Biochemical Pharmacology, Vol. 46, No. 3, pp. 395–403, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EFFECTS OF GENISTEIN, A TYROSINE KINASE INHIBITOR, ON PLATELET FUNCTIONS

GENISTEIN ATTENUATES THROMBIN-INDUCED Ca^{2+} MOBILIZATION IN HUMAN PLATELETS BY AFFECTING POLYPHOSPHOINOSITIDE TURNOVER

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(*Received* 14 *January* 1993; *accepted* 29 *April* 1993)

Abstract—Genistein, a tyrosine kinase inhibitor, had no or only slight inhibitory effects on platelet aggregation or serotonin release induced by thrombin, while intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation was substantially attenuated. It also inhibited the cyclooxygenase pathway, but this effect was not directly related to the suppressive effect of genistein on $[Ca^{2+}]_i$ elevation. In order to clarify the mechanism by which genistein suppresses Ca^{2+} mobilization, its effect was examined on inositol phospholipid metabolism. The production of inositol-1,4,5-trisphosphate was inhibited by genistein in a dose-dependent manner, while 47 kDa protein phosphorylation or phosphatidic acid formation was not affected, suggesting that genistein does not inhibit phospholipase C activity. Pretreatment of unstimulated platelets with genistein increased the amount of phosphatidylinositol-4-monophosphate [PI(4)P], while that of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] was reduced. Thrombin stimulation of genistein-pretreated cells intensified this tendency, i.e. a further increase in the amount of PI(4)P and a decrease in the amount of PI(4,5)P_2 in an inversely proportional manner. Taken together, these findings imply that genistein acted at the step of PI(4)P 5-kinase which produces PI(4,5)P_2 from PI(4)P. Protein tyrosine phosphorylation induced by thrombin was not affected by genistein, suggesting that the inhibitory effect of genistein on polyphosphoinositides was unrelated to tyrosine kinase inhibition.

Platelets which play a key role in thrombus formation, haemostasis and regeneration of vessels respond to a wide variety of stimuli. The signal transduction mechanisms that lead to platelet responses appear to be multiple, and distinct pathways probably exist for each agonist. Many cellular processes have been attributed to the action of protein kinases including protein kinase C, cAMP-dependent protein kinase and myosin light chain kinase which phosphorylate the serine or threonine residues of substrate proteins. Recently, tyrosine kinases that phosphorylate target proteins at tyrosine residues have been found in platelets [1]. Human platelets possess a high level of the tyrosine kinase activity, most of which is attributed to $pp60^{c\text{-}src}$ [2, 3]. Activation of platelets by several agonists increase the level of tyrosine phosphorylation resulting in the appearance of a new set of tyrosine-phosphorylated proteins [4–7]. Ligand binding to the glycoprotein IIb/IIIa which plays a critical role in platelet aggregation appears to regulate protein tyrosine phosphorylation [8, 9]. Tyrosine phosphorylation may also be induced by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$†) elevation or by Ca^{2+} depletion in intracellular Ca^{2+} storage sites [10]. These findings suggest the possible involvement of tyrosine kinase in the signal transduction system in platelets, although the precise role of tyrosine phosphorylation in platelet activation remains elusive.

Specific inhibitors of protein kinases provide useful tools for the investigation of the role of protein kinases in cellular functions. Several tyrosine kinase inhibitors have been developed and have served to identify the specific actions of tyrosine kinases in a wide variety of cell types [11–13]. Genistein, a potent inhibitor of tyrosine kinase, was discovered in a fermentation broth of *Pseudomonas spp* [11]. The effects of genistein have been evaluated on platelet functions; it inhibited platelet activation and tyrosine phosphorylation induced by platelet-activating factor [14]. It was also reported that genistein suppressed the inositol phospholipid metabolism induced by U-46619, a thromboxane A_2 mimetic, suggesting that tyrosine phosphorylation is associated with the turnover of inositol phospholipids [15]. In a preliminary study, we found that genistein attenuated intracellular Ca^{2+} ($[Ca^{2+}]_i$) elevation induced by thrombin, which led us to investigate the mechanism by which it modifies Ca^{2+} mobilization in platelets. In this report, we present evidence to suggest that

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† Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PRP, platelet-rich plasma; fura 2-AM, fura 2 acetoxymethyl ester; I(1,4,5)P_3, inositol-1,4,5-trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol-monophosphate; PIP_2, phosphatidylinositol-bisphosphate; Hepes, 4-(2-hydroxyethyl)1-piperazine-ethanesulfonic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; STA_2, (+)-9,11-epithia-11,12-methano-thromboxane A_2; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

