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Thrombin-induced human platelet aggregation is inhibited by protein-tyrosine kinase inhibitors, ST638 and genistein

Momoyo Asahi*, Shigeru Yanagi, Shinji Ohta, Tetsuya Inazu, Keiko Sakai, Fumito Takeuchi, Takanobu Taniguchi and Hirohei Yamamura

Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11, Japan

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We have investigated the involvement of protein-tyrosine kinases in thrombin-induced aggregation of human platelets, using ST638 and genistein which are known inhibitors of protein-tyrosine kinase. Preincubation of platelets with 50 μM of ST638 or 25 μg/ml of genistein completely blocked the platelet aggregation induced with 0.05 unit/ml of thrombin. The increase of protein-tyrosine phosphorylation bands (135-, 124-, 76-, 64-, and 60-kDa) induced with thrombin was also inhibited by these inhibitors in a dose-dependent manner. These inhibitors also blocked the platelet aggregation and protein-tyrosine phosphorylation induced with thrombin in aspirin-treated platelets. Increase of the intracellular Ca^{2+} concentration induced by thrombin was also inhibited by higher concentrations of genistein. These results suggest that the protein-tyrosine phosphorylation plays a certain role in platelet activation having some relation to the intracellular Ca^{2+} concentration.

Platelet aggregation; Thrombin; Protein-tyrosine phosphorylation; Ca^{2+}; ST638; Genistein

1. INTRODUCTION

Stimulation of human platelets by physiological agonists such as thrombin and collagen is associated with a rapid turnover of inositol phospholipids through phospholipase C activation, which leads to intramembrane diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) [1–3]. These DG and IP_3, which activate protein kinase C and mobilization of intracellular Ca^{2+}, respectively, are considered to be intracellular messengers [4,5]. Thus, physiological responses of platelets are elicited concurrently with these putative signal transduction processes. Though high levels of protein-tyrosine kinase activity have been found in some non-proliferating terminally differentiated cells such as neurons and platelets [6–8], the physiological function of protein-tyrosine kinases and their regulatory mechanism in normal cells have not been clearly understood. Platelets possess several protein-tyrosine kinases such as the cellular *src* gene or *fyn* gene products [8–10] and unidentified kinases [11]. In platelets, $pp60^{c\text{-}src}$ is particularly rich [8].

Protein-tyrosine phosphorylation is transiently elevated in intact human platelets when it is stimulated with various agonists [12–18]. Cellular responses of platelets, i.e. aggregation and dense granule secretion, are also tightly correlated with the protein-tyrosine phosphorylation [13,14]. On the other hand, it has been recently found that the potent and specific protein-tyrosine kinase inhibitors such as erbstatin or genistein block the platelet activating factor (PAF)-induced platelet activation in rabbit [19,20]. In this paper, we will report the involvement of protein-tyrosine kinases in thrombin-induced aggregation in human platelets, with use of ST638 (α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide) and genistein.

Correspondence address: H. Yamamura, Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11, Japan. Fax: (81) 776-67-8102.

Abbreviations: DG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; EGF, epidermal growth factor.

*On leave from Fukui Prefectural College.

2. EXPERIMENTAL

2.1. *Materials and chemicals*

Human thrombin, prostaglandin E1 and apyrase were purchased from Sigma. Genistein and daidzein were obtained from Funakoshi Pharmaceutical Co. ST638 was a generous gift from Dr. Tadayoshi Shiraishi, Biochemical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd. Other chemicals were reagent grade. Antibodies against phosphotyrosine conjugated to human immunoglobulin were prepared by the method of EK and Heldin [21].

2.2. *Methods*

Isolation of human platelets and anti-phosphotyrosine immunoblot analysis were carried out as described previously [16]. Platelet aggregation was monitored using Chronolog Lumiaggregometer (CHRONOLOG, Corp.) as described previously [16].

For the measurement of protein-tyrosine kinase activity in the platelets extracts in vitro, washed platelets were suspended in 10 vols. of buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol and 10% glycerol). The suspension was sonicated in an ice bath with three 15 s bursts in a sonicator. Particulate material was collected by centrifugation at 100,000 × *g* for 60 min at 4°C and

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solubilized in the buffer containing 0.5% Triton X-100. The activities of particulate fractions were then assayed by measuring the phosphorylation of $E_{11}G_1$ (EDAEYAARRRG) as described previously [7], in the presence of various concentrations of protein-tyrosine kinase inhibitors, for 5 min at 30°C.

