IIIa receptor; ADP; Actin polymerization; Ca^{2+}, intracellular

1. Introduction

Platelets play an important role in thrombosis and haemostasis. Studies have shown that antiplatelet agents can prevent development of thrombotic disorders such as myocardial infarction and peripheral vascular diseases (Dinerman and Mehta, 1990; Schwartz et al., 1990). A number of antiplatelet drugs such as aspirin, prostaglandin analogues, ticlopidine and glycoprotein IIb/IIIa receptor antagonists have been used clinically (Armstrong, 1996; Coller, 1990; Vorchheimer et al., 1999). Each antiplatelet drug has its own mechanism of antiplatelet action.

TA-993 [(−)-*cis*-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-8-methyl-2-(4-methylphenyl)-1,5-benzothiazepin-4(5*H*)-one maleate] is a 1,5-benzothiazepine derivative like diltiazem, a well-known Ca^{2+} channel antagonist, although it is an (L)-enantiomer while diltiazem is a (D)-enantiomer. TA-993 exerts potent action on platelets but only very weak Ca^{2+} channel antagonistic cardiovascular effects (Odawara et al., 1996). It has recently been demonstrated that TA-993 inhibited thrombus formation in various models in vivo (Kaburaki et al., 1998a,b; Narita et al., 1995; Odawara et al., 1996). MB3 (deacetyl and *N*-monomethyl TA-993) is the most potent antiplatelet agent among the metabolites of TA-993 in vitro (Odawara et al., 1996). Although it has already been reported that other 1,5-benzothiazepine derivatives including diltiazem showed antiplatelet action (Addonizio et al., 1986; Meth et al., 1983; Odawara et al., 1994), their mechanisms are not clear.

ADP is an important platelet stimulant that is stored in platelets and released upon platelet activation (Holmsen and Weiss, 1979). ADP-induced platelet activation plays an important role in haemostasis and thrombosis (Maffrand et al., 1988). In the present study, we investigated the mechanisms of inhibition by TA-993 and MB3 in ADP-induced platelet aggregation.

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2. Materials and methods

2.1. Materials

TA-993 [(−)-*cis*-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-8-methyl-2-(4-methylphenyl)-1,5-benzothiazepin-4(5*H*)-one maleate] and MB3 (deacetyl and *N*-monomethyl TA-993) were synthesized at Tanabe Seiyaku, (Saitama, Japan). Acridinium derivative I [4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate] and fura-2 acetoxymethylester (AM) were purchased from Dojindo Laboratories (Kumamoto, Japan). Human fibrinogen (fraction I, type I), ADP, sodium nitroprusside, DNA (calf thymus, type 1), and DNase I (beef pancreas, DN 100) were purchased from Sigma (St. Louis, MO, USA). Arg-Gly-Asp-Ser (RGDS) was obtained from Peninsula Laboratories (Belmont, CA, USA). Prostaglandin E_1 and EDTA were obtained from Nacalai Tesque (Tokyo, Japan). All other chemicals were of the reagent grade.

2.2. Preparation of gel-filtered platelets

Human blood samples were obtained by venipuncture from drug-free, normal male volunteers and treated with 0.1 vol. of 3.8% trisodium citrate. Platelet-rich plasma was prepared by centrifugation of the blood at $150 \times g$ for 10 min at room temperature. Gel-filtered platelets were prepared from platelet-rich plasma by chromatography on a Sepharose 2B (Pharmacia, Sweden) column using modified Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) containing 3.5% bovine serum albumin and 2% glucose for elution. Platelets were counted electronically with a cell counter (CELLTAC MEK-5158, Nihon Koden, Japan). The final platelet concentration was adjusted to about 3×10^8/ml.

2.3. Preparation of fixed platelets

Gel-filtered platelets were stimulated by 20 μM ADP for 10 min. They were then fixed by treatment with 0.8% paraformaldehyde for 30 min at room temperature. The platelets were washed with modified Tyrode's buffer three times.

