A New Anti-Platelet Drug, E5510, Has Multiple Suppressive Sites during Receptor-Mediated Signal Transduction in Human Platelets

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ABSTRACT—The mode of action of E5510, 4-cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid, which has very potent anti-platelet activities, was investigated by examining its effects on the biochemical responses in the process of human platelet activation. In a whole-cell system, E5510 inhibited the increased turnover of inositol phospholipids arising from phospholipase C activation, arachidonic acid release from phospholipids by phospholipase A₂, mobilization of intracellular free Ca²⁺, protein kinase C activation, and thromboxane A₂ production. In a cell-free system, E5510 inhibited cyclooxygenase activity and cyclic AMP-dependent phosphodiesterase activity in a dose-dependent manner. An elevation of cyclic AMP in platelets was also observed at a relatively high concentration of E5510. It was suggested that receptor-mediated turnover of inositol phospholipids, intracellular Ca²⁺ increase, arachidonic acid release from phospholipids and protein kinase C activation might be indirectly inhibited by the increased cyclic AMP level in platelets. Thromboxane A₂ production in the whole-cell system was very strongly inhibited by E5510, and the IC₅₀ for this effect was 100 times lower than that of direct inhibition of cyclooxygenase in the cell-free system. It was concluded that although the primary mode of action of E5510 is the inhibition of the cyclooxygenase pathway of positive signal transduction in platelets, E5510 has another mode of action by increasing platelet cyclic AMP, which can act as a negative messenger in platelet signal transduction, and these multiple sites of action synergistically antagonize platelet cellular activation.

E5510, 4-cyano-5,5-bis(4-methoxyphenyl)-4-pentenoic acid, is a very powerful platelet aggregation inhibitor (1). This compound strongly inhibits human platelet aggregation induced by collagen, ADP, epinephrine, platelet-activating factor, AA or thrombin in

vitro. This inhibitory action is similar in some respects to that of CO inhibitors such as ASA and U53059 (2, 3), in terms of the preferential suppression of secondary platelet aggregation. However, there exists an absolute difference between E5510 and CO inhibitors in that

Abbreviations used are: ADP, adenosine 5'-diphosphate; AA, arachidonic acid; CO, cyclooxygenase; ASA, acetylsalicylic acid; PI turnover, turnover of inositol phospholipids; PLC, phospholipase C; PA, phosphatidic acid; IP₃, inositol trisphosphate; PKC, protein kinase C; PLA₂, phospholipase A₂; TX, thromboxane; BSA, bovine serum albumin; PRP, platelet-rich plasma; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; SDS, sodium dodecyl sulfate; PDE, phosphodiesterase; SSVM, sheep seminal vesicular microsomes.

E5510 dose-dependently inhibits thrombin-induced platelet aggregation, which is not inhibited by CO inhibitors (1). The mode of action of this compound against platelet activation has not yet been clearly established.

Biochemical events in platelets respond to both positive and negative signals (4, 5). In short, extracellular positive signals which bind to specific receptors on the platelet membrane promote the PI turnover through activation of PLC, which results in rapid production of diacylglycerol and inositol phosphates (6–8). As diacylglycerol is readily converted to PA, an increase in PA is observed after platelet activation (9). IP_3 and diacylglycerol, intermediates of PI turnover, induce intracellular Ca^{2+} mobilization (10, 11) and PKC activation (12), respectively. The intracellular Ca^{2+} increase promotes the release of AA from platelet phospholipids through activation of PLA_2 (13). The released AA is converted into TXA_2 which can act as a feedback promoter and induce further stimulation of PLC, leading to acceleration and amplification of platelet activation. TXA_2 acts as a powerful positive signal for non-activated platelets (14). On the contrary, negative signals such as PGI_2 activate platelet adenylate cyclase and increase cyclic AMP content in the platelets. This increase in cyclic AMP inhibits the receptor-linked degradation of inositol phospholipids and the Ca^{2+} mobilization, which result in activation of PLA_2 and PKC during activation of platelets by the positive signals, and suppresses the cellular response of platelets (12, 15).

