0026-895X/88/010058-06$02.00/0

MOLECULAR PHARMACOLOGY, 33:58–63

Desensitization of Receptor-Coupled Activation of Phosphoinositide-Specific Phospholipase C in Platelets: Evidence for Distinct Mechanisms for Platelet-Activating Factor and Thrombin

WILLIAM J. MORRISON and SHIVENDRA D. SHUKLA

Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

Received July 22, 1987; Accepted October 19, 1987

SUMMARY

Platelet-activating factor (PAF) receptor-coupled activation of phosphoinositide-specific phospholipase C (PLC) was studied in platelets that were made refractory, by short-term pretreatments, to either PAF or thrombin. Generation of [^{3}H]inositol triphosphate ([^{3}H]IP_3) was monitored specifically for this purpose. [^{3}H]Inositol-labeled rabbit platelets that were incubated (10 min) with increasing concentrations of PAF and subsequently challenged by the same concentration of PAF had greatly diminished PLC activity ([^{3}H]IP_3 production) as compared to controls. Platelets incubated (10 min) with fixed concentrations of PAF and then challenged with increasing concentrations of PAF had log-dose response curves of [^{3}H]IP_3 production progressively shifted to the right (i.e., to higher concentrations) and were depressed as the PAF pretreatment was increased from 0.5 to 10 nM. Platelets pretreated with 10 nM PAF became completely refractory to further PAF stimulation of PLC. Washing the pretreated platelets with either buffer or buffer containing 0.5% bovine serum albumin did not restore the PAF sensitivity. After the same pretreatments (i.e. 0.5–10 nM PAF for 10 min), platelets remained fully responsive to thrombin (2 units/ml)-stimulated production of [^{3}H]IP_3. Platelets pretreated with increasing concentrations of thrombin (0.15–2 units/ml) for different times (5–40 min) became refractory to both thrombin and PAF. It is concluded that PAF receptor-coupled activation of PLC becomes refractory (desensitized) in platelets preexposed to PAF, whereas platelets pretreated with thrombin are desensitized to both thrombin and PAF. It is proposed that thrombin has two transmembrane pathways leading to the activation of PLC, one shared with PAF and another utilizing separate mechanistic inputs.

Isolated rabbit platelets take up [^{3}H]inositol and incorporate it into membrane phosphoinositides. They produce [^{3}H]IP_1, [^{3}H]IP_2, [^{3}H]IP_3, and [^{3}H]IP_4 when stimulated by either PAF or thrombin. The primary mechanism involves activation of PLC and hydrolysis of the membrane PIP_2 to IP_3 and diacylglycerol (1, 2). The amount of IP_3 produced depends on the degree of PLC stimulation and on the availability of PIP_2 (2). PAF causes the hydrolysis of [^{3}H]PIP_2 and an immediate (5 sec) peak in [^{3}H]IP_3 which quickly decays to a level just above basal (3, 4). Thrombin causes prolonged hydrolysis of [^{3}H]PIP_2 and a gradual (2 min) rise in [^{3}H]IP_3 that later decreases toward the baseline to a level well above basal (3, 5).

Both PAF and thrombin cause rabbit platelets to aggregate (6, 7). PAF-induced aggregation becomes specifically desensitized (i.e., unresponsive) to PAF after being pretreated with subthreshold concentrations of PAF, but retains the capacity to react (aggregate) to other agents, including thrombin (6). Rabbit platelets pretreated with the PAF receptor antagonist CV-3988 and then washed retain the capacity to aggregate normally to PAF (7). Platelets pretreated with PAF and then washed become unresponsive (desensitized) to further PAF stimulation but retain the ability to respond to other agents (7). This type of specific PAF desensitization has been observed in human polymorphonuclear leukocytes (8) and in human platelets where the attenuated aggregatory response (desensitization) was associated with a shift in the number of high affinity PAF-binding sites to low affinity sites (9). Other studies using human platelets have demonstrated an actual loss of specific PAF-binding sites following pretreatments with higher concentrations of PAF (10).

Specific thrombin binding has been shown for platelets,

This work was supported by National Institutes of Health Grant 1RO1 DK 35170.