2.4. Platelet aggregation

Gel-filtered platelets were used for ADP (10 μM)-induced platelet aggregation in the presence of 200 nM fibrinogen and 1 mM Ca^{2+} with an aggregometer (Model PAT-606, MC Medical, Japan). Aggregation was initiated by addition of 200 nM fibrinogen.

2.5. Preparation of acridinium–fibrinogen

Acridinium-labeled human fibrinogen (acridinium–fibrinogen) was prepared according to the previous method (Katoh et al., 1995). In brief, the mixture of fibrinogen and acridinium was incubated at 4°C for 30 min with stirring, and then applied to a Sephadex G-50 (Pharmacia) column (2.5 × 40 cm). Chemiluminescence emission of acridinium was induced by addition of 0.1 N NaOH containing 0.06% H_2O_2. The quantity of chemiluminescence was determined as a transient signal with a 1251 Luminometer (Bio-Orbit, Finland). Specific activity of acridinium–fibrinogen was 2000 ± 200 (S.E.M.) mV s/mg protein ($N = 5$). Acridinium–fibrinogen was stored at 4°C without freezing. Under these conditions, it was stable for at least 2 weeks.

2.6. Fibrinogen binding studies with gel-filtered platelets

Fibrinogen binding to gel-filtered platelets was measured as previously described (Katoh et al., 1995). Briefly, gel-filtered platelets (300 μl) were stimulated by ADP (10 μM) at room temperature without stirring in the presence of a test compound dissolved in 1 mM Ca^{2+}-supplemented modified Tyrode's buffer. Acridinium–fibrinogen (200

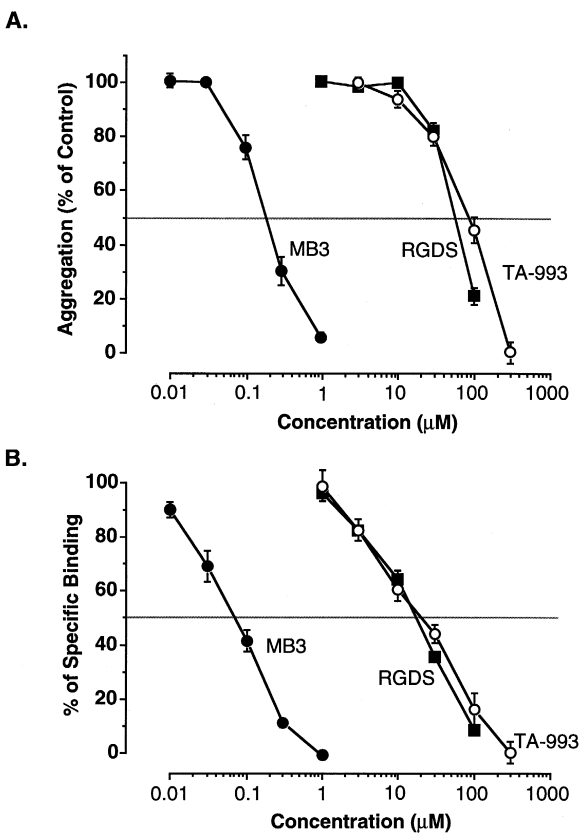

Fig. 1. Inhibitory effects of TA-993, MB3 and RGDS on ADP-induced platelet aggregation (A) and fibrinogen binding to ADP-stimulated platelets (B). Gel-filtered platelets were suspended in modified Tyrode's buffer containing 1 mM $CaCl_2$, and were preincubated with various concentrations of test compounds and 200 nM fibrinogen at 37°C for 3 min. Aggregation was initiated by addition of 10 μM ADP. Fibrinogen binding was determined as described in Materials and methods. Data are expressed as means ± S.E.M. of five blood donors.

