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Platelet-Activating Factor Stimulation of Tyrosine Kinase and Its Relationship to Phospholipase C in Rabbit Platelets: Studies with Genistein and Monoclonal Antibody to Phosphotyrosine

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SUMMARY

Platelet-activating factor (PAF) is a proinflammatory lipid that has platelet-stimulating property. PAF receptor-coupled activation of phosphoinositide-specific phospholipase C (PLC) and phosphorylation of several proteins has already been established in our laboratory. To investigate further the molecular mechanism and relationship between activation of PLC and protein phosphorylation, we have used Genistein (a putative inhibitor of tyrosine-specific protein kinases), phosphotyrosine antibody, and phosphoamino acid analysis to probe the involvement of tyrosine kinase in this process. Washed rabbit platelets were loaded with *myo*-[2-^{3}H]inositol and challenged with PAF (100 nM) after pretreatment with Genistein. PLC-mediated production of radioactive inositol monophosphate, inositol diphosphate, and inositol triphosphate was monitored. PAF alone caused stimulation of PLC activity ([^{3}H]inositol triphosphate production), whereas pretreatment with Genistein (0.5 mM) diminished PAF-stimulated PLC activity to basal level. Genistein also blocked PAF-stimulated platelet aggregation at this dose. In contrast to Genistein, staurosporine which inhibits protein kinase C, potentiated PAF-stimulated [^{3}H]inositol triphosphate production. Genistein substantially inhibited the combined effects of staurosporine and PAF on inositol triphosphate production. Genistein also reduced PAF-induced phosphorylation of M_r 20,000 and 50,000 proteins. Phorbol 12-myristate 13-acetate-induced M_r 40,000 protein phosphorylation was also affected by Genistein. The above results suggested that Genistein inhibited tyrosine kinase at an early stage of signal transduction by inhibiting PLC. This, in turn, decreased the activation of protein kinase C and, therefore, caused a reduction in M_r 40,000 protein phosphorylation. The inhibition of PLC by Genistein raised the possibility of involvement of tyrosine kinase in PAF receptor-coupled PLC activation. Western blot analysis using monoclonal antibody to phosphotyrosine demonstrated that PAF stimulated the tyrosine phosphorylation of two major proteins of 50,000 and 60,000 molecular weight. When platelets were challenged with PAF after treatment with either Genistein or CV-6209 (a PAF receptor antagonist), the reactivity of these proteins to monoclonal antibody was inhibited. Phosphoamino acid analysis of M_r 50,000 and 60,000 proteins confirmed that PAF increased the phosphorylation of tyrosine residues in both M_r 50,000 and 60,000 proteins and that this was inhibited by Genistein. Thus, PAF caused a receptor-dependent phosphorylation of tyrosine residues on M_r 50,000 and 60,000 proteins. Based on these observations, it is concluded that tyrosine kinase is involved in the PAF receptor-coupled PLC activation and signal transduction mechanism.

PAF, an ether phospholipid, is a potent platelet-activating agent (1-3) and stimulates phosphoinositide-specific PLC, which in turn produces DG and IP_3 (4, 5). Elevated levels of IP_3 within the cytoplasm stimulates Ca^{2+} release from intracellular stores (6, 7). DG remains within the membrane and stimulates PKC activity (7, 8), which leads to phosphorylation of several proteins in platelets. Recently, we established that activation of PLC and the phosphorylation of proteins by PAF is a receptor-coupled event (10, 11) and that these processes are desensitized in platelets preexposed to PAF (9, 10). It was also demonstrated that PAF-stimulated PLC activity is negatively affected by PKC activation and that inhibition of PKC by staurosporin did not prevent desensitization of PLC by PAF (12). In order to assess the role of protein phosphorylation in this process, we have investigated the involvement of tyrosine kinase in PAF actions. In fact, certain growth factors, e.g., epidermal growth factor or platelet-derived growth factor, have

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ABBREVIATIONS: PAF, platelet-activating factor; PLC, phospholipase C; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloracetic acid; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; IP, inositol monophosphate; DG, diacylglycerol; G protein, GTP-binding protein; PMA, phorbol 12-myristate 13-acetate.

been shown to simulate phosphoinositide turnover through activation of PLC (see Ref. 13). Therefore, a functional link between tyrosine kinase and PLC is conceivable. Protein-tyrosine kinases are involved in proliferation and transformation of cells (14–16). Recently, nonproliferating terminally differentiated cells like blood platelets have been shown to contain high protein-tyrosine kinase activities (17, 18). It was also demonstrated recently that thrombin, a physiological activator, causes elevation of platelet phosphotyrosine levels by stimulating tyrosine-specific protein kinases (19–21).

Here, we report on the investigation of the involvement of tyrosine kinases in PAF receptor-coupled processes, using Genistein [a putative inhibitor of tyrosine-specific protein kinases (22)], phosphotyrosine monoclonal antibody and phosphoamino acid analysis as probes.

Experimental Procedures

Materials. PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was supplied by Bachem (Torrance, CA). *myo*-[2-^{3}H]Inositol (specific radioactivity, 15 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]PAF containing a C16 alkyl chain (40.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Dowex-1 resin (AG 2-X8, 200–400 mesh) was obtained from Bio-Rad (Richmond, CA). Carrier-free ^{32}P, [^{32}P]NAD, and Genistein were purchased from ICN Radiochemicals and Biochemicals (Irvine, CA). Staurosporine was purchased from Kyowa Medax Co. (Tokyo, Japan). PMA was obtained from LC Services Corporation (Woburn, MA). mAb to phosphotyrosine was supplied by Boeringer Mannheim (Indianapolis, IN). Pertussis toxin was obtained from List Biological Research (Cambridge, CA). All other chemicals and solvents used were of the highest analytical grade available.