The purpose of this paper is to elucidate the mode of action of E5510 in inhibiting receptor-mediated platelet activation. We studied the effects of this compound on positive signal transduction and negative signal transduction in human platelets.

MATERIALS AND METHODS

Materials

E5510, U53059, OPC13013 (Cilostazol) and BW755C were synthesized in our laboratory.

ASA, mepacrine (quinacrine 2HCl) and PGE_1 were purchased from Iwaki Pharmaceutical (Tokyo, Japan), Sigma (St. Louis, U.S.A.) and Funakoshi Pharmaceutical Co., Ltd. (Tokyo, Japan). Drugs were dissolved in dimethyl sulfoxide and diluted to the desired concentration with saline containing 0.1% BSA. Fura2/AM was from Molecular Probes (Junction City, U.S.A.). $[1-^{14}C]$ Arachidonic acid (54.9 mCi/mmol), $[^{32}P]Na_2HPO_4$ (0.9–1.1 Ci/mmol) and $[2,8-^3H]$ adenosine 3',5'-cyclic phosphate (32.1 Ci/mmol) were purchased from NEN (Boston, U.S.A.). Thrombin from bovine plasma (77 U/mg) was obtained from Miles Lab. (Elkhart, U.S.A.). All other reagents used here were analytical grade.

Preparation of human platelets and aggregation measurement

Human venous blood of healthy male volunteers who had not received any medication for more than two weeks was anti-coagulated with one-tenth volume of 3.8% sodium citrate and centrifuged at $100 \times g$ for 10 min at room temperature to obtain PRP was washed twice with 0.1% EDTA saline and finally suspended in Ca^{2+} -free Tyrode solution containing 0.1% BSA at the cell concentration of 5×10^8 cells/ml. Silicon-coated glassware or polypropylene tubing was used for preparing PRP and in the following experiments.

Platelet aggregation was measured according to Born's turbidimetric method (16) with an aggregometer (Hematracer, Nicho Bioscience Co., Ltd., Tokyo, Japan), after preincubation of a 200- μ l aliquot of washed platelets and 25 μ l of drug solution for 3 min at 37°C. To induce platelet aggregation, 25 μ l of thrombin dissolved in Tyrode solution was added to the reaction mixture. The intensity of platelet aggregation was measured according to the method reported previously (1), and the effects of test drugs were expressed as % inhibition values compared to the control.

Preparation of radioisotope-labeled platelets

$[1-^{14}C]$ AA was added to PRP to give the concentration of 1.7 μ Ci/ml PRP, and the

mixture was incubated at 37°C for 1 hr. The platelets were washed twice with 15 mM Tris-buffered saline (pH 7.4) containing 5 mM KCl, 1 mM EGTA, and 5.5 mM glucose and finally suspended in the same buffer without EGTA to give the cell concentration of 3×10^8 platelets/ml.

³²P-Prelabeled platelets were obtained by incubating platelets with 15 mM Tris buffer (pH 7.4) containing 129 mM NaCl, 2.8 mM KCl, 1 mg/ml glucose, 8.9 mM NaHCO₃ and 1.1 mCi [³²P]-Na₂HPO₄ at 37°C for 30 min and then suspending them in 15 mM Tris buffer (pH 7.4) containing 135 mM NaCl, 5 mM KCl and 5.5 mM glucose at the cell concentration of 4×10^8 platelets/ml (17).

Determination of AA release (PLA₂ activity) in thrombin-stimulated platelets

A mixture of 0.8 ml of [¹⁴C]AA-labeled platelet suspension, 0.1 ml of drug solution or vehicle and 0.05 ml of 20 mM CaCl₂ was preincubated at 37°C for 3 min in the presence of 100 μM BW755C, which completely inhibited both CO and lipoxygenase (18), and the platelets were activated by adding 0.05 ml of 1 U/ml thrombin. After 3 min, the reaction was terminated by the addition of 0.1 ml of ice-cold 0.1 N HCl. Lipids were extracted into 4 ml of chloroform: methanol (2:1) according to the method of Bligh and Dyer (19). [¹⁴C]-Labeled eicosanoids were separated by thin layer chromatography on a silica gel plate (Whatman, LK6-DF, Clifton, U.S.A.) in a solvent system consisting of the organic phase from ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100) (20). The areas corresponding to AA, 12-HETE, TXB₂ and HHT were scraped off and the radioactivities were measured. The increase in the sum of the radioactivities in AA, 12-HETE, TXA₂ and HHT was considered to reflect the PLA₂ activity.