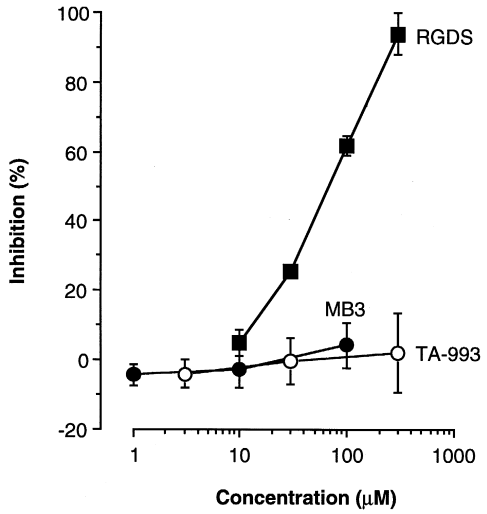

Fig. 2. Inhibitory effects of TA-993, MB3 and RGDS on fibrinogen-induced aggregation in ADP-stimulated fixed platelets. The fixed platelets were suspended in modified Tyrode's buffer, and were preincubated with various concentrations of test compounds at 37°C for 3 min. Aggregation of the fixed platelets was initiated by addition of 200 nM fibrinogen. Data are expressed as means ± S.E.M. of four to five blood donors.

nM) was added to the mixture and incubated for 10 min. After the incubation, the reaction mixture was removed and layered onto 20% sucrose, and platelet bound-fibrinogen was separated from free fibrinogen by centrifugation at 15,000 × *g* for 3 min with a Beckman microfuge. The platelet pellet in the tip of each tube was cut off with a razor blade and the quantity of chemiluminescence of acridinium–fibrinogen bound to the platelets was measured by adding 0.1 N NaOH containing 0.06% H_2O_2 in a luminometer. Nonspecific binding of acridinium–fibrinogen to the platelets was assessed in the presence of 10 mM EDTA (Peerschke et al., 1980).

2.7. Platelet cyclic AMP and cyclic GMP levels

Platelet-rich plasma was prepared from citrated human blood as described above. Platelet-rich plasma and test compounds were incubated at 37°C for 2 min. The reaction was stopped by addition of 10% perchloric acid, and the reaction mixture was centrifuged and the resulting supernatant was assayed for cyclic AMP and cyclic GMP levels with the cyclic nucleotide radioimmunoassay kits (Yamasa, Japan).

2.8. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Intracellular Ca^{2+} assays were performed by the methods described by Erne et al. (1984) and Grynkiewicz et al. (1985) with slightly modification. In brief, platelets were loaded with 4 μM fura-2 AM by incubating platelet-rich plasma for 20 min at 37°C, and these were then centrifuged. The supernatant was discarded and the cells were resuspended in Ca^{2+} free-HEPES buffer (145 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES, pH 7.4). The external Ca^{2+} concentration was adjusted by addition of $CaCl_2$ to 1 mM and the cells were incubated at 37°C for 3 min before the addition of ADP. The calibration of intracellular fluorescence as a function of Ca^{2+} was performed by the method described by Tsien et al. (1982). Fluorescence signal of fura-2-loaded platelets, by excitation at 340 nm, and emission at 497 nm, was recorded with a calcium ion analyzer (FS-100, Kowa, Japan). TA-993 and MB3 did not affect the fluorescence signal of fura-2.

2.9. Measurement of actin polymerization

Gel-filtered platelets were incubated for 3 min at 37°C in the presence of test compounds and fibrinogen (100

