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Desensitization of Platelet-Activating Factor-Stimulated Protein Phosphorylation in Platelets

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SUMMARY

Treatment of ^{32}P-labeled rabbit platelets with platelet-activating factor (PAF) caused a time- and dose-dependent phosphorylation of several proteins including five major phosphorylated proteins with apparent molecular weights of 20,000, 35,000, 40,000, 65,000, and 150,000. Both PAF and thrombin caused a rapid increase followed by a decrease in phosphorylation of proteins, indicating the occurrence of a phosphorylation-dephosphorylation process. Four separate PAF receptor antagonists, CV-3988, CV-6209, SRI-63-441, and SRI-63-675 drastically reduced the PAF-stimulated protein phosphorylation. The order of potency was SRI-63675 > SRI-63441 ≥ CV-6209 > CV-3988. These antagonists had no effect on thrombin-stimulated protein phosphorylation. Pretreatment of platelets with PAF (0.1 nM) completely abolished any further protein phosphorylation by the same concentration of PAF. PAF pretreatment shifted the dose response of protein phosphorylation by about 2 log units, to the right. When platelets were treated with PAF (10 nM) for 10 min, this abolished phosphorylation of proteins by any concentration of PAF. These studies indicated a homologous desensitization of protein phosphorylation. Interestingly, PAF-pretreated platelets still exhibited phosphorylation of proteins by thrombin. On the other hand, a lack of protein phosphorylation by PAF or thrombin was observed in platelets preexposed to thrombin and this demonstrated a heterologous desensitization. It is concluded that phosphorylation of proteins by PAF is a PAF receptor-coupled event and that this process is desensitized in platelets preexposed to PAF. The fact that both the activation of phosphoinositide-specific phospholipase C and the phosphorylation of proteins are desensitized in PAF-pretreated platelets suggests that a close "regulatory" intercommunication between these processes exists.

PAF, an ether phospholipid, is a potent platelet-activating agent (1–3). One of the earliest events associated with PAF interaction with platelets is phosphoinositide-specific PLC-mediated production of inositol triphosphate (4, 5). This process is coupled to PAF receptor binding, inasmuch as PAF-specific receptor antagonists inhibit it.[1] Phosphorylation of cellular proteins has been suggested to be an important mechanism for signal transmission in various types of stimulus-response coupling (6, 7). In the case of phosphoinositide turnover, the diglyceride activates protein kinase C (8–10), which phosphorylates several proteins. For example, in platelets, the M_r 40,000 protein is rapidly phosphorylated in this manner (11).

Recently, we established that pretreatment of rabbit platelets with PAF caused homologous desensitization of PAF receptor-coupled activation of PLC (12). However, PAF-desensitized platelets still generated IP_3 when stimulated with thrombin. On the contrary, thrombin pretreatment caused heterologous desensitization of PLC to both PAF and thrombin. Interestingly, human platelets also display desensitization of thrombin receptor-mediated hydrolysis of phosphoinositides (13).

In our continuing objective to identify mechanism(s) coupling PAF receptor binding to stimulated PLC activity, we have investigated the phosphorylation of proteins under conditions in which PLC is desensitized to PAF. The results described here throw important light on the significance of protein phosphorylation in PAF desensitization.

Experimental Procedures

Materials. PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3 phosphorylcholine) was supplied by Bachem (Torrance, CA) and was routinely checked for purity by silica gel G thin layer chromatography, in a solvent mixture of chloroform/methanol/water (65:35:6, v/v). Thrombin and PMA were supplied by Sigma Chemical Co. (St. Louis, MO) and LC Services (Woburn, MA), respectively. Carrier-free ^{32}P was bought from ICN Biomedicals (Costa Mesa, CA). All other chemicals and solvents used were of analytical reagent grade.

Preparation of platelets and labeling with ^{32}P. Freshly drawn rabbit blood was used to prepare platelets by methods described before

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[1] Morrison, W. J., and S. D. Shukla. Antagonism of platelet activating factor receptor binding and stimulated phosphoinositide specific phospholipase C in rabbit platelets. Submitted for publication.

ABBREVIATIONS: PAF, platelet-activating factor; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,-*N*,-*N*′,-*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PKC, protein kinase C.