Isolation of platelets and their labeling with [^{3}H]inositol. Blood was withdrawn from the central ear artery of New Zealand white rabbits and platelets were isolated essentially as described earlier (10). Washed rabbit platelets, at a concentration of 1.5×10^9 cells/ml of Tyrode's-gelatin buffer (pH 6.5) without Ca^{2+} (containing 0.1 mM EGTA), were incubated with *myo*-[2-^{3}H]inositol (10 μCi/ml) at 37° for 90 min. Cells were centrifuged at 830 × *g* for 15 min at 24°. The platelet pellet was resuspended in Tyrode's gelatin buffer, pH 7.2, containing 0.1 mM EGTA, to a concentration of 3×10^8 cells/ml.

Stimulation of platelets with various agents and analysis of inositol polyphosphates. Labeled platelets were incubated with appropriate concentrations of PAF and other agents (see text) at 37° for the indicated time, and the reactions were terminated with 0.1 ml of perchloric acid (70%, w/v). After termination, the reaction mixtures were centrifuged at 100 × *g* for 5 min and then the supernatant was neutralized with a solution of 2.0 M KOH (prepared in 75 mM HEPES). This was recentrifuged at 1000 × *g* for 5 min and the supernatant was analyzed for radioactive inositol polyphosphate content. Separation of IP, IP_2, and IP_3 was based on Dowex-1 chromatography of the samples, as described elsewhere (10). Aliquots (3 ml) of each fraction were mixed with 15 ml of Budget-Solve scintillation cocktail (Research Products International, Mount Prospect, IL), and their radioactivities were determined using a programmable Beckman LS 7500 liquid scintillation system.

Effect of pertussis toxin treatment on PAF-stimulated [^{3}H] inositol phosphates production. *myo*-[^{3}H]inositol-labeled platelets (3.5×10^8 cells/ml) were incubated with saponin (15 μg/ml), NAD (0.2 mM), EGTA (10 μM), ATP (1 mM), and pertussis toxin (5 μg/ml, preincubated with 2.0 mM dithiothreitol for 30 min at 37°) for 30 min at 37°. After the incubation, platelets were challenged with PAF and [^{3}H]inositol phosphates were analyzed (see above). In some experiments [^{32}P]NAD was used to determine the protein substrate for pertussis toxin. In this case, the ^{32}P-labeling of protein was monitored as described below.

Assay for protein phosphorylation. Washed platelets (1.5×10^9 cells/ml) in Tyrode's buffer, pH 6.5, were labeled with carrier-free ^{32}P (100 μCi/ml) for 90 min at 37°. Labeled platelets were washed in Tyrode's buffer, pH 7.2, and the cell density was adjusted to 7.5×10^8 cells/ml. Duplicate aliquots (1 ml) were challenged with the drugs for the times indicated for each experiment. The reactions were terminated by addition of 100 μl of TCA (50%, v/v) and were centrifuged at 2000 × *g* for 5 min. The supernatant was aspirated and the resulting pellets were washed with 5% TCA and then resuspended in a solubilizing buffer containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0625 M Tris, pH 6.8 (11). The samples were incubated overnight, neutralized with Trizma base, and subjected to SDS-PAGE, using 8.75% acrylamide, under the conditions described by Laemmli (23). The gel was stained with 0.5% Coomassie brilliant blue, destained and dried under vacuum and heat using a Bio-Rad slab gel dryer (model 224; Bio-Rad, Richmond, CA). Protein bands were identified after 5–6 hr of autoradiography and, in some experiments, were cut, incubated overnight in Budget-Solve scintillation cocktail (Research Products International), and counted in an LS-7500 liquid scintillation system.

[^{3}H] PAF binding assay. [^{3}H]PAF binding assays were performed according to the method of Morrison and Shukla (11). Rabbit platelets were suspended in a Ca^{2+}-free Tyrode's-gelatin buffer, pH 7.2, containing 0.1 mM EGTA. Specific [^{3}H]PAF binding was taken as the difference between total binding to platelets and nonspecific binding. Nonspecific binding was measured in the presence of 10 μM unlabeled PAF or 10 μM CV6209. For binding assays, 1-ml aliquots containing $1–3 \times 10^8$ cells/ml were incubated in triplicate with [^{3}H]PAF (50 pM) and 10 μM concentrations of PAF or the antagonist CV6209, for 10 min at 25°, in polystyrene test tubes. All assays were terminated by vacuum filtration through Whatman (GF/B) glass microfiber filters, using a Brandel cell harvester (model 24). The filters were rapidly rinsed twice with 5 ml of ice-cold 50 mM Tris buffer, pH 8.0, soaked overnight in 10 ml of scintillation cocktail, and counted in a Beckman LS-7500 scintillation counter.

Platelet aggregation. Platelets were suspended in Tyrode's-gelatin buffer, pH 7.2, containing 1.33 mM Ca^{2+}, and their aggregation patterns were monitored (5) using PAF (1×10^{-9} M) with or without Genistein (0.25 and 0.5 mM), in a Chronolog Aggregometer model 330 (Havertown, PA).