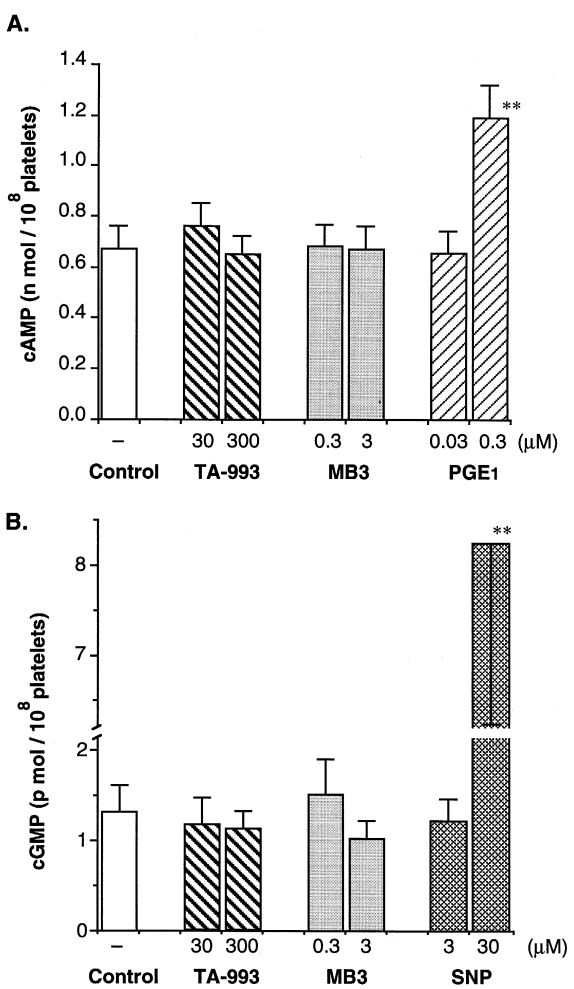

Fig. 3. Effects of TA-993 and MB3 on resting cyclic AMP (A) and cyclic GMP (B) levels in platelets. Platelet-rich plasma and test compounds were incubated at 37°C for 2 min. The reaction was stopped by addition of 10% perchloric acid. cyclic AMP and cyclic GMP levels in the platelets were assayed by the respective radioimmunoassay kits. Data are expressed as means ± S.E.M. of three to four blood donors. $^{**}P < 0.01$ versus control.

nM). They were then agitated with ADP (10 μM) at 37°C for 3 min. Incubation was terminated by addition of an equal volume of a lysis buffer (modified Tyrode's buffer containing 0.2% Triton X-100 and 2 mM EDTA). The amount of actin that was present in a filamentous form was determined by the DNase I inhibition assay as described by Fox et al. (1981). In brief, the mixture of 15 μl of cell lysate and 5 μl of DNase I solution was added to 3 ml of DNA solution and the absorbance at 260 nm was measured with a 557 Hitachi spectrophotometer. TA-993 and MB3 did not directly inhibit the DNase I activity.

2.10. Statistical analysis

Data are expressed as means ± S.E.M. Differences between groups were analysed using one-way analysis of variance (ANOVA) and a multiple comparison test. Statistical significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Effect on ADP-induced platelet aggregation and fibrinogen binding to ADP-stimulated gel-filtered platelets

TA-993, MB3 and RGDS inhibited ADP (10 μM)-induced platelet aggregation in a concentration-dependent manner with IC50 (μM) values of 88.6 ± 11.1, 0.19 ± 0.02, and 57.1 ± 2.7, respectively (Fig. 1A) and inhibited fibrinogen binding to the ADP-stimulated platelets with IC50 (μM) values of 20.1 ± 4.4, 0.065 ± 0.003, and 15.0 ± 1.8, respectively (Fig. 1B). The rank orders of potency of these compounds were similar between the inhibition of platelet aggregation and that of fibrinogen binding: MB3 was 300–500 times more potent than TA-993 and RGDS in both systems.

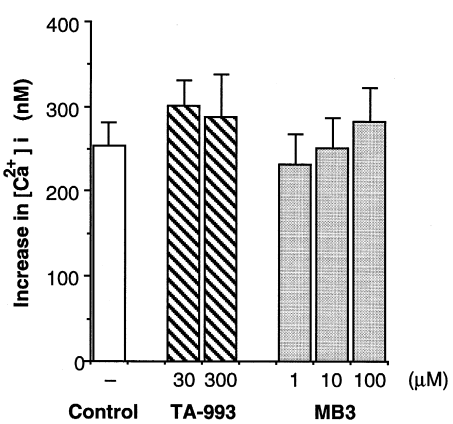

Fig. 4. Effects of TA-993 and MB3 on the ADP-induced increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in fura-2-loaded human platelets. Fura-2-loaded platelets were incubated with test compounds at 37°C for 3 min and stimulated with 10 μM ADP in the presence of 1 mM $CaCl_2$. Data are expressed as means ± S.E.M. of four blood donors.