For the measurement of intracellular Ca^{2+} concentration, platelets (1 ml, 3–5 × 10^8 cells/ml) were placed on cuvettes at 37°C with stirring, and fluorescence was monitored in a Shimadzu fluorescence spectrometer RF-5000 with standard monitor setting of 340 and 380 nm excitation and 490 nm emission. Platelets were loaded with Fura2-AM at a final concentration of 5 μM for 30 min at 37°C and washed with HEPES-buffered saline. Washed platelets were preincubated for 2 min in the absence or presence of genistein and stimulated by thrombin and the analysis continued for 3 min. In the presence of ST638 we could not measure the intracellular Ca^{2+} concentration because of having fluorescence itself.

3. RESULTS

In the previous articles [14,16–18] we have shown that protein-tyrosine phosphorylation of several proteins is enhanced by the treatment of agonists such as thrombin, collagen, calcium ionophore A23187, vanadate with H_2O_2 and wheat germ agglutinin in human platelets. We have chosen ST638 and genistein which have distinct inhibitory mechanisms of action to protein-tyrosine kinases to investigate whether protein-tyrosine kinases are required for the platelet aggregation induced with thrombin in human platelets. Fig. 1 shows a dose-response for ST638 or genistein inhibition of platelet aggregation induced with thrombin at a concentration of 0.05 unit/ml. Thrombin-induced aggregation was blocked by these inhibitors in a dose-dependent manner. The concentration of ST638 or genistein which inhibited platelet aggregation to the basal level was 50 μM or 25 μg/ml, respectively.

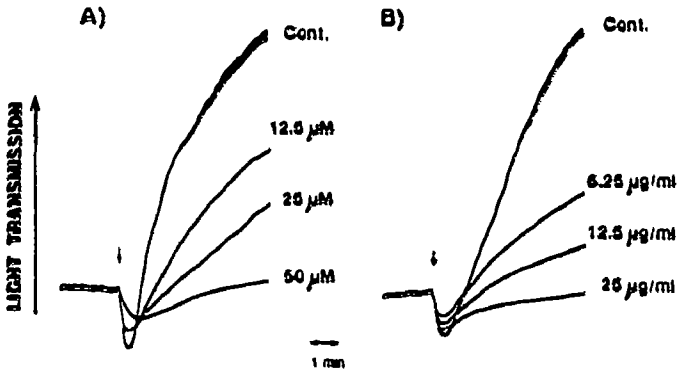

Fig. 1. Effects of ST638 or genistein on platelet aggregation induced with thrombin in intact human platelets. Platelets were preincubated for 2 min in the presence of indicated concentration of (A) ST638 (0–50 μM) (B) genistein (0–25 μg/ml) before the stimulation by thrombin (0.05 unit/ml). Then aggregation was measured as described in section 2. The arrow indicated the addition of thrombin. Cont. = in the absence of inhibitors.

In the next experiments we examined the inhibitory effect for the protein-tyrosine phosphorylation in platelets induced by thrombin at a concentration of 0.05 unit/ml with the treatment of ST638 or genistein by Western blotting using anti-phosphotyrosine antibodies. As shown in Fig. 2, five phosphotyrosine protein bands (135-, 124-, 76-, 64- and 60-kDa) which were stimulated by thrombin were reduced by the addition of ST638 or genistein. This inhibitory effect was correlated with that of platelet aggregation.