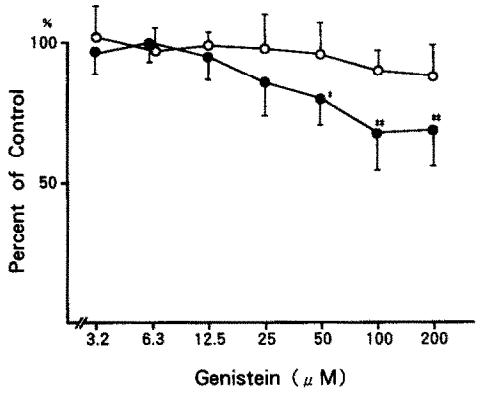


Fig. 1. Effects of genistein on thrombin-induced platelet aggregation and serotonin release. For the measurement of aggregation, 0.4 U/mL thrombin was added to a platelet suspension which had been preincubated with genistein for 5 min at 37°, and the maximum per cent decrease in optical density over the control was recorded. For the measurement of serotonin release, [^{3}H]serotonin-labeled platelets were preincubated with genistein for 5 min at 37°, and 0.4 U/mL thrombin was added to activate the cells. The reaction was terminated 10 min after stimulation, and the per cent release of serotonin over the total intracellular content was measured. The data are presented as the means ± SD of three experiments. Open circles, aggregation; closed circles, serotonin release. (*, P = 0.02; **, P = 0.01.)

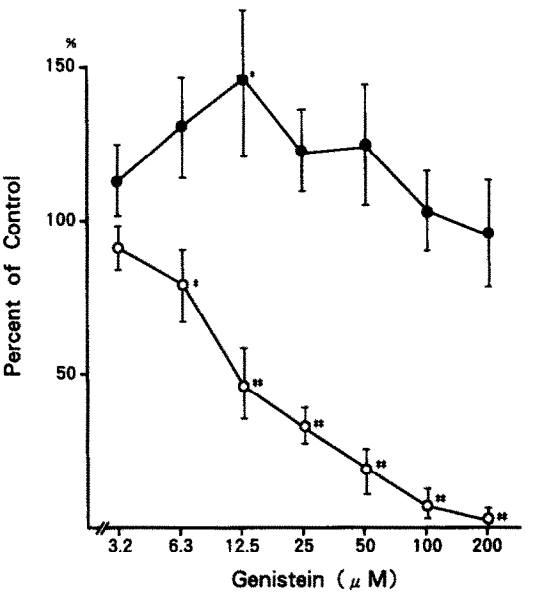


Fig. 2. Effects of genistein on arachidonic acid metabolism. Platelet suspensions used for the measurement of aggregation were used as samples for arachidonic acid metabolism. After thrombin (0.4 U/mL) activation of platelets, lipids were extracted from the samples and arachidonic acid metabolites were analysed with HPLC. The data are presented as the means ± SD of three experiments. Open circles, HHT (a cyclooxygenase product); closed circles, 12-HETE (a 12-lipoxygenase product). (*, P = 0.05; **, P = 0.01.)

genistein attenuates $[Ca^{2+}]_i$ elevation by interfering with the polyphosphoinositide turnover, which results in the impaired production of inositol-1,4,5-trisphosphate [I(1,4,5)P_3].

MATERIALS AND METHODS

Agents. Fura 2-acetoxymethyl ester (fura 2-AM) was obtained from Dojin Laboratories (Kumamoto, Japan). Genistein was from the Funakoshi Co. (Tokyo, Japan). [^{3}H]Serotonin, [^{3}H]phosphatidylinositol-4-monophosphate [PI(4)P] and other phosphoinositides were purchased from Amersham Japan (Tokyo, Japan). STA_2 [(+)-9,11-epithia-11,12-methano-thromboxane A_2] was a generous gift from Ono Pharmaceuticals (Osaka, Japan). Modified 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)/Tyrode's buffer containing 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM Hepes pH 7.25, and 5.5 mM glucose, was passed through 0.45 μm Millipore filters, and stored at 4° until use.

Platelet separation. Citrate anti-coagulated venous blood was obtained from healthy human donors who had not received any medication for a minimum of 2 weeks preceding the experiment. The blood was centrifuged at 160 *g* for 15 min to obtain platelet-rich plasma (PRP). Platelets were washed twice with Hepes/Tyrode's buffer with 0.2 μM prostaglandin I_2 and resuspended in modified Hepes/Tyrode's buffer containing 100 μM Ca^{2+} at a concentration of 2×10^5 cells/μL, unless otherwise stated.

Preparation of fura 2-loaded platelets and measurement of fura 2-detected $[Ca^{2+}]_i$ *changes.* To PRP

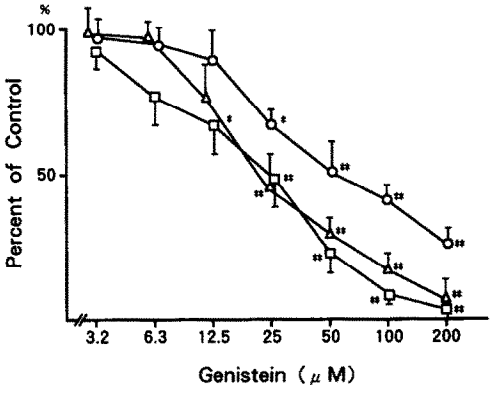


Fig. 3. Effects of genistein on $[Ca^{2+}]_i$ elevation induced by various activators. Fura 2-loaded platelets were preincubated with genistein for 5 min at 37°. Thrombin (0.4 U/mL), STA_2 (10^{-6} M), or platelet-activating factor (10^{-7} M) was added to the platelet suspension, and the change in $[Ca^{2+}]_i$ was continuously measured. The maximum $[Ca^{2+}]_i$ elevation in ratio to the control was measured for each genistein concentration. The data are presented as the means ± SD of three experiments. Open circles, thrombin; open triangles, STA_2; and open boxes, platelet-activating factor. (*, P = 0.02; **, P = 0.01.)