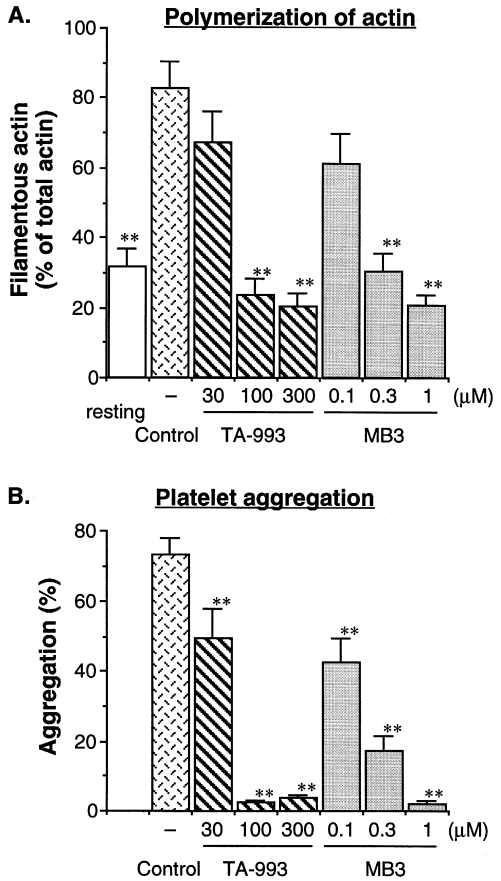

Fig. 5. Effects of TA-993 and MB3 on ADP-induced polymerization of actin (A) and platelet aggregation (B) in human gel-filtered platelets. The platelets were incubated with test compounds and 100 nM fibrinogen at 37°C for 2 min and stimulated with 10 μM ADP for 3 min. Incubation was terminated by the addition of a lysis buffer. The actin polymerization in the platelets was determined by the DNase I inhibition assay. Data are expressed as means ± S.E.M. of six blood donors. $^{**}P < 0.01$ versus control.

3.2. Effect on fibrinogen-induced aggregation of ADP-stimulated fixed platelets

The aggregation percent of fixed platelets initiated by the addition of 200 nM fibrinogen was 43.2 ± 3.3% ($N = 5$). RGDS showed concentration-dependent inhibition with an IC50 (μM) value of 69.2 ± 5.8 (Fig. 2). TA-993 and MB3 did not inhibit the fibrinogen-induced aggregation of fixed platelets even at the concentrations, which blocked platelet aggregation and fibrinogen binding (Fig. 2).

3.3. Effect on resting cyclic AMP and cyclic GMP levels in platelets

As shown in Fig. 3, the cyclic AMP and cyclic GMP levels of resting platelets were 0.67 ± 0.09 nmol/10^8 platelets and 1.3 ± 0.3 pmol/10^8 platelets, respectively. Both prostaglandin E_1 (0.3 μM) and sodium nitroprusside

(30 μM) significantly increased the cyclic AMP and cyclic GMP levels, respectively, whereas neither TA-993 nor MB3 affected the cyclic AMP and cyclic GMP levels. The activities of cyclic AMP and cyclic GMP phosphodiesterase were also not affected by either compound (data not shown).

3.4. Effect on ADP-induced increase in $[Ca^{2+}]i$ in fura-2-loaded platelets

ADP caused an increase in $[Ca^{2+}]_i$, which peaked within 10 s and then declined slowly. TA-993 and MB3 hardly affected $[Ca^{2+}]_i$ in resting platelets (data not shown) and the ADP-induced increase at the peak of $[Ca^{2+}]_i$ (Fig. 4), though platelet aggregation was almost completely inhibited.

3.5. Effect on ADP-induced polymerization of actin in platelets

ADP caused polymerization of actin from 32% to 83% in platelets. TA-993 and MB3 inhibited ADP-induced polymerization of actin at concentrations higher than 100 μM and 0.3 μM, respectively (Fig. 5A). They also showed similar patterns of concentration-dependency in their inhibitory effects on platelet aggregation (Fig. 5B). However, TA-993 and MB3 did not appear to promote actin depolymerization when they were added to the solution including extracted polymerized actin (data not shown).