ABBREVIATIONS: IP_1, inositol monophosphate; IP_2, inositol diphosphate; IP_3, inositol triphosphate; IP_4, inositol tetraphosphate; BSA, bovine serum albumin; EC_{50}, effective concentration causing 50% activity; G_i, inhibitory guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PAF, platelet-activating factor; G_p, putative guanine nucleotide-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

although it was not essential for platelet activation (11). Platelets became refractory to thrombin-induced hydrolysis of phosphoinositide following 10–20 min of exposure to thrombin but maintained a PLC-sensitive pool of phosphoinositides and the ability to hydrolyze it when stimulated by collagen (5). Thrombin desensitization has also been demonstrated for ATP secretion in prostacyclin-inhibited platelets following brief exposure to low concentrations of thrombin (12). The studies mentioned show that platelets become desensitized to specific stimulatory agents but remain responsive to other agents and thereby regulate their response to external stimuli (i.e., maintain homeostasis). Although these end responses were at different levels (binding and aggregation for PAF; phosphoinositide hydrolysis and ATP secretion for thrombin), there were several intervening molecular interactions. Thus, the observed desensitization associated with these responses could be the result of changes at one or more points along the coupled stimulus-response pathway.

Several studies have shown that platelet PAF receptors couple to PLC through a putative guanine nucleotide-binding protein (G_p) that was insensitive to pertussis toxin and therefore separate from the toxin-sensitive guanine nucleotide-binding protein (G_i) (13, 14). Other studies have shown that PAF interacts with receptors that inhibit adenylate cyclase activity and is sensitive to pertussis toxin, thus indicating an interaction with G_i (15, 16). Many studies have shown that platelet thrombin receptors couple to PLC through pertussis toxin-insensitive G_p and to adenylate cyclase through toxin-sensitive G_i (17–21).

Earlier evidence obtained in this laboratory has shown that PAF and thrombin differentially activate PLC in rabbit platelets and that the stimulation of the PLC by these two stimuli causes IP_3 production via hydrolysis of a common pool of PIP_2 (3). Our research since then has focused on the exact point where the two stimulatory agents, PAF and thrombin, converge mechanistic inputs into rabbit platelet PLC. The question of whether PAF and thrombin use the same or different PLCs to hydrolyze PIP_2 has been approached but remains poorly understood. The objective of this study was to elucidate the regulatory features of PAF receptor-coupled activation of PLC using rabbit platelets which were made refractory (desensitized), by short-term pretreatment, to either PAF or thrombin.

Materials and Methods

PAF (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was supplied by Bachem (Torrance, CA) and contained a C16 alkyl chain. Purity of PAF was monitored by thin layer chromatography on silica gel G plates in a solvent mixture of chloroform/methanol/water (65:35:6, v/v). Thrombin was bought from Sigma Chemical Co. (St. Louis, MO) and had an activity of 1000 NIH units/ml. *myo*-[2-^{3}H] Inositol (specific radioactivity, 15 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Dowex-1 resins (AG 2-X8, 200–400 mesh) were obtained from Bio-Rad Laboratories (Richmond, 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 isolated essentially as described before (3, 4). Washed rabbit platelets, at a concentration of 1.5×10^9 cells/ml of Tyrode's-gelatin buffer, pH 6.5 (without Ca^{2+}), 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 PAF or thrombin. Washed rabbit platelets labeled with [^{3}H]inositol were stimulated with PAF or thrombin under a variety of experimental protocols as described in the text. All of the incubations were conducted in polypropylene test tubes. Unless otherwise stated, 2 ml of [^{3}H]inositol-labeled platelets, 3×10^8 cells/ml of Tyrode's-gelatin buffer (pH 7.2) containing 0.1 mM EGTA were stimulated with specific concentrations of PAF (dissolved in 150 mM NaCl containing BSA, 2.5 mg/ml) or thrombin for selected time periods. The incubations were conducted at 37°, stopped with 0.1 ml of perchloric acid (70%, w/v), and mixed rapidly. The mixture was centrifuged at 1000 × *g* for 5 min, the pellet was retained for lipid extraction, and the supernatant was neutralized with a solution of 2.0 M KOH (prepared in 75 mM Hepes). The suspension was kept at 4° until the resulting $KClO_4$ precipitate was discarded by centrifugation and the supernatant was collected and analyzed for radioactive inositol polyphosphate contents.