Immunoblot with mAb to phosphotyrosine. Washed platelets (1×10^9 cells/ml) were permeabilized with saponin (15 μg/ml) and were incubated with ATP (2 mM), sodium molybdate (10 μM), and sodium vanadate (100 μM) for 10 min at 37° (24). Vandate and molybdate were used to inhibit phosphatase (24). These platelets were stimulated with PAF (1×10^{-9} μM) or thrombin (1 unit/ml), after pretreatment with Genistein (500 μM) or the PAF antagonist CV6209 (10 μM) at different time intervals. The reactions were terminated by addition of 0.1 ml of TCA (50%, v/v), reaction mixtures were centrifuged, and pellets were washed with 5% TCA. They were then resuspended in solubilizing buffer and subjected to SDS-PAGE as described above. The separated proteins were transferred to nitrocellulose paper (Bio-Rad), according to the method of Towbin *et al.* (25). After transfer onto the nitrocellulose paper, the paper was soaked in Tris-buffered saline containing 3% gelatin, for 30 min. It was subsequently washed with Tris-buffered saline containing 0.05% Tween 20 (TTBS) and soaked overnight in a 1:200 dilution of mAb to phosphotyrosine (Boeringer Mannheim). It was washed again with TTBS, reacted for 2 hr with goat anti-mouse peroxidase-conjugated IgG (Bio-Rad) diluted to 1:500 in TTBS, and then rinsed with TTBS followed by Tris-buffered saline. The color was developed by reaction with color reagent (Bio-Rad). In some papers, after incubation with mAb to phosphotyrosine, the nitrocellulose paper was soaked with ^{125}I-Protein A (ICN). It was then washed and dried, and radioactive bands were visualized by autoradiography for 24 hr on Kodak X-ray O-Mat films.

Phosphoamino acid analysis. Phosphorylated proteins were detected by autoradiography of dried SDS-PAGE gels (see above). In some cases, gels were treated with NaOH (1 M) for 1 hr, followed by

incubation in 7% acetic acid/7% methanol (v/v) at 26° for 2 hr in a shaker (26). The gels were dried and then phosphorylated proteins in those dried gels were detected by autoradiography. The phosphoamino acids were analyzed by the method of Neufeld *et al.* (27). Sections of the gels containing M_r 50,000 and 60,000 phosphoproteins were cut out and the fragments were rehydrated in 10% methanol. The cellophane support was removed and the fragments were dried at 70° for 1 hr. The fragments were incubated in 1 ml of medium containing 50 mM NH_4HCO_3, 0.1% SDS, and 10 mM 2-mercaptoethanol (28), for 16 hr at 20° (29). The supernatants were collected and were hydrolyzed with 6 N HCl for 2 hr at 110°. The acid hydrolysates were lyophilized (Speed-Vac concentrator; Hetovac, Laurel, MD). The concentrated residue was washed two times, i.e., by resolubilization in 200 μl of distilled water and relyophilization. Residues were dissolved in 10–30 μl of distilled water and applied to an Eastman Chromagram cellulose sheet (20 × 20 cm^2; catalog no. 13254 or 13255). Thin layer plates were run in a Chromagram developing apparatus (Gelman Apparatus, model 51325-1), in a solvent solution of propionic acid/1 M ammonium hydroxide/isopropyl alcohol (45/17.5/17.5; v/v). Standard phosphoamino acids (Sigma Chemical Co.) were run in parallel and detected by ninhydrin spray (0.3% in 1-butanol). ^{32}P-labeled phosphoamino acids were detected by autoradiography and were compared with standards.

Results and Discussion

Effect of Genistein on PAF-induced aggregation. PAF-induced aggregation was inhibited by Genistein in a dose-dependent manner. Fig. 1 and Table 1 show that treatment of platelets for 30 sec with 250 μM Genistein caused a partial inhibition of PAF-stimulated aggregation, whereas doses of 500, 750, and 1000 μM inhibited aggregation to near basal level. This suggested that Genistein, a putative inhibitor of tyrosine-specific protein kinases (22), inhibited PAF-induced aggregation.

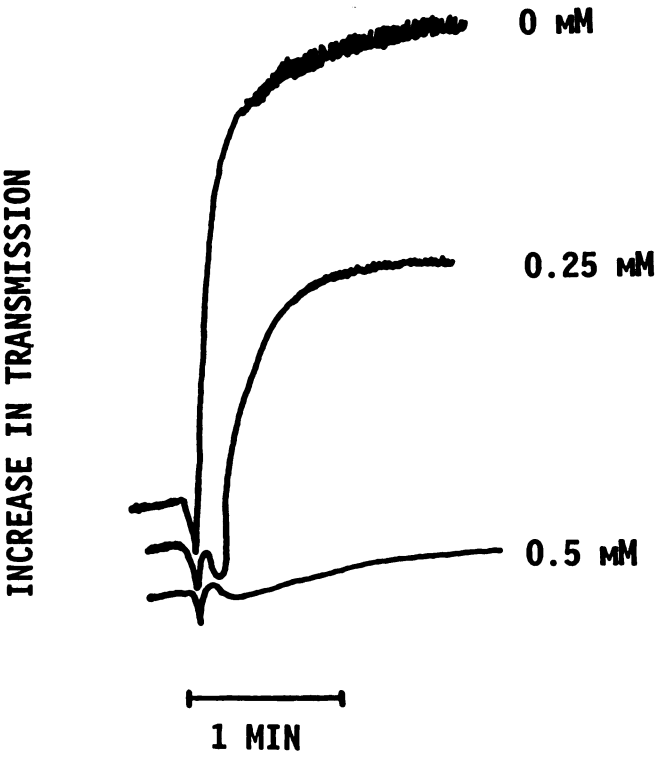

Fig. 1. Effect of Genistein on PAF-stimulated aggregation. Washed rabbit platelets (5 × 10^8/ml) were suspended in a Tyrode's-gelatin buffer, pH 7.2, containing 1.33 mM Ca^{2+}, and their aggregation pattern was monitored using PAF (100 nM), after a 30-sec pretreatment with Genistein (0.5 and 0.25 mM), in a Chronolog aggregometer model 330 (Havertown, PA).