4. Discussion

In this study, we have demonstrated that TA-993 and its metabolite MB3 inhibit platelet aggregation through the following antiplatelet mechanisms; (1) indirect blockade of glycoprotein IIb/IIIa receptors and (2) prevention of actin polymerization in ADP-stimulated human platelets. They exerted no influence on the intracellular Ca^{2+} concentration, and cyclic AMP and cyclic GMP levels.

TA-993 and MB3 inhibit platelet aggregation induced by ADP as well as by collagen, thrombin, epinephrine, platelet-activating factor and U-46619. MB3 has the most potent inhibitory action among the TA-993 metabolites (Odawara et al., 1996), and is thought to mainly contribute to the inhibition of platelet aggregation by TA-993 in vivo in various animal models (Kaburaki et al., 1998a,b; Narita et al., 1995; Odawara et al., 1996). TA-993 and MB3 also inhibited fibrinogen binding to the ADP-stimulated platelets concentration-dependently (Fig. 1). These results demonstrate that TA-993 and MB3 inhibit platelet aggregation through inhibition of fibrinogen binding to glycoprotein IIb/IIIa receptors in human platelets. We then examined the effect of TA-993 and MB3 on fibrinogen-induced aggregation in ADP-treated fixed platelets. The assay using fixed platelets is specific for direct inhibitors of fibrinogen binding, as the fixing process inactivates all enzymes and energy requiring processes and thus prevents signal transduction, and therefore, fixed platelets do not respond to ADP (Allain et al., 1975). TA-993 and MB3 did not inhibit fibrinogen-induced aggregation in ADP-treated fixed platelets, but RGDS did, suggesting that the benzothiazepines inhibit the activation of glycoprotein IIb/IIIa receptors and subsequent fibrinogen binding in intact platelets.

The drugs that increase either cyclic AMP or cyclic GMP levels are powerful inhibitors of platelet activation (Cox et al., 1992; Ko et al., 1994), but they do not have effects on fixed platelet (Cox et al., 1992). TA-993 and MB3 have been shown to be potent, broad spectrum antiplatelet compounds (Odawara et al., 1996). However, TA-993 and MB3 affected neither intracellular cyclic AMP nor cyclic GMP levels in resting platelets. The increase in $[Ca^{2+}]_i$ is a characteristic feature of platelet aggregation and is believed to play an important role as a second messenger for platelet activation (Kroll and Schafer, 1989). TA-993 and MB3 again had no effect on the ADP-induced $[Ca^{2+}]_i$ increase. Therefore, these results indicate that the antiplatelet action of TA-993 and MB3 could not be attributed to effects on the intracellular cyclic AMP and cyclic GMP levels and Ca^{2+} concentration in platelets.

Several reports suggested an association of glycoprotein IIb/IIIa receptors with cytoskeletal proteins such as filamentous actin in stimulated platelets (Ariyoshi and Salzman, 1996; Fox, 1993; Phillips et al., 1980). Inhibitors of actin polymerization, such as cytochalasin D and mycalolide-B, inhibited the conformational change of glycoprotein IIb/IIIa receptors in collagen- and ADP-stimulated platelets without inhibition of the increase in $[Ca^{2+}]_i$ in ADP-stimulated platelets (Ariyoshi and Salzman, 1996; Sugidachi et al., 1998). Our result, i.e. inhibition of actin polymerization by TA-993 and MB3, is consistent with the above data. In addition, we also previously reported that TA-993 and MB3 not only inhibited ADP-induced primary and secondary aggregation but also caused rapid disaggregation when added 1 min after ADP in human platelets (Odawara et al., 1996). Taken together, these observations suggest that TA-993 and MB3 may inhibit the conformational change of glycoprotein IIb/IIIa receptors via the inhibition of actin polymerization. However, cytochalasin D and mycalolide-B inhibit the contraction of vascular smooth muscles (Hori et al., 1993) and depolymerize the filamentous actin in platelets and vascular smooth muscle cells (Saito et al, 1994; Sugidachi et al., 1998), and their low doses amplify collagen-induced platelet aggregation (Haslam et al., 1975; Sugidachi et al., 1998). Since TA-993 and MB3 did not show such effects in the present and previous studies (Odawara et al., 1996), it is likely that the mechanism by which TA-993 and MB3 inhibit actin polymerization is different from those of cytochalasin D and mycalolide-B. Furthermore, TA-993 and MB3 decrease fluorescence polarization in 1,6-di-