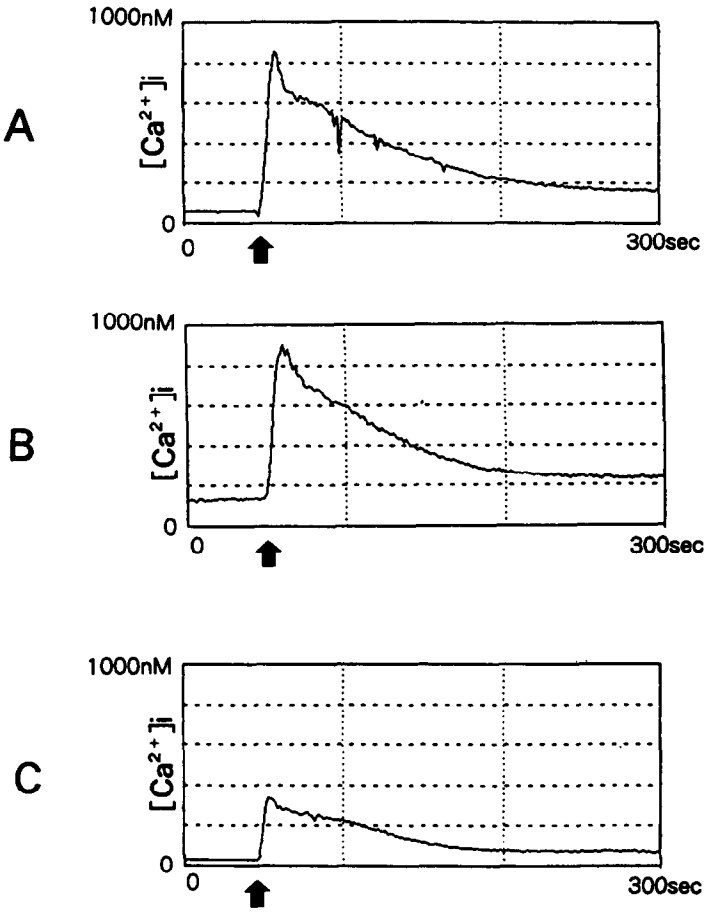


Fig. 4. Effects of aspirin and genistein of $[Ca^{2+}]_i$ changes induced by thrombin. Fura 2-loaded platelets were preincubated either with 0.5 mM aspirin, 100 μM genistein, or with an equivalent volume of saline for 5 min. Thrombin (0.4 U/mL) was added at the time indicated by an arrow, and the change in $[Ca^{2+}]_i$ was continuously measured. (A) The control; (B) pretreatment with 0.5 mM aspirin; (C) pretreatment with 100 μM genistein. The data are representative of three experiments.

obtained as described above, fura 2-AM at a final concentration of 3 μM was added, and the mixture was incubated at 37° for 30 min. After incubation, platelets were washed twice with modified Hepes/Tyrode's buffer and resuspended in the same buffer at a concentration of 1×10^5 cells/μL. Fura 2 fluorescence was measured with a Hitachi F-2000 fluorescence spectrophotometer, with an excitation wavelength alternating every 0.5 sec from 340 to 380 nm, and the emission wavelength set at 510 nm. $[Ca^{2+}]_i$ values were determined from the ratio of fura-2 fluorescence intensity at 340 nm excitation and 380 nm excitation [16].

Serotonin release. PRP was incubated with [^{3}H]-serotonin (1 μCi/mL of PRP, 10–20 Ci/mmol) for 30 min at 37°. After incubation, the cells were washed twice and resuspended at a concentration of 2×10^5 cells/μL in modified Hepes/Tyrode's buffer containing 2 μM imipramine. After the reaction was terminated with 1% formaldehyde, the samples were rapidly centrifuged and the radioactivity of the supernatants was determined by liquid scintillation counting. The release of [^{3}H]serotonin was quantified by expressing the released label as a percentage of the total radioactivity incorporated into platelets.

Aggregation. Platelet aggregation was measured as a change in optical density of platelet suspensions. Briefly, 1 mL of a platelet suspension (2×10^5 cells/μL) was added into a cuvette, and the change in optical density after agonist stimulation was continuously monitored with a Sysmex A-100 platelet aggregometer (Kobe, Japan). The data are presented as the maximum per cent decrease in optical density over that of the control.