Analysis of inositol polyphosphates. Separation of IP_1, IP_2, IP_3, and IP_4 was based on Dowex-1 chromatography of the samples as described by Shukla *et al.* (3) with the following modifications: 1.5 cm of Dowex was used instead of 5 cm, and this allowed for complete separation of all phosphoinositols without elution overlap. Aliquots (5 ml) of each fraction were mixed with 10 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.

Washing of pretreated platelets. Platelets pretreated with 10 nM PAF were washed with 2 volumes of Tyrode's-gelatin buffer, pH 7.2, containing 0.1 mM EGTA and, in one experiment, 0.5% BSA. The suspension was then centrifuged at 830 × *g* for 15 min at 24°. The resulting pellet was resuspended in Tyrode's-gelatin-EGTA buffer, pH 7.2, to give a final concentration of 3×10^8 cells/ml. The platelets were then rechallenged with increasing concentration of PAF for 5 sec. The phosphoinositols and phosphoinositides were analyzed by the methods described above.

Extraction and analysis of phosphoinositides. The 1000 × *g* pellet obtained after stimulating the platelets with PAF or thrombin was suspended in 980 μl of H_2O, then mixed with 3.75 ml of chloroform/methanol/12 N HCl (200:400:1.6, v/v) and allowed to stand at room temperature for 20 min. This was followed by the addition of 1.25 ml of chloroform and 1.2 ml of water. After mixing, the mixture was centrifuged at 1000 × *g* for 5 min. A one-ml aliquot was then taken from the lower phase and dried under N_2 at 37°. The dried lipids were resuspended in 65 μl of chloroform/methanol (1:1, v/v), layered onto a high performance thin layer chromatography plate, and developed in a solvent mixture containing chloroform/methanol/20% methylamine (60:36:10, v/v). Phospholipids were identified by visualization under ultraviolet light after spraying with 1-toluidino-2-naphthelene-sulfonic acid (1 mM). The bands were scraped, added to 10 ml of Budget-Solve liquid scintillation cocktail, and counted in a Beckman LS-7500 scintillation counter.

Results

The following sections describe the experimental results after platelets were: (i) pretreated with PAF and then rechallenged with the same or increasing concentrations of PAF; (ii) pretreated with PAF and then rechallenged with a fixed concentration of thrombin; (iii) pretreated with thrombin and then rechallenged with increasing concentrations of PAF; and (iv) pretreated with thrombin and rechallenged with increasing concentrations of thrombin. Essentially, two experimental protocols were followed.

First, washed rabbit platelets labeled with [^{3}H]inositol were pretreated with various concentrations of PAF for 10 min at 37° and were thus desensitized. Subsequent to this 10-min pretreatment, cells were rechallenged with selected and increas-

ing concentrations of PAF for 5 sec or with thrombin (2 units/ml) for 2 min. The lipid precursor phosphoinositides, PIP_2, phosphatidylinositol-4-phosphate, and PI, and the products of phosphoinositide turnover (i.e., PLC activity), namely, the water-soluble inositol phosphates, IP_1, IP_2, IP_3, and IP_4, were monitored.

In another series of experiments, platelets were exposed to various concentrations of thrombin for 20 min at 37° and were thus desensitized. Subsequent to this 20-min pretreatment, cells were rechallenged with selected and increasing concentrations of PAF for 5 sec or with increasing concentrations of thrombin for 2 min. The precursors and products of phosphoinositide turnover (i.e., PLC activity) were then monitored. It should be noted that PAF and thrombin cause a peak rise in IP_3 at 5 sec and 2 min, respectively (3, 4), and, therefore, these time frames were utilized for the rechallenge protocol.