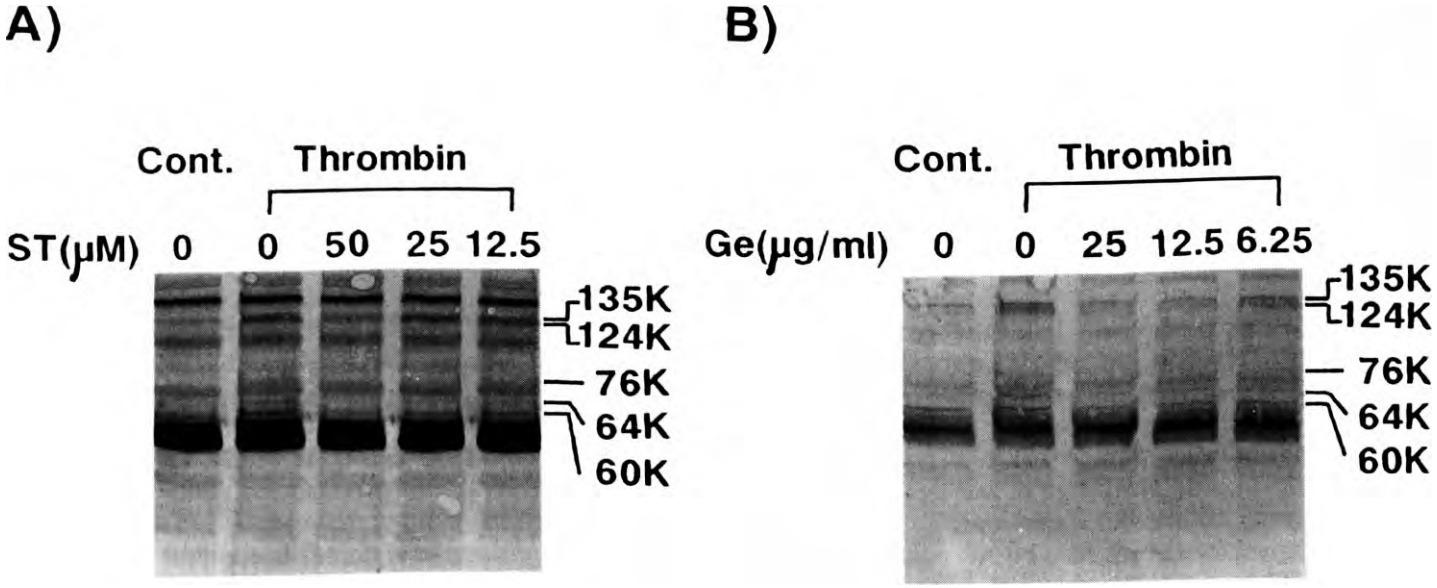

Fig. 2. Inhibition of ST638 or genistein on thrombin-induced protein-tyrosine phosphorylation by immunoblotting. Platelets were stimulated by thrombin (0.05 unit/ml) for 2 min after 2 min preincubation in the presence of the indicated concentration of (A) ST638 or (B) genistein. Samples were subjected to SDS–10% polyacrylamide gel electrophoresis, transferred to polyvinyliden difluoride membranes, probed with antiphosphotyrosine antiserum as described previously [16]. ST, ST638 (μM); Ge, genistein (μg/ml); Cont. = no stimulation by thrombin. The lines indicate the positions of proteins with molecular masses of 135-, 124-, 76-, 64-, and 60-kDa that underwent tyrosine phosphorylation in response to thrombin stimulation.

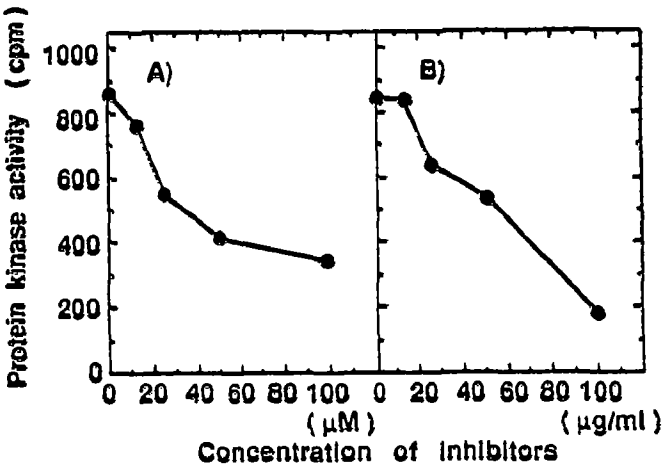

Fig. 3. Effects of ST638 or genistein on the protein-tyrosine kinase activity in the human platelet extracts in vitro. Particulate fractions of platelet extracts were assayed for protein-tyrosine kinase activity by measuring the phosphorylation of $E_{11}G_1$ as a substrate. The kinase activities were assayed in the presence of (A) ST638 (0–100 μM) and (B) genistein (0–100 μg/ml) as described in section 2. These results were representative of three experiments.