Measurement of arachidonic acid metabolites by HPLC. Lipids were extracted from platelet samples used for aggregation measurement, and evaporated under nitrogen stream. Arachidonic acid metabolites contained in the residues were then subjected to reversed-phase HPLC using TSK-Gel ODS-80T_m (4.6 × 150 mm, Toyo Soda, Tokyo, Japan). The mobile phase consisted of methanol/water/acetic

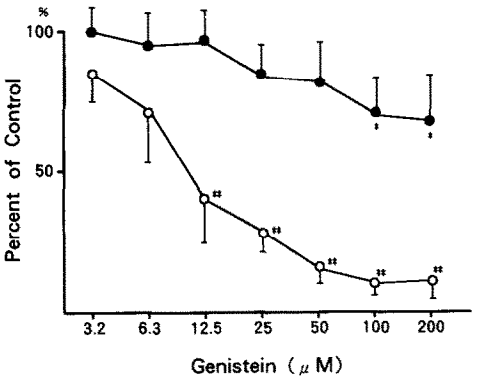


Fig. 5. Effects of genistein on phosphatidic acid formation or inositol 1,4,5-trisphosphate production induced by thrombin. Platelets were preincubated with genistein for 5 min at 37°. After incubation, thrombin (1 U/mL) was added to a platelet suspension to activate platelets. The reaction was terminated 5 sec after activation for the measurement of inositol-1,4,5-trisphosphate, or 4 min after activation for the measurement of phosphatidic acid. Open circles, inositol-1,4,5-trisphosphate; closed circles, phosphatidic acid. The data are presented as the means ± SD of three experiments (percentage over the control). (*, P = 0.02; **, P = 0.01.)

acid (75:25:0.01, by vol.) at a flow rate of 1 mL/min. Column effluents were monitored at 235 nm for 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). The amounts of arachidonic acid metabolites were quantified by comparing the peak area with that of the internal standard.

Measurement of I(1,4,5)P_3. Platelets were suspended in the Hepes/Tyrode's buffer at a concentration of 5×10^6 cells/μL. After reaction, 100 μL of 10% trichloroacetic acid were added to the platelet suspension to stop the reaction and the mixture was kept on ice for 15 min. The mixture was centrifuged at 10,000 *g* for 5 min, and the resulting supernatant was treated five times with 5 mL of water-saturated ethylether to extract trichloroacetic acid. The remaining ether was further removed *in vacuo*, and the I(1,4,5)P_3-containing residue was neutralized with KOH to pH 7.4. The amount of I(1,4,5)P_3 was measured with an I(1,4,5)P_3 assay kit (Amersham Japan).

Labeling with [^{32}P]orthophosphate, measurement of phosphatidic acid and protein phosphorylation. Platelets in PRP obtained from 200 mL of blood were washed once with Hepes/Tyrode's buffer and resuspended in 5 mL phosphate-free Hepes/Tyrode's buffer. One millicurie of [^{32}P]orthophosphate was added to the platelet suspension and the mixture was incubated for 90 min at 37°. The platelets were washed twice, and resuspended in Hepes/Tyrode's buffer containing 100 μM Ca^{2+}. After reaction, lipids were extracted by the Bligh–Dyer method and the chloroform phase dried under nitrogen. The evaporated residue was redissolved in chloroform containing unlabeled phosphatidic acid and applied to thin-layer chromatographs using the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (90:50:20:100, by vol.) as solvent. The spot corresponding to phosphatidic acid was identified with iodine vapor and scraped off for the determination of radioactivity. For the measurement of protein phosphorylation, the ^{32}Pi-loaded cells were dissolved in Laemmli sample buffer. The mixture was boiled for 3 min, and applied to gel electrophoresis using 12.5% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was dried and radioactive bands on the gel were detected by autoradiography.

Extraction and deacylation of inositol phospholipids and HPLC analysis of deacylated inositol phospholipids (glycerophosphoinositides). Platelets loaded with ^{32}Pi were suspended at a concentration of 5×10^5 cells/μL. Lipids in the sample were extracted by the Bligh–Dyer method, and dried under N_2. Deacylation of inositol phospholipids was performed essentially according the method described by Clarke and Dawson [17], which utilized methylamine for deacylating inositol phospholipids. Glycerophosphoinositides in samples were analysed by strong anion exchange HPLC on two connected TSK-gel SAX columns (Toso, Japan). The gradient was shifted from 10 mM $NH_4H_2PO_4$ (pH 3.8) (Pump A) to 1.75 M $NH_4H_2PO_4$ (pH 3.8) (Pump B) with a constant flow rate of 1 mL. The samples were applied in 10 mM $NH_4H_2PO_4$, and the column was washed with 100% pump A for 10 min followed by a linear gradient of 0–5% pump B for 1 min, 5–10% for 139 min, 10–20% for 1 min, 20–33% for 99 min, 33–80% for 10 min, 80–100% for 1 min, and 100% for 19 min. Radioactivity in the eluate was monitored on-line with a LB503 HPLC radioactivity monitor (Berthold, Germany), and the data were analysed on a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan). Glycerophosphoinositides were identified by comparing the elution times of ^{3}H-labeled standard inositol phospholipids that had been deacylated.