Pretreatment with PAF. Fig. 1 shows that the hydrolysis of [^{3}H]PIP_2 by increasing concentrations of PAF causes a dose-dependent production of [^{3}H]IP_3. [^{3}H]IP_3 levels increased 100% after 0.1 nM PAF and 700% after 100 nM PAF. [^{3}H]IP_3 levels increased from 20% to 80% over basal after platelets were pretreated with PAF for 10 min and then rechallenged with the same concentration of PAF for 5 sec. [^{3}H]IP_2 production reflected the changes seen with [^{3}H]IP_3, whereas [^{3}H]IP_1 and [^{3}H]IP_4 levels showed little change in either the untreated or PAF-pretreated platelets. The question of whether the time frame for peak rise in IP_3, subsequent to pretreatment and rechallenge, had been shifted to longer times was answered by assaying [^{3}H]IP_3 production from 5 to 120 sec, and no such shift was found (data not shown). The results demonstrated that the cell's PLC became desensitized to further stimulation by PAF.

Similar results were obtained when platelets were incubated with fixed concentrations of PAF and then challenged with increasing concentrations of PAF. [^{3}H]IP_3 levels were expressed as -fold increase over basal since amounts of the PIP_2 labeled with [^{3}H]inositol in the platelet plasma membrane varied somewhat among experiments. Significant increases in [^{3}H]IP_3 were seen after challenging the platelets with 0.5 nM PAF for 5 sec. Significant reductions in [^{3}H]IP_3 production were seen after pretreating platelets with 0.5 nM PAF for 10 min and then rechallenging with increasing concentrations of PAF for 5 sec. Fig. 2 shows that PAF-induced desensitization was dose dependent. The efficacy of PAF decreased approximately 50% and its EC_{50} value nearly doubled after platelets had been pretreated with 0.5 nM PAF for 10 min. The EC_{50} value increased 10-fold after cells were pretreated with 5 nM PAF (10 min), whereas the efficacy decreased another 10%. After pretreating the cells with 10 nM PAF for 10 min, the stimulated [^{3}H]IP_3 production was completely abolished.

[^{3}H]PIP_2 levels decreased after platelets were challenged with increasing concentrations of PAF for 5 sec (Fig. 3). The maximum decrease was approximately 30–40% after being challenged with 1 nM PAF for 5 sec. Basal levels of [^{3}H]PIP_2 increased from 20% to 80% after the cells were pretreated with increasing concentrations of PAF for 10 min and then rechallenged with the same concentration of PAF. Similar patterns were seen in phosphatidylinositol-4-phosphate labeling in control and pretreated platelets. Large decreases were seen in [^{3}H]PI in cells that had been challenged with increasing concentrations of PAF for 5 sec, whereas little change was observed under the same conditions in the PAF-pretreated platelets.

The question of whether PAF was blocking the receptor and thereby preventing activation of PLC was approached by washing the platelets after they were pretreated with PAF. Half of the pretreated platelets and half of the control platelets were washed twice in Tyrode's buffer prior to challenging with PAF. Both washed and unwashed control platelets showed dose-dependent increases in [^{3}H]IP_3. Pretreated platelets that had been exposed to 10 nM PAF for 10 min were completely refractory to further PAF stimulation. The same results were obtained after the pretreated platelets were washed with Tyrode's