(4, 5). Washed rabbit platelets (1.5×10^9 cells/ml of Tyrode's gelatin buffer, pH 6.5, containing 0.1 mM EGTA) were labeled with ^{32}P (100 μCi/ml) for 1 hr at 37°. The cells were centrifuged at $850 \times g$ for 15 min and suspended in Tyrode's gelatin buffer, pH 7.2, containing 0.1 mM EGTA, at a concentration of 0.75×10^9 cells/ml. These platelets were used immediately.

Protein phosphorylation experiments. Phosphorylation of proteins was monitored under various protocols by assaying the ^{32}P-radioactivity in polypeptide bands separated by SDS-PAGE. In general, 1 ml of ^{32}P-labeled platelets (0.75×10^9 cells), suspended in Tyrode's gelatin buffer, pH 7.2, containing 0.1 mM EGTA (i.e., no extracellular Ca^{2+}), were incubated in polypropylene test tubes with the appropriate concentration of the agent (see below), at 37° in duplicate. The reactions were terminated with 0.1 ml of 50% (w/v) trichloroacetic acid after desired time periods. The mixture was centrifuged at $1500 \times g$ for 15 min. The supernatant was carefully aspirated out. The pellet was washed once with 5 ml of 5% (w/v) trichloroacetic acid by centrifugation, as above, and was solubilized with 0.2 ml of a cocktail medium containing glycerol, 2-mercaptoethanol bromophenol blue, and SDS (see Ref. 14). To each tube, 25 μl of 1 M Trizma base solution was added for neutralization. The tubes were then heated at 95° for 5 min to achieve solubilization.

PAGE. Solubilized samples were subjected to SDS-PAGE essentially by the method of Laemmli (14). The concentration of acrylamide was 10%. Standard samples of known molecular weight were run in parallel. The gels were stained with Coomassie blue, dried in a Bio-Rad gel dryer, and autoradiographed (6–8 hr) using Kodak XAR-2 films. The radioactive bands were cut out, transferred into vials, and counted with 10 ml of Budgetsolve cocktail (Research Products International, Mount Pleasant, IL) in a Beckman LS 7500 scintillation counter.

Results and Discussion

The following sections describe the results of experiments in which phosphorylation of proteins was studied in ^{32}P-labeled rabbit platelets treated with PAF, thrombin, or four PAF antagonists. The relevance of the protein phosphorylation to PAF receptor-coupled desensitization mechanisms are discussed.

Pattern of protein phosphorylation by PAF. Treatment of ^{32}P-labeled rabbit platelets with PAF (1×10^{-8} M) caused a time-dependent rapid phosphorylation of several proteins (see Figs. 1 and 4). In this study we monitored five major phosphorylated proteins with apparent molecular weights of 20,000, 35,000, 40,000, 65,000, and 150,000. The level of ^{32}P incorporation into these proteins showed a maximum at 10 sec and decreased thereafter to an elevated basal level by 5 min. In comparison, thrombin (1 unit/ml)-stimulated phosphorylation of proteins peaked at 10 to 15 sec and the radioactivity decreased rather slowly thereafter. With both PAF and thrombin, the decreased levels, at 5 min and 15 min, respectively, were always above the basal radioactivity (Fig. 1). Phosphorylation of proteins by (50μg/ml) PMA showed a peak at 2–5 min and remained elevated for 10–15 min. A gradual decrease in the radioactivity was observed after 15 min (results not shown). These results demonstrated that, in addition to the reported M_r 20,000 and 40,000 proteins (15, 16), PAF stimulated rapid phosphorylation of a number of other proteins, which was followed by a decrease in phosphorylation (i.e., dephosphorylation).