TABLE 1

Aggregation studies of platelets treated with PAF and Genistein

Washed rabbit platelets were treated with 100 nM PAF (P) or pretreated with different concentration of Genistein (G) followed by 100 nM PAF (P). Their aggregation response, as monitored by a decrease in light transmission, was recorded using a Chronolog aggregometer. DMSO, dimethyl sulfoxide.

Incubations	Aggregation
	%
Control (DMSO)	0
P (100 nM)	100
G (250 μM), 30 sec → P	68.5 ± 7.2
G (500 μM), 30 sec → P	27.14 ± 2.85
G (750 μM), 30 sec → P	18.50 ± 1.92
G (1000 μM), 30 sec → P	7.10 ± 0.81

TABLE 2

[^{3}H]Inositol phosphate levels after treatment with different agents

Platelets were challenged with 100 nM PAF (P), 500 μM Genistein (G), 3 μM staurosporin (S), and (1 unit/ml) thrombin (T), and the [^{3}H]inositol phosphates were analyzed (see Experimental Procedures). The arrows indicate preincubations at 37° with one drug followed by treatment with another. Values are expressed as the mean ± standard error from four separate experiments and were compared using control as 100%.

Incubations	IP_3	IP_2	IP
		% of control	
Control	100	100	100
G, 30 sec	109.5 ± 6.54	101 ± 7.5	118 ± 7.77
P, 5 sec	222.5 ± 17.5	268 ± 53.03	254 ± 26.87
G, 30 sec → P, 5 sec	108.5 ± 6.5	114.5 ± 15.09	123 ± 12.02
G, 1 min → P, 5 sec	119 ± 3	98.5 ± 7.42	156 ± 28.28
G, 5 min → P, 5 sec	108.5 ± 6.01	99.5 ± 8.68	144.5 ± 22.48
T, 30 sec	199 ± 6.74	216 ± 49.5	413.67 ± 84.4
G, 30 sec → T, 30 sec	160.5 ± 8.55	145 ± 21.21	435.6 ± 99.83
S, 10 min	115 ± 18	139 ± 27.58	103 ± 7.78
S, 10 min → G, 1 min → P, 5 sec	132.5 ± 15.5	213 ± 10.6	103.5 ± 1.11
S, 10 min → P, 5 sec	291 ± 17	484.5 ± 6.94	589.5 ± 56.25

Influence of Genistein on PAF-stimulated increase in [^{3}H]inositol phosphates. Washed rabbit platelets labeled with [^{3}H]inositol were pretreated with Genistein (500 μM) for different time intervals (30 sec, 1 min, and 5 min), and cells were then challenged with PAF for 5 sec. [^{3}H]IP_3 levels were increased by PAF alone, whereas Genistein pretreatment inhibited PAF-induced [^{3}H]IP_3 production (Table 2). Genistein pretreatment at different doses (500, 375, 250, 125, and 62.5 μM) for 30 sec also inhibited PAF-induced [^{3}H]IP_3 production in a dose-dependent manner (Table 3). Genistein pretreatment only partially reduced thrombin-induced [^{3}H]IP_3 production. This fact indicates that [^{3}H]IP_3 production induced by thrombin might be mediated partly through tyrosine kinase involvement, and this supports the recent report of elevation of phosphotyrosine levels by thrombin treatment (19–21). Table 2 also shows that pretreatment with staurosporin (3 μM) for 10 min increased [^{3}H]IP_3 levels by 291% over basal level, whereas staurosporin pretreatment followed by Genistein treatment caused a decrease of PAF-induced [^{3}H]IP_3 production (132%). The PAF-induced increase in [^{3}H]IP_3, [^{3}H]IP_2, and [^{3}H]IP was blocked by Genistein pretreatment. Staurosporin pretreatment increased PAF-induced inositol phosphate production and this was inhibited by Genistein. Similar results were also observed for [^{3}H]IP_2 and [^{3}H]IP levels.

These results demonstrated that pretreatment with Genistein completely blocked PAF-stimulated PLC activity. Pre-

TABLE 3

[^{3}H]Inositol phosphate levels after treatment with different doses of Genistein

Platelets were challenged with 100 nM PAF (P) after treatment with different concentrations of Genistein (G) and their [^{3}H]inositol phosphates were analyzed (see Experimental Procedures). The arrows indicate preincubations with one agent followed by treatment with another. Values are expressed as the mean ± standard error from four separate experiments and were compared using control as 100%. DMSO, dimethyl sulfoxide.

Incubation	IP_3	IP_2	IP
	% of control		
Control (DMSO)	100	100	100
G (500 μM), 30 sec	86 ± 7.3	115.3 ± 6.63	117 ± 4.04
P, 5 sec	248.66 ± 23	334.6 ± 12.4	413 ± 6.02
G (500 μM), 30 sec → P, 5 sec	102.6 ± 1.95	145 ± 8.5	179 ± 3.47
G (375 μM), 30 sec → P, 5 sec	122 ± 12.4	158 ± 6.11	193 ± 4.96
G (250 μM), 30 sec → P, 5 sec	147 ± 15.03	182.6 ± 6.84	231 ± 9.45
G (125 μM), 30 sec → P, 5 sec	193.33 ± 15.32	222.66 ± 8.72	286 ± 6.76
G (62.5 μM), 30 sec → P, 5 sec	220.33 ± 7.86	284 ± 15.74	331 ± 7.19

TABLE 4

Specific binding of [^{3}H]PAF to platelets pretreated with Genistein

Rabbit platelets were suspended in Ca^{2+}-free Tyrode's-gelatin buffer, pH 7.2, containing 0.1 mM EGTA. Specific binding was taken as the difference between total binding and nonspecific binding. Nonspecific binding was measured by the addition of 10 μM unlabeled PAF or 10 μM PAF antagonist CV6209 (see Experimental Procedures). Platelets were preincubated for 5 min with Genistein (G) at a dose of 250 or 500 μM before binding assays were conducted. Values are expressed as the mean ± standard error from two separate experiments.