Determination of PA accumulation in thrombin-stimulated human platelets

The ³²P-prelabeled platelet suspension (4×10^8 cells/ml, 0.4 ml) was preincubated with

drug solution or vehicle at 37°C for 5 min and activated by the addition with 50 μl of 5 U/ml thrombin. After 20 sec of incubation at 37°C under stirring (1000 rpm), the reaction was stopped by adding 2 ml of CHCl₃/methanol/conc. HCl (20:40:1), and phospholipids were extracted and analyzed by the method of Tysnes et al. (21). The radioactivity in the PA fraction was measured as an index of PI turnover (9, 22).

Measurement of intracellular [Ca²⁺] ([Ca²⁺]_i) with fura2

Loading of platelets with fura2 (2 μM) was carried out as described by Rao et al. (23). After the cells had been washed free of excess dye, they were resuspended in Ca²⁺-free Tyrode solution containing 2 mM EGTA (1.5×10^8 platelets/ml). The fura2-loaded platelet suspension (3 ml) was preincubated with or without drug (3 μl) at 37°C for 10 min; then thrombin was added at the final concentration of 0.05 U/ml, and continuous fluorescence measurements were made in a Shimadzu RF-540 spectrofluorometer. Ca²⁺ concentration was calculated by the procedure of Grynkiewicz et al. (24).

40-K protein phosphorylation after stimulation of platelets by thrombin

The mixture of 40 μl of ³²P-labeled platelets (5×10^8 cells/ml) and 5 μl of drug solution or vehicle was incubated at 37°C for 5 min and stimulated by 5 μl of 5 U/ml thrombin. After 1 min, the reaction was stopped by adding 25 μl of 2% SDS solution containing 63 mM Tris, 10% glycerol, and 0.01% bromophenol blue (pH 6.8). The radioactive platelet proteins were analyzed by SDS-polyacrylamide slab gel electrophoresis under the conditions described by Laemmli (25). The gel was stained, dried on a filter paper and exposed to X-ray film to prepare an autoradiograph, and the phosphorylation of protein with a molecular weight of about 40 K was determined according to the established method (7). The degree of phosphorylation was considered to reflect the PKC activity (7).

Measurement of PDE and cyclic AMP content in platelets

Washed human platelets ($3-5 \times 10^8$ cells/ml) suspended in 50 mM Tris/HCl (pH 7.5) were sonicated (50 W, 30 sec) and ultracentrifuged for 60 min at $100,000 \times g$. PDE activity was measured according to the method of Thompson and Strada (26), using the supernatant fraction described above as the enzyme preparation (10 μ g protein) and [3 H]cyclic AMP as a substrate (100,000 cpm) under the condition of 10 min incubation at 37°C with or without drug.

Platelet cyclic AMP was determined using a cyclic AMP radioimmunoassay kit (NEN, Boston, U.S.A.) based on a protein binding method, after incubation of 0.45 ml of platelet suspension in Tyrode solution (5×10^8 cells/ml) with 0.05 ml of drug solution or vehicle at 37°C for 3 min.

Measurement of TXB₂ production in platelets

A mixture of 85 μ l of washed platelets (5×10^8 cells/ml) suspended in 0.05 M Tris/HCl saline (pH 7.6) and 5 μ l of drug solution or vehicle was preincubated at 37°C for 5 min. The reaction was started by the addition of 10 μ l of 200 μ M [14 C]AA (0.2 μ Ci) and terminated by adding 300 μ l of stopping solution, consisting of ethylacetate/methanol/1 M citric acid (30:4:1), after 1 min. The reaction mixture was dried over 0.5 g of Na₂SO₃ and centrifuged ($1,100 \times g$, 5 min). An aliquot of the supernatant solution (50 μ l) was analyzed by thin layer chromatography according to the same method described above, and the radioactivity in TXB₂ was determined.