Effect of PAF antagonists. It was necessary to establish the specificity of the protein phosphorylation in relation to PAF receptor occupancy. This was investigated using four PAF receptor antagonists, CV-3988, CV-6209, SRI 63-441, and SRI

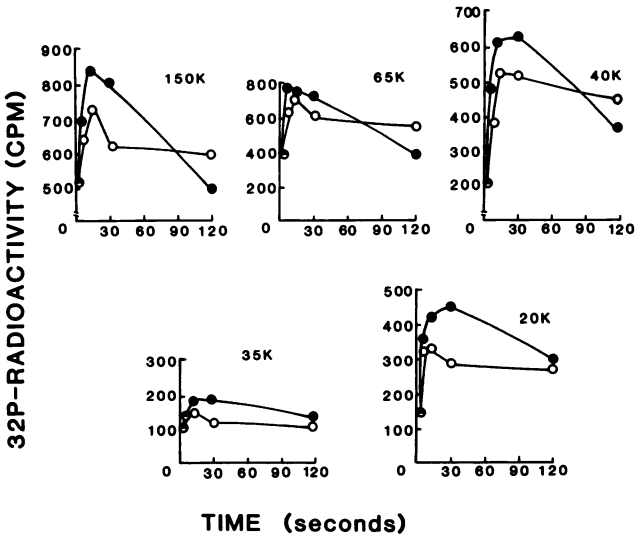

Fig. 1. Time course of protein phosphorylation by PAF. ^{32}P-labeled rabbit platelets were challenged with PAF (1×10^{-8} M) (●) or thrombin (1 unit/ml) (○) for different times and the radioactivity in different proteins was determined after SDS-PAGE by protocols described in Experimental Procedures. Results from one of four separate experiments, all of which showed similar patterns, are presented. Values in cpm represent those for 0.19×10^9 cells.

63-675. These antagonists themselves did not stimulate protein phosphorylation. Pretreatment of ^{32}P-labeled rabbit platelets with these antagonists drastically reduced the PAF-stimulated phosphorylation of M_r 20,000, 40,000, 65,000, and 150,000 proteins (Fig. 2). The overall order of potency was SRI 63-675 > SRI 63-441 ≥ CV-6209 > CV-3988. The phosphorylation of the M_r 35,000 protein by PAF was inhibited only 30–50% by CV-6209 or by the two SRI derivatives and less than 10% by CV-3988. The significance of this differential inhibition of the M_r 35,000 protein remains to be explained. It is of interest to note that these four antagonists have been observed to inhibit PAF-stimulated aggregation and specific [^3H]PAF binding (17, 18).[1] Experiments described here demonstrate that antagonists that inhibit PAF receptor binding also inhibited the phosphorylation and, thus, indicate that phosphorylation of proteins by PAF is mediated through the PAF receptor. Because both the PLC activation and protein phosphorylation are inhibited by these antagonists,[1] a relationship between these two processes is likely.

In another experiment, it was observed that treatment of ^{32}P-labeled platelets with either of the above four antagonists (10^{-5} M) at 37° for 5 min did not inhibit the phosphorylation of proteins by thrombin (1 unit/ml; results not shown). Thus, these antagonists inhibited the phosphorylation of protein by PAF but not that by thrombin and demonstrated a specificity for PAF.

Phosphorylation of proteins in platelets treated with PAF. The preceding section established that PAF stimulated protein phosphorylation in a time-, dose- (see Fig. 3), and receptor-specific manner. We next addressed the relevance of this process in the desensitization of platelets. To this end, a series of experiments was conducted based on protocols in which the desensitization of PAF receptor-coupled PLC has been previously observed (12).

^{32}P-labeled platelets were treated with 0.1 nM PAF for 10 min and then challenged with increasing concentrations of