Incubations/treatments	Specific [^{3}H]PAF binding	
	dpm/10^9 cells	*pmol of PAF/10^9 cells*
Control (total specific binding)	5913 ± 62.5	0.0662 ± 0.005
G (250 μM), 5 min	5872 ± 43.2	0.0658 ± 0.007
G (500 μM), 5 min	5710 ± 47.8	0.0640 ± 0.006

TABLE 5

Effect of pertussis toxin on PAF-stimulated [^{3}H]inositol phosphate levels

In these studies, *myo*-[^{3}H]inositol-labeled rabbit platelets were incubated with saponin (15 μg/ml), NAD (0.2 mM), ATP (1 mM), and activated pertussis toxin (PT) (see Experimental Procedures) for 30 min at 37°. After this incubation, platelets were challenged with 100 nM PAF (P) for 5 sec and the [^{3}H]inositol phosphates were analyzed (see Experimental Procedures). Values are expressed as the mean ± standard error from four separate experiments and were compared using control as 100%.

Incubation	IP_3	IP_2	IP
	% of control		
Control	100	100	100
P, 5 sec	180 ± 23	307 ± 15	226 ± 18
PT, 30 min	120 ± 7	98 ± 4	105 ± 8
PT, 30 min → P, 5 sec	135 ± 17	273 ± 6	225 ± 34

TABLE 6

Pattern of protein phosphorylation after treatment with different agents

^{32}P-Labeled rabbit platelets were treated using selected protocols (see text) and the phosphorylation pattern of M_r 20,000, 40,000, and 50,000 proteins was monitored (see Experimental Procedures). PAF (P) (100 nM), Genistein (G) (500 μM), and thrombin (T) (1 unit/ml) were used. Arrows indicate treatment with one drug followed by another. Results are presented as mean ± standard error from four separate experiments. DMSO, dimethyl sulfoxide.

Incubation	Phosphorylation		
	M_r 20,000	*M_r 40,000*	*M_r 50,000*
	% of control		
Control (DMSO)	100	100	100
P, 10 sec	168 ± 7.07	417 ± 38.18	136.5 ± 12.37
G, 1 min	99.5 ± 1.23	93.5 ± 5.3	80 ± 6.32
G, 1 min → P, 10 sec	112.5 ± 4.0	258.5 ± 4.6	96 ± 6.32
G, 5 min → P, 10 sec	102 ± 10.6	251.5 ± 10.35	89.75 ± 2.67
G, 1 min → T, 15 sec	135 ± 4.94	250.5 ± 24.39	99.5 ± 4.6
T, 15 sec	207 ± 36.76	348 ± 9.19	119 ± 7.07
G, 1 min → PMA, 1 min	128 ± 7.78	357 ± 5.65	117 ± 15.2
PMA, 1 min	182 ± 27.93	498 ± 26.52	142 ± 8.43

treatment with staurosporin followed by Genistein also inhibited PAF-stimulated PLC activity. This indicates that the mechanism involved in PAF-induced PLC activation is not mediated via PKC (12). Inhibition by Genistein raised the possibility of involvement of tyrosine kinase in PAF-stimulated PLC activation. Genistein had no effect on PLC activity *in vitro* and, therefore, the observed inhibition is not due to direct interaction of Genistein with PLC (results not shown).

The possibility that Genistein could block [^{3}H]PAF binding and may, thus, inhibit the PAF responses was next considered. Values for specific [^{3}H]PAF binding to platelets in the presence of Genistein (250 or 500 μM) were same as in the control, i.e., no Genistein (Table 4). Thus, Genistein did not inhibit the specific binding of [^{3}H]PAF to platelets. Therefore, the inhibition of PAF-induced PLC activation by Genistein was likely due to the inhibition of tyrosine kinase activity in the receptor-coupled PLC activation.

Effect of pertussis toxin on PAF-stimulated PLC activity. In a related study, we investigated the involvement of G proteins in PAF receptor-coupled PLC activation. We used pertussis toxin, which ADP-ribosylates G_i protein, to monitor whether pertussis toxin-sensitive G protein was involved in the PAF-stimulated PLC activity. Although PAF-mediated PLC activation (IP_3 production) was affected to some extent by pertussis toxin treatment, this had little influence on IP_2 and IP production (Table 5). It has been observed by Banga *et al.* (30) that pertussis toxin itself can activate human platelets by causing IP_3 and phosphatidic acid production. So, it is difficult to interpret such data, because pertussis toxin may have stimulatory as well as inhibitory effects on platelets (30). Pertussis toxin substrate was identified in saponin-permeabilized rabbit platelets using [^{32}P]NAD (see Experimental Procedures). The ADP-ribosylated radioactive protein migrated on SDS-PAGE with an apparent molecular weight of 41,000 (data not shown), in agreement with the observations of Brass *et al.* (31) and Crouch and Lapetina (32). In these experiments, although the M_r 41,000 protein was ADP-ribosylated, we were not able to show appreciable pertussis toxin-sensitive G protein involvement in the PAF receptor-coupled PLC activation. PAF-stimulated [^{3}H]IP_3 production decreased in pertussis toxin-pretreated saponin-permeabilized cells, but it was not reduced to basal level. IP_2 and IP production did not show much reduction by pertussis toxin in PAF-stimulated platelets. This, therefore, raised the intriguing possibility of the involvement of mechanisms (e.g., tyrosine kinase) in addition to the G proteins in the PAF-mediated signal transduction pathway.