Measurement of CO activity

SSVM were prepared by the method of Wallach and Daniels (27). The lyophilized microsomes were used as the CO preparation after being dissolved in 0.1 M Tris/HCl buffer (pH 7.4) containing 5 mM tryptophan and 5 mM glutathione. The enzyme solution (0.2 mg/ml) with or without drug (90 μ l in total) was preincubated at 25°C for 10 min. The enzyme reaction was initiated by adding 10 μ l of

0.2 mM AA and stopped by adding 0.9 ml of ethanol/1 M citric acid (180:1) 20 min later. The mixture was centrifuged ($2,000 \times g$, 2 min) and PGE₂ content in the supernatant was assayed by using a PGE₂ radioimmunoassay kit (NEN, Boston, U.S.A.) as an index of CO activity.

Statistics

Statistical significance was evaluated by Student's *t*-test. IC₅₀ values were calculated by Lichfield-Wilcoxon's method.

RESULTS

Effect on thrombin-induced platelet aggregation

ASA alone did not inhibit this aggregation up to 500 μ M, although it completely suppressed AA-induced platelet aggregation at 100 μ M (data not shown), which suggested that the CO activity in platelets was entirely inhibited under this condition. E5510 inhibited thrombin-induced platelet aggregation both in the presence and absence of 100 μ M ASA (Fig. 1). This indicates that E5510 inhib-

Fig. 1. Effects of E5510 on thrombin-induced platelet aggregation in aspirin-treated or untreated human washed platelets in vitro. Human washed platelet suspension was preincubated with 100 μ M aspirin or vehicle for 10 min at 37°C. Platelet aggregation induced by 0.03 U/ml of thrombin was measured after 3 min of incubation with a desired concentration of E5510 solution, using both aspirin-treated and untreated human washed platelet suspensions. Each point represents the mean $\pm$ S.E. of 2 or 3 preparations. (■): effect of E5510 in aspirin-treated platelets, (□): effect of E5510 in untreated platelets, (○): effect of aspirin in untreated platelets.

its thrombin-induced platelet activation through a mechanism other than CO inhibition.

Effects on platelet signal transduction in a whole-cell system

As human platelets could be activated by thrombin even if platelet CO was inhibited by ASA (Fig. 1), it was considered that thrombin can induce cellular responses of platelets through a receptor-coupled signal transduction pathway other than AA cascade in platelets. In order to clarify further the difference between E5510 and CO inhibitors, we examined the effect of E5510 on AA release from phospholipids in platelets prelabeled with [14 C]AA after thrombin-stimulation in the presence of 100 μ M BW755C. Under these conditions, BW755C completely inhibited both CO activity (TXB₂ + HHT production) and lipoxygenase activity (12-HETE production), but AA release was not affected after thrombin-stimulation (Table 1). E5510 inhibited thrombin-induced AA release in platelets in which CO and lipoxygenase were inhibited (Fig. 2). Mepacrine, a PLA₂ inhibitor (28), and OPC13013, a PDE inhibitor (29), also showed inhibitory effects but U53059, a CO inhibitor, tended to enhance AA release rather than inhibiting it. These results led us to examine the effect of E5510 on the second messengers in platelets after the activation by thrombin.

Receptor-linked increase in PI turnover,

which was determined by measuring the accumulation of PA in response to thrombin, and the intracellular mobilization of free Ca²⁺ in thrombin-stimulated platelets were inhibited by E5510 in a concentration-dependent manner (Figs. 3 and 4).

Phosphorylation of 40-K protein in thrombin-stimulated platelets, which was considered as PKC activation after the transient increase of diacylglycerol formation and mobi-

Fig. 2. Effects of E5510, U53059, mepacrine, and OPC13013 on arachidonic acid (AA) release from phospholipids in thrombin-stimulated human platelets in the presence of BW755C. [14 C]AA-labeled platelet suspension containing 100 μ M BW755C was incubated for 3 min at 37°C with test drug solution at the desired concentration and stimulated by 0.05 U/ml of thrombin. Total AA release was measured by the same method described in Table 1. Results are the mean values of duplicate assays. CNT: control (vehicle instead of drug solution), MEP: mepacrine.