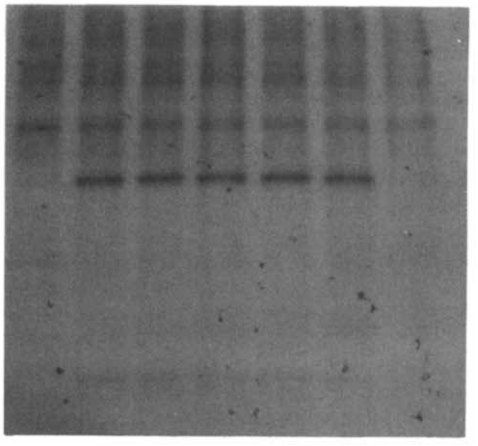
Detection of tyrosine phosphorylation by immunoblotting. Platelets were suspended in Hepes/Tyrode's buffer approximately at a concentration of 4×10^6 cells/μL. After the reaction was terminated by the addition of Laemmli sample buffer, the mixture was then boiled for 3 min. Platelet proteins were separated by 8% SDS–PAGE, and electrically transferred to the Clear Blot Membrane P (Atto, Tokyo, Japan). The immunoblots were incubated with 1 μg/mL of the monoclonal antibody PY-20 which specifically recognizes phosphotyrosine residues (ICN, CA, U.S.A.) for 3 hr. The binding of the antibody was detected using peroxidase-conjugated goat anti-mouse IgG (Cappel, PA, U.S.A) and visualized with ECL detection reagents (Amersham, U.K.).

The significance of the results was calculated by *t*-test.

RESULTS

Effect of genistein on thrombin-induced aggregation, and arachidonic acid metabolism

Genistein at any concentration tested in this study had no inhibitory effect on platelet aggregation induced by 0.4 U/mL thrombin. Serotonin release



Thr 1U/ml − + + + + + +
Genistein 0 10 20 50 100
(μ M) SSP

Fig. 6. Effects of genistein on protein phosphorylation induced by thrombin. ^{32}P-labeled platelets were preincubated with genistein for 5 min at 37°. After preincubation, thrombin (1 U/mL) was added, and the mixture was incubated for another 2 min. The reaction was terminated by the addition of SDS sample buffer, and protein phosphorylation was evaluated by SDS gel electrophoresis and subsequent autoradiography. The data are representative of three experiments. SSP represents 1 μM staurosporine, a potent protein kinase C inhibitor.

was slightly inhibited by genistein at concentrations higher than 50 μM (Fig. 1). Thrombin- (0.4 U/mL) induced production of arachidonic acid metabolites was 65.7 ± 19.2 ng/2 × 10^8 cells for HHT, a cyclooxygenase product, and 41.9 ± 12.5 ng/2 × 10^8 cells for 12-HETE, a 12-lipoxygenase product. Genistein in a dose-dependent manner inhibited the production of HHT, while the production of 12-HETE was unaffected or slightly enhanced (Fig. 2). These findings suggest that genistein has an inhibitory effect on the production of cyclooxygenase-related arachidonic acid metabolites, while the activity of phospholipase A_2 and 12-lipoxygenase was relatively unaffected.

Effect of genistein on $[Ca^{2+}]_i$ *elevation*

The resting $[Ca^{2+}]_i$ of platelets was 74 ± 23 nM as detected by the Fura 2 method. Thrombin at a concentration of 0.4 U/mL elevated $[Ca^{2+}]_i$ to the maximum level of 761 ± 180 nM. Genistein in a dose-dependent manner inhibited $[Ca^{2+}]_i$ elevation induced by thrombin (0.4 U/mL), 10^{-6} M (+)-9,11-epithia-11,12-methano-thromboxane A_2 (STA_2), a thromboxane A_2 analog or 10^{-7} M platelet-activating factor (Fig. 3). ID_{50} of genistein for thrombin-induced $[Ca^{2+}]_i$ elevation was approximately 50 μM. Higher doses of thrombin (2 U/mL =<) were unable to overcome this effect. The inhibitory effect on $[Ca^{2+}]_i$ elevation was observed in the absence of extracellular Ca^{2+} as well as in the presence of extracellular Ca^{2+}, suggesting that genistein interferes with a process(s) involving intracellular Ca^{2+} mobilization (data not shown). The inhibitory effect of genistein on intracellular Ca^{2+} mobilization could not be attributed to the suppressed production of thromboxane A_2, since 0.5 mM aspirin, which completely and irreversibly blocks cyclooxygenase, did not affect thrombin-induced $[Ca^{2+}]_i$ elevation in the same manner (Fig. 4).