Protein phosphorylation pattern. Treatment of ^{32}P-labeled rabbit platelets with PAF (1×10^{-9} M) caused rapid phosphorylation of several proteins (10). In this study, we monitored three phosphorylated proteins with apparent molecular weights of 20,000, 40,000, and 50,000. It was reported earlier that the levels of PAF-stimulated ^{32}P incorporation into M_r 20,000 and 40,000 proteins was maximum at 10 sec and decreased thereafter (10). We, therefore, studied the phosphor-

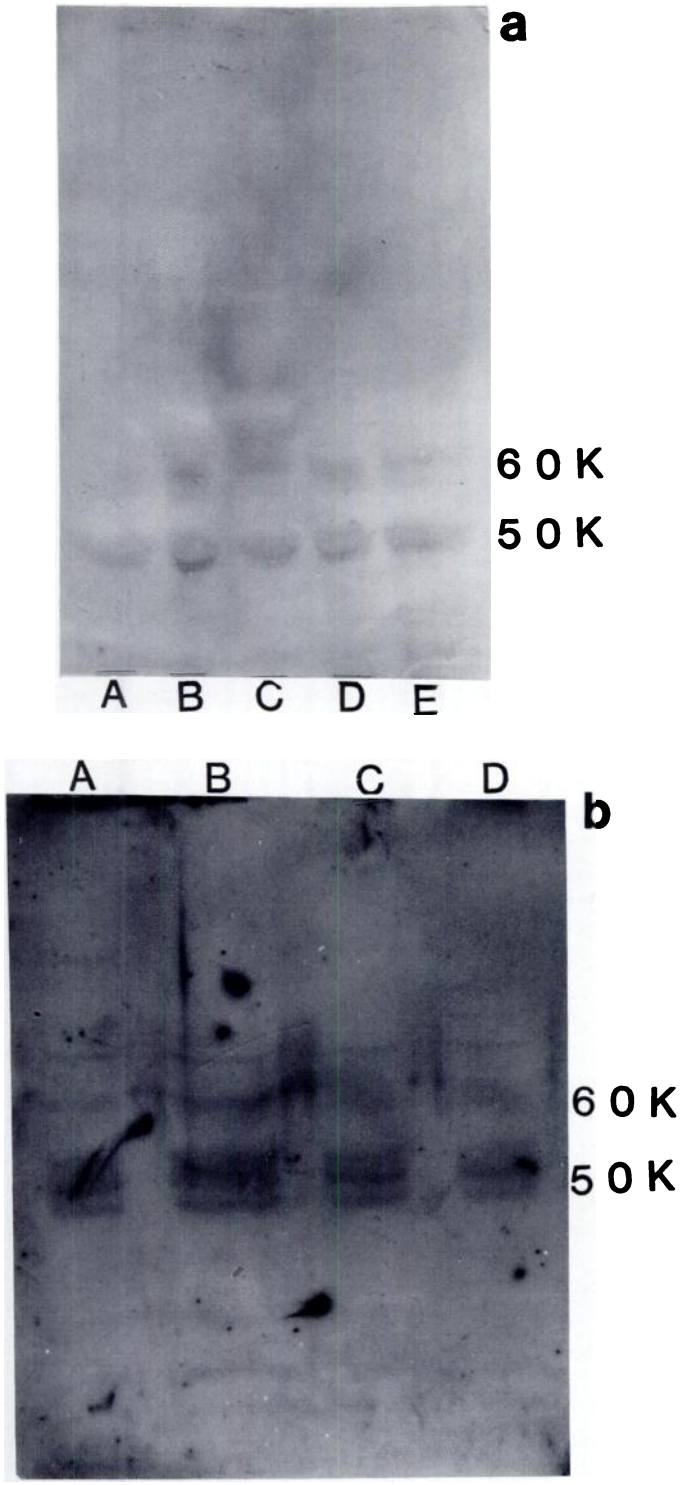

Fig. 2. Western blot analysis of platelets treated with PAF and other agents probed by a mAb to phosphotyrosine. a, After appropriate incubations, samples were subjected to SDS-PAGE and were electrophoretically transferred to nitrocellulose sheets. The sheets were then treated with mAb to phosphotyrosine, followed by incubation with goat anti-mouse IgG horseradish peroxidase conjugate. The color of the blot was developed and a photograph was taken immediately. *Lane A*, control platelets; *lane B*, PAF (100 nM)-treated platelets; *lane C*, Genistein (500 μM) pretreatment followed by PAF (100 nM) treatment; *lane D*, 1 unit/ml thrombin; *lane E*, Genistein followed by thrombin (1 unit/ml). b, Western blot analysis of PAF-treated rabbit platelets reactive to phosphotyrosine mAb and developed using ^{125}I-Protein A. Control (*lane A*), PAF-treated (*lane B*), Genistein-pretreated followed by PAF-treated (*lane C*), and antagonist (CV6209)- followed by PAF-treated (*lane D*) platelets were subjected to protocols similar to those in a. After sheets were treated with phosphotyrosine mAb, they were incubated with ^{125}I-Protein A and an autoradiograph was developed after 24 hr.

ylation of these proteins by PAF at 10 sec after Genistein (500 μM) treatment for 1 and 5 min.