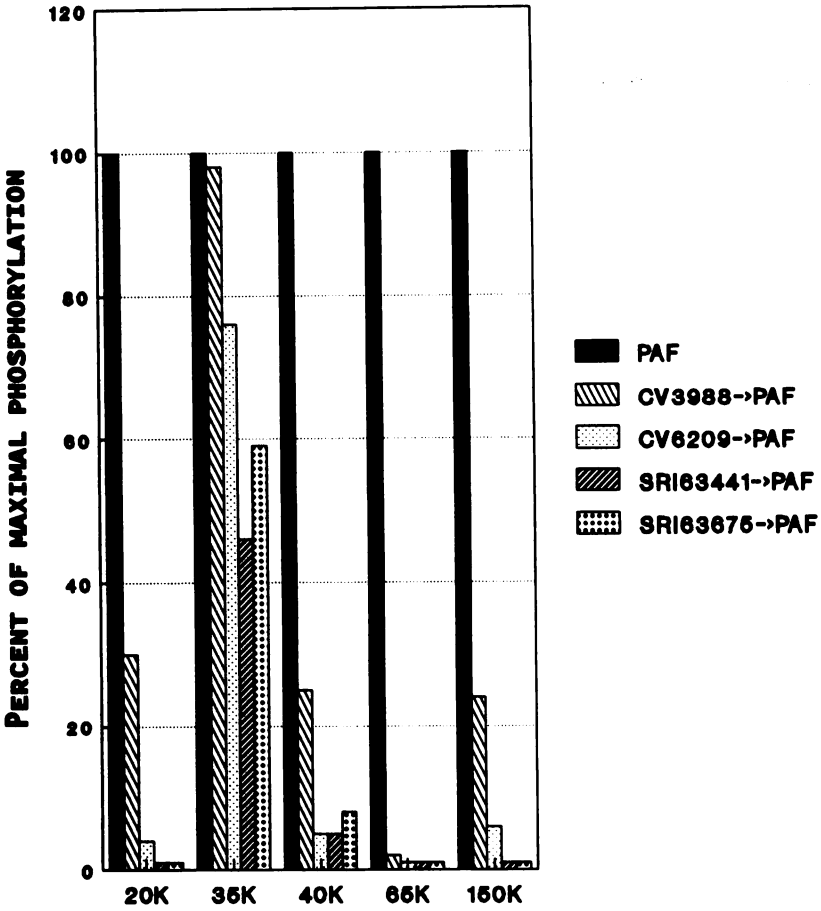

Fig. 2. Influence of PAF antagonists on protein phosphorylation. ^{32}P-labeled platelets were pretreated with antagonists (10^{-5} M, final) for 5 min at 37° and then challenged with PAF (10^{-8} M, final) for 10 sec. The phosphorylation of various proteins were monitored as described in Experimental Procedures. Each antagonist was dissolved in water and used fresh.

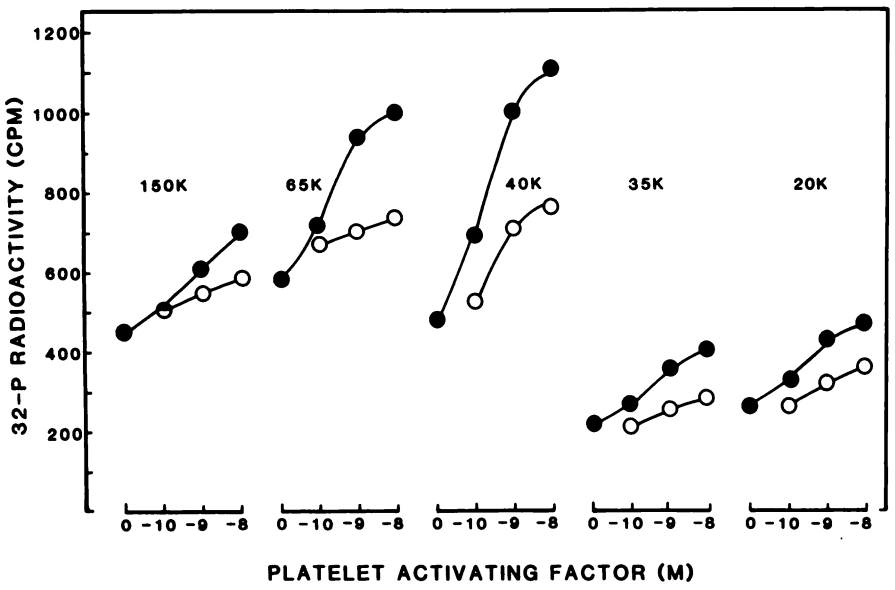

Fig. 3. Dose-related phosphorylation of proteins in platelets pretreated with PAF. ^{32}P-labeled platelets were treated with increasing concentrations of PAF for 10 sec (●) or with 10^{-10} M PAF for 10 min followed by different doses of PAF for 10 sec (○). The phosphorylation of proteins was assayed as described in Experimental Procedures. Values in cpm represent those for 0.19×10^9 cells.