Although ST638 and genistein have been known potent and specific inhibitors for protein-tyrosine kinases, it is necessary to examine whether activities of protein-tyrosine kinases in platelet extracts were inhibited by them. We assayed protein-tyrosine kinase activity by measuring the tyrosine phosphorylation of $E_{11}G_1$ which is one of the best substrates for protein-tyrosine kinases. Fig. 3 shows effects of ST638 or genistein on protein-tyrosine kinase activities in human platelet particulate fractions in vitro. The kinase activities of particulate fraction from platelet extracts were much higher than those of supernatant fractions (data not shown). Both inhibitors strongly inhibited the protein-tyrosine kinase activities from human platelets. Half maximal inhibitory concentrations of ST638 or genistein were about 15 μM or 45 μg/ml, respectively.

Recently it has been reported that the inhibitory effect of genistein on platelet activation is due to its preventive action on thromboxane A2 binding to the receptor, rather than via inhibition of protein-tyrosine phosphorylation [22]. In order to assess whether inhibition of ST638 or genistein is related to inhibition of protein-tyrosine kinase, these inhibitors and another isoflavone compound, daidzein which has no inhibitory activity for protein-tyrosine kinase [23] but inhibits thromboxane A2 binding anf thromboxane A2-induced platelet aggregation [22], were examined for aspirin-treated platelet responses. As shown in Fig. 4, in the presence of 1 mM aspirin protein-tyrosine phosphorylation induced