The results presented in Table 6 indicate that the radioactivity in M_r 20,000, 40,000, and 50,000 proteins increased due to PAF (100 nM) treatment. Levels of radioactivity were very high in the M_r 40,000 protein (417%), compared with control. The increase in the radioactivity in the M_r 20,000 (168%) and 50,000 (136.5%) proteins was also significant, in comparison with control (100%). Genistein alone did not phosphorylate M_r 20,000 or 40,000 proteins but did decrease the radioactivity in the M_r 50,000 protein below the basal (control) level. Treatment with Genistein decreased the PAF-stimulated phosphorylation of the M_r 20,000, 40,000, and 50,000 proteins significantly. Genistein reduced PAF-stimulated M_r 40,000 protein phosphorylation by about 68%. Similar inhibition by Genistein of the phosphorylation of proteins (M_r 20,000, 40,000, and 50,000) was observed when thrombin (1 unit/ml) was used as a stimulus. PMA-stimulated phosphorylation of the M_r 20,000, 40,000, and 50,000 proteins was also decreased by pretreatment with Genistein.

Table 6 demonstrated that Genistein blocked PAF-stimulated M_r 20,000 and 50,000 protein phosphorylation, whereas M_r 40,000 protein phosphorylation was partially blocked. It is known that the M_r 40,000 protein is phosphorylated by PKC (7). Inhibition of PLC activation by Genistein might be one of the early events, which in turn will inhibit the production of IP_3 and DG. In such a scenario, activity of neither Ca^{2+}-dependent nor DG-dependent kinases (i.e., myosin light chain kinase and PKC) will be observed. This may explain why Genistein inhibits phosphorylation of the M_r 20,000 (a myosin light chain kinase substrate) and 40,000 (a PKC substrate) proteins. The inhibitions are, therefore, consequences of inhibition of PAF receptor-coupled PLC by Genistein. Phosphorylation of the M_r 50,000 protein was of interest because it was most affected by Genistein treatment and it, therefore, might be considered one of the best substrates for tyrosine kinase in PAF-mediated signal transduction. PMA-mediated phosphorylation of the M_r 40,000 protein is also partly affected by Genistein (116% less than PAF treatment) and the significance of this partial inhibition remains to be explained.

Western blot analysis. Using a mAb to phosphotyrosine, we examined the phosphorylation of tyrosine residues in proteins by PAF, in the presence and absence of Genistein. Upon stimulation by appropriate protocols (see Experimental Procedures), platelets were solubilized with SDS sample buffer. Following electrophoresis and blotting onto nitrocellulose, samples were probed by a mAb for phosphotyrosine residues. Fig. 2 shows that PAF caused a dramatic increase in the mAb reactivity of two prominent bands, the M_r 50,000 and 60,000 proteins. In Fig. 2a, we used a peroxidase enzyme conjugate and in Fig. 2b we used ^{125}I-Protein A, to compare the reactivity of the mAb to phosphotyrosine. Other workers (19–21) have recently used a polyclonal antibody to phosphotyrosine, which might exhibit broader specificity, than the mAb used in the present study. Other investigators have also observed that, in human platelets, M_r 76,000, 116,000, and 170,000 proteins react to a polyclonal antibody to phosphotyrosine after challenge

with thrombin. In our study, using mAb to phosphotyrosine, it was observed that PAF increased the reactivity of the M_r 50,000 and 60,000 proteins to mAb (Fig. 2), indicating an increase in phosphotyrosine on these proteins. Treatment of platelets with Genistein or the PAF antagonist CV6209 (Fig. 2) suppressed the reactivity of the M_r 50,000 and 60,000 proteins to mAb after PAF challenge.

Phosphoamino acid analysis. To specifically identify the phosphotyrosine residues in the M_r 50,000 and 60,000 proteins, phosphoamino acid residues of these proteins were analyzed (see Experimental Procedures). As shown in Fig. 3, the ^{32}P radioactivity in phosphotyrosine residues of both the M_r 50,000 and 60,000 proteins was increased by PAF treatment. Basal [^{32}P]phosphotyrosine residues in both the M_r 50,000 and 60,000 proteins were noticeable in unstimulated platelets and this is in agreement with the high phosphotyrosine content observed by Golden and Brugge (20) in unstimulated human platelets. Genistein decreased the basal levels of ^{32}P-labeled tyrosine and serine phosphates of both the M_r 50,000 and 60,000 proteins. Pretreatment of platelets with Genistein reduced the increase in PAF-stimulated [^{32}P]phosphotyrosine residues of the M_r 50,000 and 60,000 proteins. It has been shown that phosphorylated tyrosine and threonine residues of proteins are more resistant to alkali treatment than phosphorylated serine residues (26, 28). We have also utilized similar protocols in our experiments. Treatment of gels with 1 M NaOH (see Experimental Procedures) caused loss of phosphoserine residues of both the M_r 50,000 and 60,000 proteins, without affecting tyrosine phosphates (data not shown). Genistein inhibited PAF-stimulated phosphorylation of tyrosine residues (Fig. 3). PMA also slightly induced tyrosine phosphorylation in the M_r 50,000 and 60,000 proteins, which can be partially inhibited by Genistein (Fig. 3). PMA has been reported to induce the phosphorylation of tyrosine residues of proteins in fibroblasts and in platelets (see Ref. 20). This evidence suggests that PMA stimulation of PKC may lead to the activation of cellular tyrosine kinases and highlights the complexity of PMA actions. The inhibition of PAF-induced aggregation and [^{3}H]inositol polyphosphate production by Genistein suggested the involvement of tyrosine kinase in platelet responses. We have reported earlier that staurosporine potentiates PAF-induced [^{3}H]IP_3 production by inhibiting PKC (12). If Genistein inhibited PKC, we would expect a potentiated [^{3}H]inositol phosphate production. This did not occur and, therefore, indicates that PKC was not inhibited by Genistein.