Effect of genistein on phosphatidic acid formation, protein phosphorylation and inositol trisphosphate production

Since the inhibitory effects of genistein on thrombin-induced $[Ca^{2+}]_i$ elevation suggests that the agent interferes with intracellular Ca^{2+} mobilization, we sought to determine at which step of intracellular $[Ca^{2+}]_i$-mobilizing mechanism genistein acts. It has been shown that receptor-mediated phospholipase C activation results in the production of IP_3 which directly released Ca^{2+} from internal Ca^{2+} storage sites [18]. In addition to IP_3, phospholipase C produces diacylglycerol by cleaving phosphatidylinositol-bisphosphate (PIP_2). Hence, we evaluated the effects of genistein on both products of phospholipase C. In our system, the basal IP_3 content was 0.9 ± 0.3 pmol/10^9 cells. Five seconds after 1 U/mL thrombin activation the increase of 7.9 ± 2.3 pmol IP_3/10^9 cells was observed. Genistein in a dose-dependent manner inhibited the thrombin-induced production of IP_3 (Fig. 5). On the other hand, genistein at only high concentrations (100–200 μM) slightly inhibited the production of

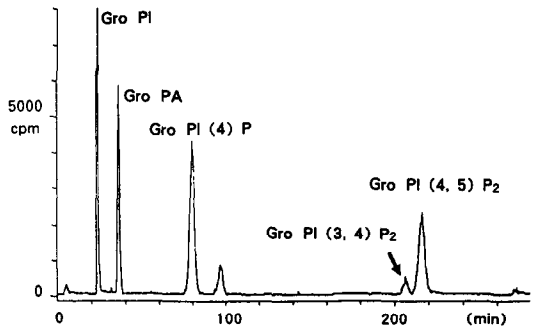


Fig. 7. Strong anion HPLC profile of deacylated inositol phospholipids. ^{32}P-labeled platelets were activated with thrombin (1 U/mL) for 5 min. Inositol phospholipids were extracted from platelets, and were deacylated by alkalinization. Deacylated inositol phospholipids were analysed with strong anion HPLC on ammonium phosphate gradients ranging from 10 mM to 1.75 M. The data are representative of a number of similar experiments.

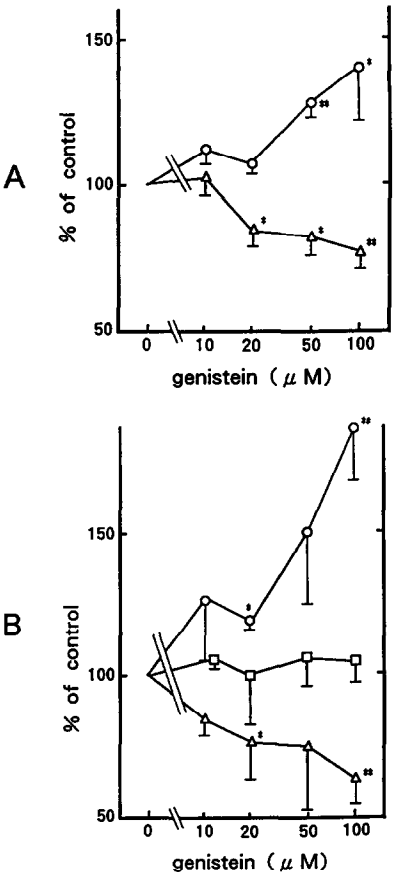


Fig. 8. (A) Effects of genistein on platelet PI(4)P and PI(4,5)P_2 at the resting stage. Platelets were incubated with genistein for 5 min at 37°. Inositol phospholipids were extracted from platelets, and deacylated by alkalinization. Deacylated inositol phospholipids were analysed with HPLC, and the peak area of PI(4)P or PI(4,5)P_2 was compared to that of the control without genistein. Open circles, PI(4)P; open triangles, PI(4,5)P_2. The data are presented as the means ± SD of three experiments. (*, P = 0.02; **, P = 0.01.) (B) Effects of genistein on platelet PI(4)P and PI(4,5)P_2 at the activated stage. Platelets were preincubated with genistein for 5 min at 37°. After preincubation, thrombin (1 U/mL) was added to the platelet suspension, and the mixture was incubated for another 5 min at 37°. Inositol phospholipids were extracted from platelets, and deacylated by alkalinization. Deacylated inositol phospholipids were analysed with HPLC, and the peak area of PI(4)P or PI(4,5)P_2 was compared to that of the control without genistein. Open circles, PI(4)P; open triangles, PI(4,5)P_2; open boxes, phosphatidic acid. The data are presented as the means ± SD of three experiments. (*, P = 0.02; **, P = 0.01.)

phosphatidic acid, a major metabolite of diacylglycerol (Fig. 5). That the production of diacylglycerol is unaffected by genistein was further confirmed by the findings that genistein had no significant effect on 47 kDa protein phosphorylation, a major substrate of protein kinase C which is activated by diacylglycerol (Fig. 6).