Table 1. Effect of BW755C on arachidonic acid release from phospholipids in thrombin-stimulated human platelets

	Thrombin	TXB ₂ + HHT	12-HETE	AA	Total AA release
Basal	-	268	245	497	1011
Control	+	407	975	2429	3811
BW755C	+	254	179	3109	3524

The figures in the table show the radioactivity in dpm. Human platelets (3×10^6 cells/tube) labeled with [14 C]-arachidonic acid were incubated with, or without 100 μ M BW755C for 3 min at 37°C and stimulated by 0.05 U/ml of thrombin. TXB₂: thromboxane B₂, HHT: 12-hydroxy-5,8,10-heptadecatrienoic acid, 12-HETE: 12-hydroxy-5,8,10,14-eicosatetraenoic acid, AA: arachidonic acid. Total AA release was calculated as the sum of the radioactivity in TXB₂ + HHT, 12-HETE and AA.

Fig. 3. Effects of E5510 and OPC13013 on phosphatidic acid production in thrombin-stimulated human ^{32}P -labeled platelets. ^{32}P -Labeled platelets (4×10^8 cells/ml) were incubated with or without drug for 5 min at 37°C and stimulated by 0.5 U/ml of thrombin for 20 sec. Phospholipids were extracted and separated by thin layer chromatography as described in Materials and Methods. The radioactivity in the phosphatidic acid spot was determined. Each column represents the mean $\pm$ S.E. of triplicate assays. Asterisks indicate statistically significant differences compared to the control ($P < 0.05$).

Fig. 4. Effects of E5510 on mobilization of $[\text{Ca}^{2+}]_i$ in fura2-loaded human platelets after thrombin stimulation. After the fura2-loaded platelet suspension (1.5×10^8 cells/ml) was preincubated with or without drug for 10 min at 37°C , the platelets were stimulated by 0.05 U/ml of thrombin. The maximum $[\text{Ca}^{2+}]_i$ increase was measured from continuous fluorescence records and calculated as described in Materials and Methods. Each column represents the mean $\pm$ S.E. of 8–14 experiments. Asterisks indicate statistically significant differences compared to the control ($P < 0.05$).

Fig. 5. Effects of E5510 and PGE₁ on phosphorylation of 40-K protein in ^{32}P -labeled human platelets after thrombin stimulation. ^{32}P -Labeled platelets (5×10^8 cells/ml) were incubated with or without drug for 5 min at 37°C and stimulated by 0.5 U/ml thrombin for 1 min. The radioactive proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, and phosphorylation of the protein with a molecular weight of about 40 K was measured as described in Materials and Methods. Each column represents the mean $\pm$ S.E. of 5–7 experiments. Asterisks indicate statistically significant differences compared to the control (**; $P < 0.01$; *; $P < 0.05$).

Fig. 6. Effects of E5510 and OPC13013 on cyclic AMP level in human platelets. Washed platelets (5×10^8 cells/ml) were incubated with or without drug for 3 min at 37°C and cyclic AMP content was determined by radioimmunoassay. Cyclic AMP level in the control was 2.8 ± 0.5 pmol/ 10^8 platelets. Each column represents the mean $\pm$ S.E. of 3–5 experiments.

lization of $[Ca^{2+}]_i$ (7), was inhibited by E5510 and PGE_1 (Fig. 5).

In order to demonstrate the effect of E5510 on the negative signal transduction in platelets, cyclic AMP content in platelets was determined after incubation of the drug with washed platelet suspension (Fig. 6). E5510 caused a dose-dependent increase in platelet cyclic AMP level.

It was reported that platelet CO products (PG endoperoxide, TXA_2), synthesized from AA which is released from phospholipids by PLA_2 and PLC after receptor-mediated platelet activation, could act as positive feedback promoters within the same platelets and activate PLC in other platelets (14, 30). Therefore, we examined the effect of E5510 on TXA_2 production after the stimulation of platelets by AA in a whole-cell system. E5510 strongly and dose-dependently inhibited TXB_2 (the stable metabolite of TXA_2) production at extremely low concentrations, giving an IC_{50} value of $0.03 \mu M$ (Fig. 7). This inhibitory potency of E5510 was almost the same as that of U53059.