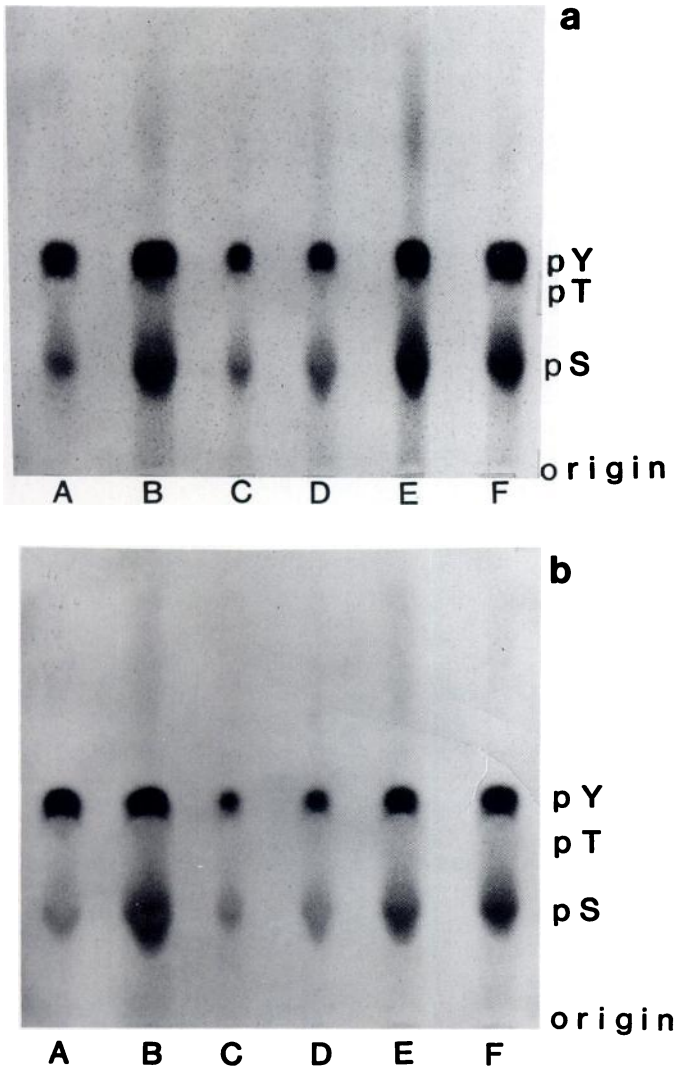

Fig. 3. Phosphoamino acid analysis of M_r 50,000 (a) and 60,000 (b) proteins. a, Phosphoamino acid residues [phosphoserine (*pS*), phosphothreonine (*pT*), and phosphotyrosine (pY)] of phosphorylated M_r 50,000 protein were monitored by autoradiography, as described in Experimental Procedures. Platelets were incubated with PAF for 10 sec, Genistein for 30 sec, and PMA for 1 min. *Lane A*, control platelets; *lane B*, PAF (100 nM)-treated platelets; *lane C*, Genistein (500 μM)-treated platelets; *lane D*, Genistein (500 μM) followed by PAF (100 nM) treatment; *lane E*, Genistein (500 μM) followed by PMA (50 μg/ml) treatment; *lane F*, PMA-treated platelets. b, Phosphoamino acid analysis of M_r 60,000 protein of control (*lane A*), PAF- (*lane B*), Genistein- (*lane C*), Genistein- followed by PAF- (*lane D*), Genistein- followed by PMA- (*lane E*), and PMA- (*lane F*) treated platelets. The conditions for this experiment were similar to those in a.

Western blot analysis with phosphotyrosine mAb showed that the M_r 50,000 and 60,000 proteins are the only proteins reactive with the mAb. The M_r 20,000 and 40,000 proteins did not react with the mAb because tyrosine residues were not phosphorylated in these proteins (7, 28). It is also known that the M_r 60,000 protein is the $pp60^{c\text{-}src}$ protein and is abundantly available in platelets (18). Thrombin and collagen, physiological activators, also elevate the number of phosphotyrosine residues and the phosphorylation of $pp60^{c\text{-}src}$ proteins in platelets; this has pointed toward the involvement of tyrosine kinase in platelet activation mechanisms (19–21). It was also recently reported by Rendu *et al.* (33) that similar changes occur in human platelet tyrosine phosphorylation of M_r 60,000 and 56,000 proteins during thrombin activation.

In the next phase, we analyzed the phosphoamino acids in the M_r 50,000 and 60,000 proteins. It was demonstrated that PAF significantly stimulated an increase in [^{32}P]phosphotyrosine levels of both the M_r 50,000 and 60,000 proteins, and this was inhibited by Genistein treatment. These results confirmed that the M_r 50,000 and 60,000 proteins are the substrates for tyrosine kinase and may be involved in PAF receptor-coupled PLC activation in rabbit platelets.

Protein tyrosine kinases have been proposed to be involved in proliferation and transformation of cells, because of their close relationship with transformation genes and several growth factors. It has also been recently proposed that growth factors directly stimulate tyrosine phosphorylation of either PLC or a tightly associated protein, and this tyrosine phos-

phorylation may have functional consequences that are related to phosphatidylinositol bisphosphate hydrolysis (13). The present study has demonstrated that increases in the phosphorylation of tyrosine residues are early events in the signal transduction pathway for stimulation of platelets by PAF. It was also suggested recently that a M_r 52,000 protein might be associated with the regulatory components for preserving the functional activity of the solubilized PAF receptor complex (34). It would be interesting to resolve whether the tyrosine kinase substrates, the M_r 50,000 and 60,000 proteins, have any functional relationship to the PAF receptor and PLC in platelets.

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