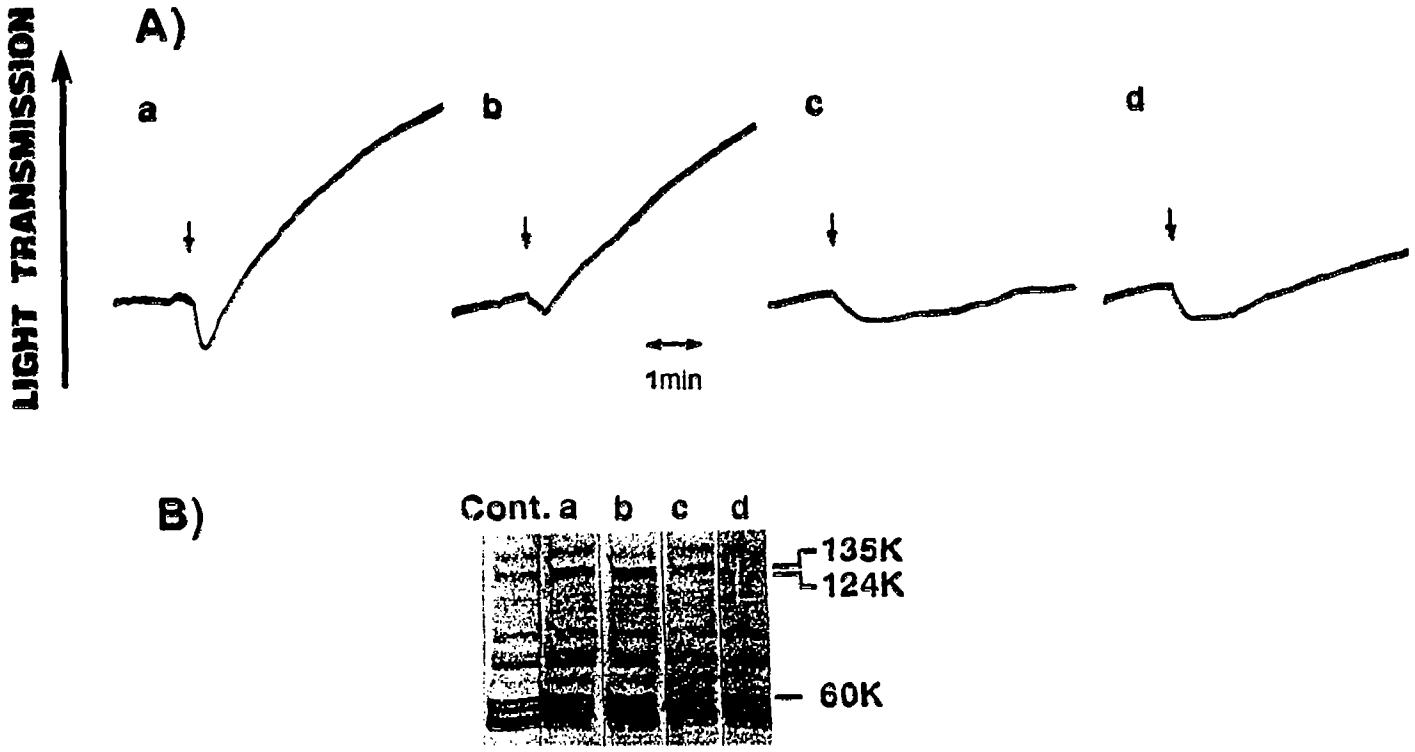

Fig. 4. Effects of daidzein, genistein, or ST638 on aspirin-treated platelet aggregation and protein-tyrosine phosphorylation stimulated by thrombin. The aspirinized-platelets which were treated with 1 mM aspirin for 30 min were preincubated without or with agents for 2 min at 37°C with stirring and then stimulated by thrombin (0.06 unit/ml, a–d). Aliquots of samples were subjected to SDS–7.5% polyacrylamide gel electrophoresis as described previously [16]. (A) Platelet aggregation and (B) protein-tyrosine phosphorylation. a, no agents; b, daidzein (50 μg/ml); c, genistein (50 μg/ml); d, ST638 (50 μM); Cont. = no stimulation by thrombin. The arrow indicates the addition of thrombin (A). The lines indicate the positions of proteins with molecular masses of 135-, 124-, and 60-kDa that underwent tyrosine phosphorylation in response to thrombin stimulation (B).

with thrombin at low concentration was observed in 135-, 124-, 76-, 64-, and 60-kDa proteins, although two phosphotyrosine protein bands of 76-, and 64-kDa increased slightly. Although 50 μM ST638 and 50 μg/ml genistein inhibited the platelet aggregation and protein-tyrosine phosphorylation induced by thrombin in aspirin-treated platelets, daidzein (50 μg/ml) could inhibit neither the platelet aggregation nor protein-tyrosine phosphorylation. These results suggested that the inhibitory effects of ST638 and genistein on thrombin-induced platelet activation were not simply due to the prevention of its binding to the thromboxane A2 receptor, but rather due to the inhibition of protein-tyrosine kinases.

In the last experiment we questioned whether these inhibitors could inhibit the increase of cellular Ca^{2+} when the cells were stimulated by thrombin. As shown in Fig. 5 intracellular Ca^{2+} induced by thrombin was inhibited by the addition of genistein in a dose-dependent manner although a much higher concentration is necessary for complete inhibition. The result suggests that protein-tyrosine phosphorylation in platelets by thrombin may have some relation to the intracellular Ca^{2+} concentration.

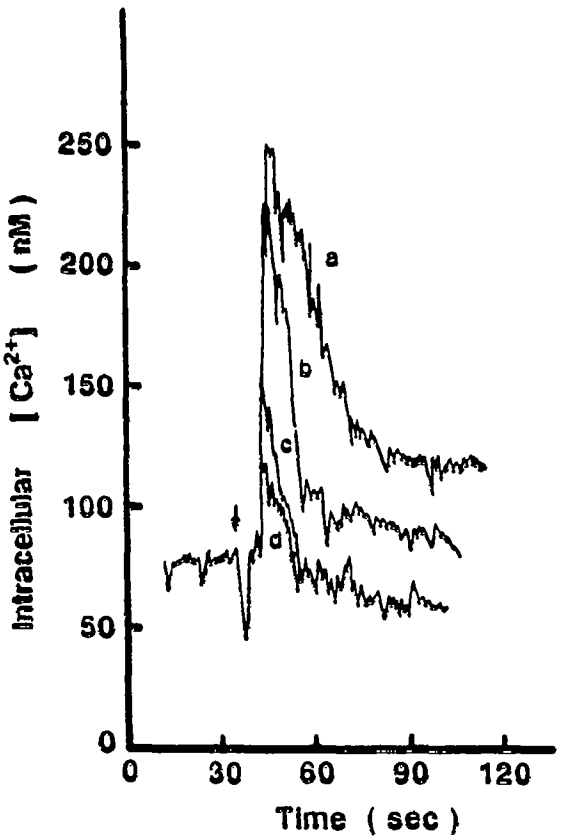

Fig. 5. Effect of genistein on thrombin-induced Ca^{2+} mobilization. Fura2-AM loaded platelets were preincubated (a) without or with genistein (b) 25 μg/ml, (c) 50 μg/ml, and (d) 100 μg/ml for 2 min at 37°C with stirring. Intracellular fluorescence was monitored by flow cytometry, and after baseline 30 s-period, thrombin (0.05 unit/ml) was added (arrow). Detailed conditions are described in section 2.