PAF for 10 sec. It was observed that pretreatment with 0.1 nM PAF completely abolished any further stimulation of protein phosphorylation by the same concentration of PAF (Figs. 3 and 4). In control incubations, in which platelets were first treated with bovine serum albumin (instead of PAF) for 10 min, the phosphorylation response to PAF remained unaltered in these platelets. Further, pretreatment of platelets with PAF shifted the dose response of protein phosphorylation by about 2 log units to the right (i.e., higher concentration). Thus, the homologous desensitization of protein phosphorylation for the major phosphorylated proteins occurred. It is worth noting that preincubation of platelets with PAF has been shown to desensitize the aggregatory response (19–21). In another protocol, treatment of ^{32}P-labeled platelets with a concentration of PAF for 10 min followed by the same concentration of PAF for 10 sec or treatment of platelets with 10 nM PAF for 10 min followed by increasing concentrations of PAF for 10 sec showed almost a total lack of protein phosphorylation response. Pretreatment

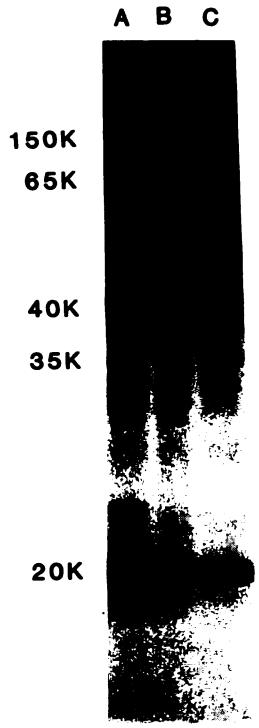

Fig. 4. Autoradiograph of phosphorylated protein in control and PAF-desensitized platelets. ^{32}P-labeled platelets were treated with PAF and their proteins were separated by SDS-PAGE and autoradiographed. The pattern of radioactive bands in the autoradiogram is shown. Apparent molecular weights of proteins are presented in thousands (*K*). The treatments were as follows: *A*, PAF (10 nM) 10 sec; *B*, PAF (10 nM) 10 min followed by PAF (10 nM) for 10 sec (desensitized); and *C*, control.

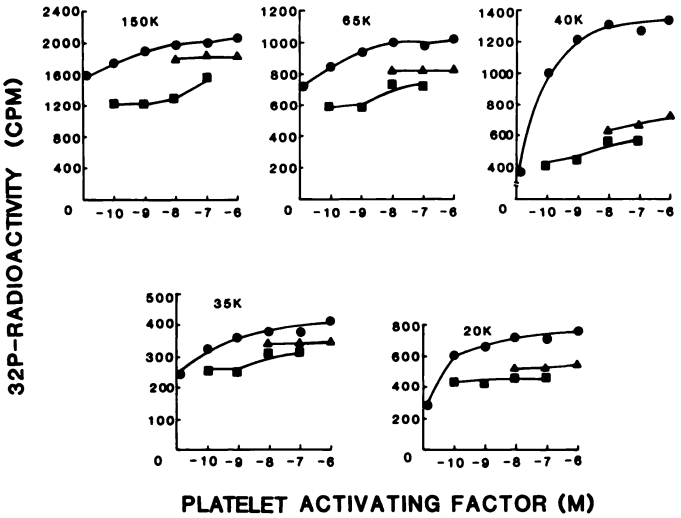

Fig. 5. Dose-related desensitization of protein phosphorylation. Rabbit platelets labeled with ^{32}P were treated either with different doses of PAF for 10 sec (●), with a concentration of PAF for 10 min followed by the same concentration of PAF for 10 sec (■), or with 10^{-8} M PAF for 10 min followed by different doses of PAF for 10 sec (▲). Phosphorylation of protein was determined employing SDS-PAGE and autoradiography followed by counting of radioactivity in protein bands (see Experimental Procedures).

of platelets with 10 nM PAF for 10 min abolished any further phosphorylation of protein by any concentration of PAF (Fig. 5).