Effect of genistein on inositol phospholipid metabolism induced by thrombin

Phospholipase C generates diacylglycerol from substrates including phosphatidylinositol (PI), phosphatidylinositol-monophosphate (PIP), and PIP_2, while IP_3 is produced only when phospholipase C utilizes PI(4,5)P_2 [19]. The sustained level of phosphatidic acid, the major metabolite of diacylglycerol, in spite of the diminished production of inositol trisphosphate suggested that the activity of phospholipase C is unaffected by genistein and that genistein modified the metabolism of inositol phospholipids. We therefore measured the effect of genistein on inositol phospholipid metabolism in thrombin-activated platelets. The HPLC profiles of deacylated inositol phospholipids clearly identified various species of inositol phospholipids (Fig. 7), including PI(3,4)P_2, a novel inositol phospholipid assumed to be closely linked to tyrosine kinase [20, 21]. Treatment of intact platelets with genistein for 5 min resulted in an increment in the amount of PI(4)P with a corresponding decrement in the amount of PI(4,5)P_2 (Fig. 8A). Longer incubation with genistein (5–20 min) did not significantly change this profile. Thrombin activation of genistein-pretreated platelets induced a further increase in the amount of PI(4)P and a further decrease in the amount of PI(4,5)P_2 (Fig. 8B). The activation-associated production of phosphatidic acid and PI(3,4)P_2 was unaffected by genistein pretreatment.

Effect of genistein on tyrosine phosphorylation induced by thrombin

Using a monoclonal antibody against phosphorylated tyrosine, we were able to demonstrate one major protein (molecular mass 60 kDa) which is tyrosine-phosphorylated in resting platelets. This probably corresponds to pp60$^{c\text{-}src}$, which is abundant in platelets and known to be autophosphorylated. Upon thrombin activations, several tyrosin-phosphorylated proteins appeared with differing time courses (data not shown), including 64, 75, 95 and

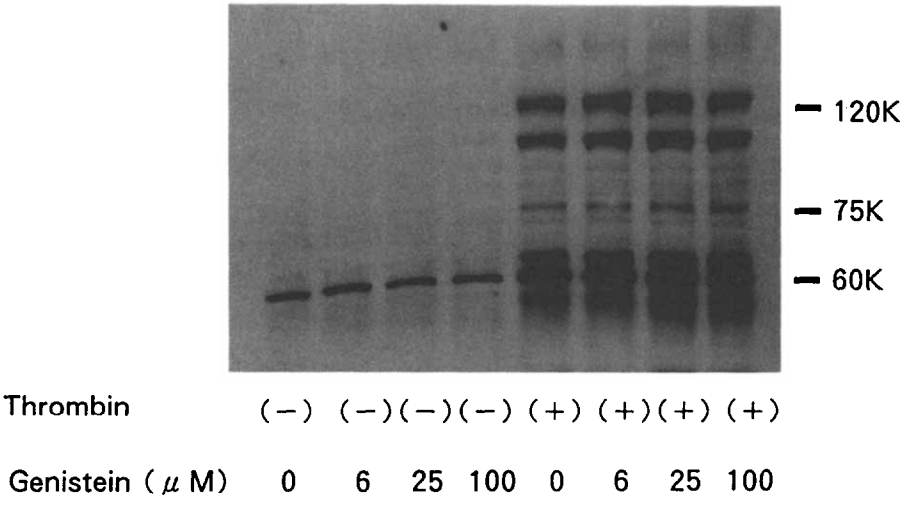


Fig. 9. Effect of genistein on tyrosine phosphorylation induced by thrombin. Platelets were preincubated with genistein for 5 min at 37°. Thrombin (1 U/mL) or an equivalent volume of saline was added to a platelet suspension, and the reaction was terminated 5 min afterwards. Tyrosine phosphorylation of platelet proteins was detected by a monoclonal antibody against phosphotyrosine (PY-20). The data are representative of three experiments.

120 kDa proteins. Pretreatment with genistein up to 200 μM had no effect on tyrosine phosphorylation of resting platelets. Genistein at any of the concentrations tested in the present study did not inhibit the appearance of the tyrosine-phosphorylated proteins induced by 1 U/mL thrombin (Fig. 9). The time course of the appearance of tyrosine-phosphorylated proteins was also not affected (data not shown).

DISCUSSION

To date, there have been a number of reports on the appearances of tyrosine-phosphorylated proteins in activated platelets [5–8]. The molecular masses of tyrosine-phosphorylated proteins and the time course of their appearance varies with different antibodies used to detect phosphotyrosine, probably due to different specificities of antibodies. With PY-20, a commercially available monoclonal antibody against phosphotyrosine, we confirmed that thrombin stimulation of platelets induced a new set of tyrosine-phosphorylated proteins. Genistein at any of the concentrations tested in the present study had no significant effects on the appearance of tyrosine-phosphorylated proteins in platelets. Our findings with PY-20 on the ineffectiveness of genistein on platelet tyrosine phosphorylation are in accord with those of Nakashima *et al.* [22]. Although we cannot totally rule out a possibility that the specificity of our antibody does not cover a set of tyrosine-phosphorylated proteins that could be inhibited by genistein, our findings along with those of Nakashima *et al.* suggest that genistein may not inhibit tyrosine kinases activated during thrombin activation. Platelets contain high tyrosine kinase activity, the majority of which is attributable to $pp60^{c\text{-}src}$. $pp59^{fyn}$ and others constitute the rest of the tyrosine kinase activity present in platelets [2, 3]. Genistein whose efficacy was evaluated on tyrosine kinases including epidermal growth factor receptors and $pp60^{v\text{-}src}$ [11] may not act on $pp60^{c\text{-}src}$ or thrombin-activated tyrosine kinases that are distinct from $pp60^{c\text{-}src}$.