4. DISCUSSION

It has been known that ST638, which is competitive with substrate protein for the protein-tyrosine kinase binding site, is a potent and specific inhibitor of protein-tyrosine kinases of oncogene and protooncogene products and epidermal growth factor (EGF) receptor kinase in vitro [24] or in intact cells [25]. Genistein also specifically inhibits the activities of oncogene product kinases and EGF receptor kinase, and its inhibitory effect is caused by competing with ATP [23]. Both inhibitors did not inhibit the enzyme activities of serine/threonine-specific protein kinase such as cAMP-dependent protein kinase and protein kinase C [23,24].

In the present study, we found that ST638 and genistein inhibited human platelet aggregation, when platelets were induced with thrombin at a concentration of 0.05 unit/ml. In our experiments, changes of phosphotyrosine protein bands of 135-, 124-, 76-, 64-, and 60-kDa were also observed by the stimulation with the same concentration of thrombin, and those protein-tyrosine phosphorylations were reduced to basal level by the pretreatment of ST638 or genistein. These inhibitory effects were closely correlated with that of platelet aggregation in a dose-dependent manner. Both inhibitors also strongly suppressed the protein-tyrosine kinase activities from the particulate fraction in human platelets in vitro. The half-maximal inhibitory concentration of ST638 toward protein-tyrosine kinase in vitro was about 15 μM which was almost equivalent to its inhibitory concentration on platelet aggregation. On the other hand genistein inhibited its activity at half-maximal inhibitory concentration of about 45 μg/ml, although it inhibited platelet aggregation at the concentration of 25 μg/ml. These different inhibitory effects may be explained by the difference of inhibitory actions between ST638 and genistein. In aspirin-treated platelets, both ST638 and genistein inhibited the thrombin-induced platelet aggregation and protein-tyrosine phosphorylation, whereas daidzein, which has no inhibitory activity for protein-tyrosine kinase but inhibits [^{3}H]U46619 binding and thromboxane A2-induced platelet aggregation [22] did not prevent thrombin-induced platelet aggregation and protein-tyrosine phosphorylation. These results suggested that the role of ST638 and genistein in thrombin-induced platelet aggregation was at least in part the inhibition of the activity of protein-tyrosine kinases. Based on these observations, it has been shown that the activation of protein-tyrosine kinase may be necessary for platelet aggregation stimulated with thrombin in human platelets. Therefore, it turns out that protein-tyrosine phosphorylation plays a certain role in human platelet aggregation having some relation to the intracellular Ca^{2+} concentration (Fig. 5).

It is well known that the thrombin is the most potent agonist of human platelets. The human platelets start to aggregate at the concentration of thrombin around 0.03 unit/ml [22] and maximal aggregation is obtained at the concentration of 0.5–1.0 unit/ml. The inhibition of human platelet aggregation and tyrosine phosphorylation by protein kinase inhibitors can only be seen in the low concentration of thrombin, and in the presence of a higher concentration of thrombin (1 unit/ml) neither tyrosine phosphorylation nor platelet aggregation is inhibited by these inhibitors. The reason why a higher concentration of thrombin-induced aggregation was not inhibited by protein-tyrosine kinase inhibitors is considered to be as follows. First, there are a lot of protein-tyrosine kinases in human platelets, so if a higher concentration of thrombin activates many protein-tyrosine kinases, the inhibitors could not inhibit all the activity of protein-tyrosine kinases. Second, there are at least two different mechanisms of activation of platelets by thrombin: protein-tyrosine kinases are essential for the platelet activation in the case of lower concentration of thrombin but in the case of higher concentration of thrombin phosphatidylinositol breakdown seems to be directly activated [4]. Further studies are currently underway to understand the signal transduction mechanism through protein-tyrosine phosphorylation in human platelet activation.

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