Influence of thrombin and PMA on PAF-stimulated protein phosphorylation. The interrelationship between PAF-stimulated protein phosphorylation and that caused by thrombin or PMA was next investigated. ^{32}P-labeled platelets were treated with PAF, thrombin, or PMA (50μg/ml) for 5, 15, or 30 min, respectively, and then challenged with PAF (10 sec), thrombin (15 sec), or PMA (15 min) in various combinations. The time selected for pretreatments corresponded to the point at which phosphorylation had decreased close to the basal level (see Fig. 1). The time frame for the rechallenge was that in which a maximum phosphorylation response was observed. The results of these carefully selected sequential treatment protocols are summarized in Table 1. It was noted that, whereas pretreatment of platelets with PAF desensitized them to PAF (Fig. 1), the phosphorylation response for thrombin or PMA was still present. On the other hand, pretreatment of platelets with thrombin greatly diminished the PAF-stimulated phosphorylation (Table 1). Because the levels of ^{32}P-phosphorylated proteins remained high for a prolonged time after PMA pretreatment, any subsequent effect of PAF or thrombin was masked by such high levels of radioactivity (results not shown). Interestingly, PAF- or thrombin-pretreated platelets still showed an appreciable increase by PMA.

These results demonstrated that desensitization of protein phosphorylation by PAF was homologous in nature whereas that by thrombin was heterologous. It is relevant to note that the pattern of desensitization of phosphorylation responses is similar to that seen with desensitization of phosphoinositide-specific PLC (12). Phosphorylation of proteins by PAF and thrombin could be due to kinase activities including Ca^{2+}-calmodulin- and Ca^{2+}-phospholipid -dependent kinases. The mechanism by which PAF-desensitized cells are able to exhibit thrombin-stimulated protein phosphorylation is not known. The possibility of involvement of separate PKCs for PAF and thrombin exists. In this context, it is noteworthy that seven isoforms of PKC have been reported in the literature (22). The importance of PAF-stimulated rapid phosphorylation and dephosphorylation of proteins is also poorly understood. Based on the results presented here, its involvement in the desensitization process is obvious. Recently, it was elegantly demonstrated that agonist-induced phosphorylation of β_2-adrenergic receptors is involved in the mechanism of desensitization (23). In the case of platelet-derived growth factor, the occupancy of receptors appears to control the kinase activity of the receptor

TABLE 1

Desensitization of protein phosphorylation in platelets pretreated with thrombin

^{32}P-labeled rabbit platelets were treated with PAF (10^{-8} M) or thrombin (1 unit/ml) for 10 or 15 sec, respectively. In another set of experiments, platelets were treated with PAF (5 min) followed by thrombin (15 sec) or thrombin (5 min) followed by PAF (10 sec). The phosphorylation pattern of the *M*, of 40,000 protein was monitored (see Experimental Procedures). PAF at 10 nM and 1 unit/ml thrombin were used. Results are presented as mean ± standard deviation (number of experiments). *Arrows* indicate treatment with one agent followed by the other.

Incubation	Phosphorylation of *M*, 40,000 Protein
	% of control
Control	100
PAF, 10 sec	268 ± 48 (6)
Thrombin, 15 sec	230 ± 20 (6)
PAF, 5 min	128 ± 11 (3)
PAF, 5 min → thrombin, 15 sec	209 ± 67 (5)
Thrombin, 5 min	149 ± 10 (3)
Thrombin, 5 min → PAF, 10 sec	140 ± 12 (3)
Thrombin, 5 min → thrombin, 15 sec	146 ± 15 (3)

and the activity of phosphatase (24). Whether an analogous scenario for the association between the PAF receptor and a putative kinase exists would, therefore, be a matter of considerable interest.

In summary, it is concluded that phosphorylation of proteins by PAF is a PAF receptor-coupled process. This process is desensitized in platelets preexposed to PAF. Interestingly, phosphorylation of proteins by thrombin is operational in PAF-desensitized platelets. The fact that both the activation of phosphoinositide-specific PLC (12) and the phosphorylation of proteins are desensitized in platelets pretreated with PAF suggests that a close 'regulatory' intercommunication between these processes exists. The intricacies of this relationship together with the biochemical mechanism for the homologous and heterologous desensitization of protein phosphorylation awaits further investigation.

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