Genistein attenuated $[Ca^{2+}]_i$ elevation induced by various agonists, although this effect appears to be unrelated to the tyrosine kinase inhibition in platelets. We sought to elucidate the mechanism by which genistein inhibits Ca^{2+} mobilization in platelets, by evaluating the effect of genistein on each step of the signal transduction system leading to $[Ca^{2+}]_i$ elevation. A large body of evidence has shown that the receptor-mediated $[Ca^{2+}]_i$ mobilization occurs by receptor-ligand interaction which activates GTP-binding proteins. Then subunit(s) of GTP-binding proteins activate phospholipase C. Phospholipase C catalyses the cleavage of $PI(4,5)P_2$, resulting in the production of diacylglycerol and $I(1,4,5)P_3$ and the latter releases Ca^{2+} from internal Ca^{2+} storage sites.

We sought to follow the last step. Genistein was found to suppress the release of Ca^{2+} from internal Ca^{2+} storage sites, since it was equally effective in the presence or the absence of extracellular Ca^{2+} which precludes Ca^{2+} influx. Genistein in a dose-dependent manner diminished $I(1,4,5)P_3$ production, suggesting that genistein acts at a step linked to phospholipase C which serves to produce $I(1,4,5)P_3$ and diacylglycerol. Subsequently, we found that genistein affected neither the formation of phosphatidic acid, a major metabolite of diacylglycerol, nor 47 kDa protein phosphorylation that reflects protein kinase C activation mediated by diacylglycerol. Although phospholipase D activation may in theory contribute to the formation of

phosphatidic acid, it has been shown to make a minor contribution to the overall production of phosphatidic acid in platelets [23] and that the amount of phosphatidic acid produced reflects phospholipase C activation [24, 25]. These findings suggest that genistein inhibits neither phospholipase C nor the signal transduction pathways that lie between thrombin-receptor interaction and phospholipase C activation. That genistein had little inhibitory effects on thrombin-induced aggregation or serotonin release also provides indirect evidence for the intact signal transduction pathways leading to the production of diacylglycerol, since protein kinase C activation mediated by diacylglycerol plays a key role in those functions.

$I(1,4,5)P_3$ is produced only from $PI(4,5)P_2$, while PI, PI(4)P and $PI(4,5)P_2$ can all serve as substrate for the diacylglycerol production by phospholipase C [19]. Since $PI(4,5)P_2$ constitutes a small percentage of the total phosphoinositides, it is rapidly consumed upon cell activation unless PI(4)P 5-kinase provides a new supply of $PI(4,5)P_2$ from PI(4)P [26]. This implies that the activation of PI(4)P 5-kinase is essential for the sufficient production of $I(1,4,5)P_3$ to induce a "full picture" of Ca^{2+} mobilization. We found in the present study that genistein treatment reduced the amount of $PI(4,5)P_2$ and the production of $I(1,4,5)P_3$ in response to thrombin, while the amount of PI(4)P was increased in an inverse proportion. Thus, if we postulate a single site of action on polyphosphoinositide turnover for genistein, the most likely site would be at the enzyme that catalyses phosphorylation of PI(4)P, i.e. PI(4)P 5-kinase, to form $PI(4,5)P_2$. Conversely, it may be argued that genistein has multiple sites of action on polyphosphoinositide turnover, increasing the amount of PI(4)P, reducing the amount of $PI(4,5)P_2$, and attenuating the production of $I(1,4,5)P_3$ or facilitating its catabolism. While we have no direct evidence to support any of these concepts, we consider it likely that genistein, by inhibiting PIP(4) 5-kinase, reduced the amount of $I(1,4,5)P_3$ produced in response to thrombin activation with resultant attenuation of $[Ca^{2+}]_i$ mobilization. Our findings are in partial accord with the effects of genistein on the polyphosphoinositide turnover in A431 or BALB 3T3 cells [27]; genistein inhibited hydrolysis of PIP_2 and $I(1,4,5)P_3$ production without significant effects on phospholipase C. The production of diacylglycerol was reduced in this system, however, suggesting that the mode of action exerted by genistein may be different in these cells.

In addition to the inhibitory effect on polyphosphoinositide turnover, we have found that genistein inhibits cyclooxygenase. Genistein attenuated the production of a cyclooxygenase-related arachidonic acid metabolite, but that of the 12-lipoxygenase pathway was unaffected. Although unrelated to our findings, Nakashima *et al.* found that genistein is a non-competitive blocker of thromboxane A_2 receptors [22]. Moreover, genistein is reported to inhibit the activity of several enzymes other than tyrosine kinase [28, 29]. Thus, genistein, in addition to its effect on tyrosine kinase, appears to possess several unrelated actions on platelet functions. These findings imply that the interpretation of data obtained with "specific inhibitors" must be carefully assessed.

Acknowledgement—This work was supported in part by a research grant from the Ministry of Education, Culture and Science in Japan.

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