phenyl-1,3,5-hexatriene-labeled platelets and enhance the interaction of 1-anilino-8-naphthalene sulfonate with platelet membranes (our unpublished observations), suggesting that the inhibition of actin polymerization by TA-993 and MB3 may be attributable to the increase in fluidity of platelet membranes. Further studies are needed to elucidate the antiplatelet mechanisms of TA-993 and MB3.

In conclusion, we demonstrated that TA-993 and MB3 inhibited the activation of glycoprotein IIb/IIIa receptors through inhibition of actin polymerization and subsequent fibrinogen binding, by which they inhibited platelet aggregation induced by ADP. TA-993 and MB3, both 1, 5-benzothiazepine derivatives like diltiazem, may be useful compounds for studying the mechanisms of platelet activation.

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References

Addonizio, V.P., Fisher, C.A., Strauss, J.F., Wachtfoger, Y.T., Colman, R.W., Josephson, M.E., 1986. Effects of verapamil and diltiazem on platelet function. Am. J. Physiol. 250, H366–H371.

Allain, J., Cooper, H., Wanger, R., Brinkhous, K., 1975. Platelets fixed with paraformaldehyde: a new reagent for assay of von willebrand factor and platelet aggregation factor. J. Lab. Clin. Med. 75, 318–328.

Ariyoshi, H., Salzman, E.W., 1996. Association of local Ca^{2+} grandients with redistribution of glycoprotein IIb–IIIa and F-actin in activated human blood platelets. Arterioscler., Thromb., Vasc. Biol. 16, 230–235.

Armstrong, R.A., 1996. Platelet prostanoid receptors. Pharmacol. Ther. 72, 171–191.

Coller, B.S., 1990. Platelets and thrombolytic therapy. N. Engl. J. Med. 322, 33–42.

Cox, D., Motoyama, Y., Seki, J., Aoki, T., Dohi, M., Yoshida, K., 1992. Pentamidine: a non-peptide GPIIb/IIIa antagonist — in vitro studies on platelets from humans and other species. Thromb. Haemostasis 68, 731–736.

Dinerman, J.L., Mehta, J.L., 1990. Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance. J. Am. Coll. Cardiol. 16, 207–222.

Erne, P., Bolli, P., Burgisser, E., Buhler, F.R., 1984. Correlation of platelet calcium with blood pressure: effect of antihypertensive therapy. N. Engl. J. Med. 310, 1084–1088.

Fox, J.E.B., 1993. The platelet cytoskeleton. Thromb. Haemostasis 70, 884–893.

Fox, J.E.B., Dockter, M.M., Phillips, D.R., 1981. An improved method for determining the actin filament content of nonmuscle cells by the DNase 1 inhibition assay. Anal. Biochem. 117, 170–177.

Grynkiewicz, G., Peonie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Haslam, R.J., Davidson, M.M., MacClenaghan, M.D., 1975. Cytochalasin B, the blood platelet release reaction and cyclic GMP. Nature 253, 455–457.

Holmsen, H., Weiss, H.J., 1979. Secretable storage pools in platelets. Annu. Rev. Med. 30, 119–134.

Hori, M., Saito, S., Shin, Y.Z., Ozaki, H., Fusetani, N., Karaki, H., 1993. Mycalolide-B, a novel and specific inhibitor of actomyosin ATPase isolated from marine sponge. FEBS Lett. 322, 151–154.