Effect on platelet signal transduction in a cell-free system

The direct action of E5510 on CO activity

Fig. 7. Inhibitory effects of E5510, U53059 and aspirin on thromboxane B_2 (TXB_2) biosynthesis in human platelets. Washed platelets (5×10^8 cells/ml) were incubated with or without drug for 5 min at $37^\circ C$ and $[^{14}C]$ arachidonic acid ($0.02 \text{ Ci}/20 \mu M$) was added. After 1 min, the reaction was stopped, and TXB_2 was measured as described in Materials and Methods.

was studied by determining the conversion of AA to PGE_2 by SSVM as the source of CO. The PGE_2 production rate in the control was $24 \text{ ng } PGE_2/\text{min}/\text{mg SSVM}$. Dose-dependent inhibition was seen with E5510 and U53059 (Fig. 8), and the IC_{50} values were $3 \mu M$ and $0.03 \mu M$, respectively. U53059 was 100 times more potent than E5510 in inhibiting CO activity. A PDE inhibitor, OPC13013, did not have any inhibitory effect on CO activity.

Cyclic AMP is well-known as a negative second messenger in platelets (12). The steady level of cyclic AMP in platelets is regulated by adenylate cyclase and PDE, the synthetic and degradative enzymes, respectively. We studied the effect of E5510 on these enzymes. E5510 did not modify the activity of adenylate cyclase (data not shown), but PDE activity ($6 \text{ pmol}/\text{min}/\text{mg prot.}$ in the control) was inhibited by E5510, giving an IC_{50} value of about $10 \mu M$ (Fig. 9).

DISCUSSION

It was reported that CO inhibitors such as ASA and indomethacin are not able to inhibit thrombin-induced platelet activation (14). The thrombin-induced initial PLC activation in

Fig. 8. Inhibitory effects of E5510, U53059 and OPC13013 on cyclooxygenase activity. Cyclooxygenase activity was measured using sheep seminal vesicular microsomes as the enzyme source and $20 \mu M$ arachidonic acid as the substrate as described in Materials and Methods. PGE_2 production was taken as a measure of cyclooxygenase activity. Results are expressed as % inhibition compared to the control, and each plot represents the mean $\pm$ S.E. of 2–4 experiments.

Fig. 9. Inhibitory effects of E5510, U53059 and OPC13013 on cyclic AMP-dependent phosphodiesterase (PDE). Phosphodiesterase activity was measured using human platelet cytosolic fraction as the enzyme source and [^3H]cyclic AMP as the substrate, as described in Materials and Methods. Each plot represents the mean of duplicate assays.

platelets is dissociated from AA metabolism, as evidenced by inability of ASA to inhibit thrombin-stimulated production of diacylglycerol and PA production in platelets (14, 31). Our present finding that ASA did not inhibit thrombin-induced platelet aggregation is consistent with these reports, and we confirmed that E5510 clearly inhibited-thrombin-induced platelet aggregation even in ASA-treated human platelets in which CO activity was completely suppressed. This strongly indicated that E5510 might have a different site of action from AA cascade in the process of receptor-mediated signal transduction in platelets. In order to clarify the mode of action of E5510 other than CO inhibition, we studied the effects of this compound on biochemical responses in the process of platelet signal transduction in a whole-cell system and a cell-free system.