Kaburaki, M., Doi, H., Narita, H., Odawara, A., Yasoshima, A., Murata, S., 1998a. Antithrombotic action of TA-993, a new 1,5-benzothiazepine derivative, in a canine model of femoral arterial thrombosis. J. Cardiovasc. Pharmacol. 31, 470–477.

Kaburaki, M., Inoue, H., Doi, H., Yasuhara, M., Narita, H., 1998b. Cardiovascular effects of 1,5-benzothiazepine derivatives having a L-*cis* and D-*cis* configuration in anesthetized dogs. Biol. Pharm. Bull. 21, 50–55.

Katoh, M., Chishima, S., Kiuchi, N., Ikeo, T., Sasaki, Y., 1995. A new method for the assay of exposed platelet fibrinogen receptor using a chemiluminescent label. Thromb. Haemostasis 74, 1546–1550.

Ko, F.N., Wu, C.C., Kuo, S.C., Lee, F.Y., Teng, C.M., 1994. YC-1, a novel activator of platelet guanylate cyclase. Blood 84, 4226–4233.

Kroll, M.H., Schafer, A.I., 1989. Biochemical mechanisms of platelet activation. Blood 74, 1181–1195.

Maffrand, J.P., Bernat, A., Delebassee, D., Defreyn, G., Cazenave, J.P., Gordon, J.L., 1988. ADP plays a key role in thrombogenesis in rats. Thromb. Haemostasis 59, 225–230.

Meth, P., Meth, J., Ostrowski, N., Brigmon, L., 1983. Inhibitory effects of diltiazem on platelet activation caused by ionophore A23187 plus ADP or epinephrine in subthreshold concentration. J. Lab. Clin. Med. 102, 332–339.

Narita, H., Kaburaki, M., Doi, H., Yasoshima, A., Murata, S., 1995. Antithrombotic effect of TA-993, a novel 1,5-benzothiazepine derivative, in conscious rats. Jpn. J. Pharmacol. 68, 397–404.

Odawara, A., Katoh, M., Karasawa, T., Tamura, K., Sasaki, Y., 1994. Inhibitory effect of clentiazem (TA-3090) on platelet aggregation-alone and in combination with aspirin or ticlopidine. Thromb. Res. 75, 109–119.

Odawara, A., Kikkawa, K., Katoh, M., Toryu, H., Shimazaki, T., Sasaki, Y., 1996. Inhibitory effects of TA-993, a new 1,5-benzothiazepine derivative, on platelet aggregation. Circ. Res. 78, 643–649.

Peerschke, E.I., Zucker, M.B., Grant, R.A., Egan, J.J., Johnson, M.M., 1980. Correlation between fibrinogen binding to human platelets and platelet aggregability. Blood 55, 841–847.

Phillips, D.R., Jennings, L.J., Edwards, H.H., 1980. Identification of membrane proteins mediating the interaction of human platelets. J. Cell. Biol. 86, 77–86.

Saito, S., Watabe, S., Ozaki, H., Fusetani, N., Karaki, H., 1994. Mycalolide B, a novel actin depolymerizing agent. J. Biol. Chem. 269, 29710–29714.

Schwartz, S.M., Heimark, R.L., Majesky, M.W., 1990. Developmental mechanisms underlying pathology of arteries. Physiol. Rev. 70, 1177–1209.

Sugidachi, A., Ogawa, T., Asai, F., Saito, S., Ozaki, H., Fusetani, N., Karaki, H., Koike, H., 1998. Inhibition of rat platelet aggregation by mycalolide-B, a novel inhibitor of actin polymerization with a different mechanism of action from cytochalasin-D. Thromb. Haemostasis 79, 614–619.

Tsien, R.Y., Pozzan, T., Rink, T.J., 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellular trapped fluorescent indicator. J. Cell. Biol. 94, 325–334.

Vorchheimer, D.A., Badimon, J.J., Fuster, V., 1999. Platelet glycoprotein IIb/IIIa receptor antagonists in cardiovascular disease. JAMA 281, 1407–1414.