In the whole-cell system, our previous report (1) showed that E5510 inhibited AA release from phospholipids after stimulation of platelets by thrombin, and it was found here that thrombin-stimulated AA release from platelet phospholipids was suppressed even under the condition of both CO and lipoxygenase inhibition by BW755C. This strongly in-

dicated that E5510 influences second messengers of platelet signal transduction such as PI turnover, mobilization of $[\text{Ca}^{2+}]_i$ and PKC activation, other than AA cascade. Actually, E5510 inhibited PA accumulation which could be considered as an index of initial PLC activation and acceleration of PI turnover in thrombin-stimulated platelets (14, 22), and it also inhibited thrombin-induced mobilization of $[\text{Ca}^{2+}]_i$ in platelets. As the increase in $[\text{Ca}^{2+}]_i$ results in the activation of PLA_2 (13), the inhibition of AA release from phospholipids is due to the suppression of mobilization of $[\text{Ca}^{2+}]_i$ which is caused by IP_3 formation during the activation of PI turnover (10, 11). PKC activation, which was estimated by determining phosphorylation of 40-K protein in thrombin-stimulated platelets, was inhibited by E5510 and PGE_1 . Inhibition of PKC by PGE_1 is reported to be due to cyclic AMP elevation in platelets by adenylate cyclase activation coupled with PGE_1 receptor (12, 32), and the inhibition by E5510 was supposed to be due to cyclic AMP elevation by PDE inhibition. These results suggested that E5510 antagonized thrombin-induced platelet activation by inhibiting receptor-mediated PLC activation and subsequent events in signal transduction, either directly or indirectly.

In the cell-free system, PDE activity in the cytosolic fraction of platelets was directly inhibited by E5510 in a dose-dependent manner. This result corresponded well with the fact that cyclic AMP level in platelets was dose-dependently elevated by E5510. It is well-established that the increased cyclic AMP level in platelets causes a decrease in cytosolic free Ca^{2+} by phosphorylating two microsomal proteins (24-K and 22-K proteins), resulting in the enhancement of Ca^{2+} -activated ATPase (12, 33, 34). It was also reported that thrombin-mediated PLC activation is suppressed by the increased cyclic AMP in platelets (15, 35), probably acting at the G-protein level (36). These findings indicate that cyclic AMP can act as a negative messenger in the process of platelet activation (5). Therefore, it was concluded that the inhibition by E5510 of

sequential steps of signal transduction such as PI turnover, mobilization of $[Ca^{2+}]_i$, and PKC activation, was mediated by its increasing action on platelet cyclic AMP caused by the inhibition of PDE in thrombin-stimulated platelets.

In the cell-free system, E5510 also directly inhibited CO activity in a concentration-dependent manner. The IC_{50} value of E5510 in inhibiting CO was $3 \mu M$ and that of U53059 was $0.03 \mu M$. Our previous report (1) demonstrated that the IC_{50} values for inhibition of AA-induced platelet aggregation by E5510 and U53059 were 0.7, and $0.03 \mu M$, respectively. The potency of E5510 for the inhibition of the platelet aggregation was stronger than that for the inhibition of CO activity in the cell-free system, while the potencies of U53059 for inhibition of the platelet aggregation and CO activity were the same. These evidences are supported by the present findings that the activation of the CO pathway in the whole-cell system, which was measured in terms of TXB_2 production, was very strongly inhibited by E5510 (IC_{50} : $0.03 \mu M$), compared to its inhibition of CO activity in the cell-free system (IC_{50} : $3 \mu M$). U53059, however, inhibited the CO pathway both in whole-cell and cell-free systems to the same extent. Consequently, it was suggested that this exaggerated inhibition in the whole-cell system by E5510 could be elucidated by its increasing action on platelet cyclic AMP in addition to the direct suppression of the CO pathway.

Overall, there was an indirect inhibition by E5510 of thrombin-mediated initial PLC activation, followed by IP_3 and diacylglycerol formation, intracellular free Ca^{2+} mobilization leading to PLA_2 activation and AA cascade promotion, and PKC activation. This indirect inhibition arose from cyclic AMP elevation in platelets through the direct suppression of PDE by E5510. The AA cascade, especially TXA_2 production, which can act as a powerful positive signal and feedback promoter of PLC resulting in acceleration of platelet activation, was strongly inhibited by E5510. Therefore, it was concluded that E5510 has multiple sites of

action in receptor-mediated signal transduction: that is, CO inhibition and PDE inhibition. CO inhibition can reduce the positive signal transduction and PDE inhibition can promote the negative signal transduction in the biochemical responses of platelets. These multiple modes of action of E5510 synergistically operate in platelets to antagonize the cellular activation.

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