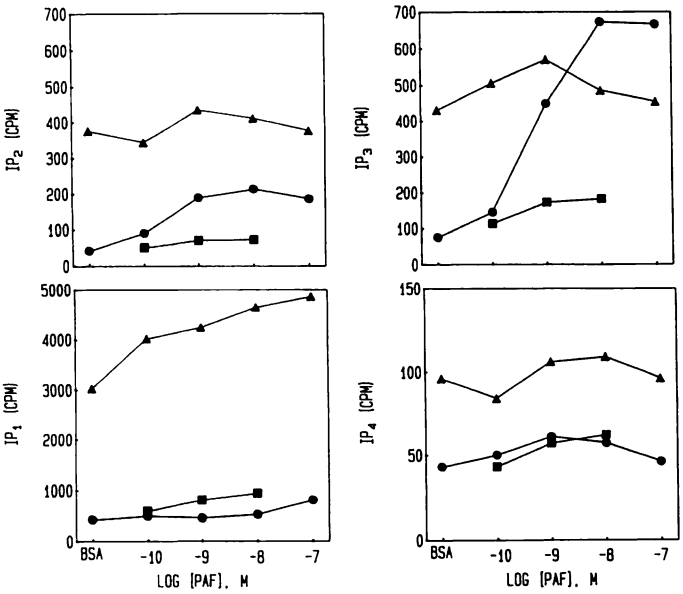

Fig. 1. PAF and thrombin-stimulated inositol phosphate production in rabbit platelets pretreated with PAF. Platelets (3×10^8/ml) were prelabeled with [^{3}H]inositol (see Materials and Methods) and then challenged with either PAF or thrombin. (●), 5-sec response to increasing concentrations of PAF; (■), platelets were pretreated with PAF for 10 min at 37° and then rechallenged with the same concentration of PAF for 5 sec; (▲), platelets were pretreated with the concentrations of PAF shown for 10 min at 37° and then rechallenged with thrombin (2 units/ml) for 2 min at 37°. Inositol phosphates were isolated by column chromatography and their radioactivity was measured by liquid scintillation counting (see Materials and Methods).

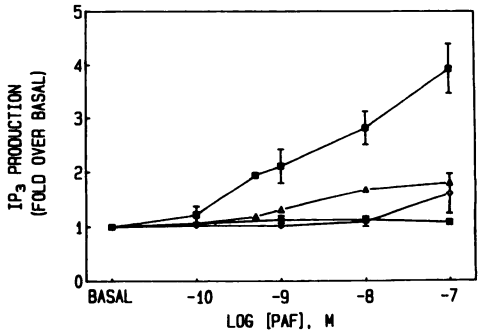

Fig. 2. Dose-dependent desensitization of PAF-stimulated [^{3}H]IP_3 production in rabbit platelets. [^{3}H]Inositol-prelabeled platelets (3×10^8/ml) were pretreated with 0 (■), 0.5 nM (△), 5 nM (◇), or 10 nM (●) PAF for 10 min at 37° and then challenged with increasing concentrations of PAF for 5 sec. [^{3}H]IP_3 was measured as described above and then normalized to basal production in the presence of 0.25% BSA in saline which was used as the carrier for PAF. Values are presented as the mean ± standard error (*bars*) and the number of separate experiments were 7, 2, 3, and 4 for 0, 0.5 nM, 5 nM, and 10 nM concentrations, respectively.

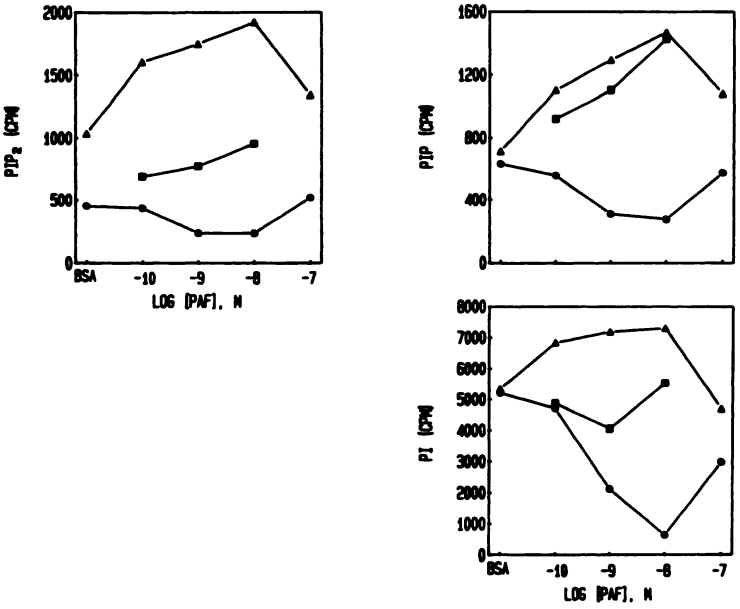

Fig. 3. PAF and thrombin-stimulated [^{3}H]inositol labeling of phosphoinositides in rabbit platelets pretreated with PAF. Platelets (3×10^8/ml) were prelabeled with [^{3}H]inositol and challenged as outlined in Fig. 1. Lipids were extracted and analyzed by thin layer chromatography and radioactivity was measured by liquid scintillation counting (see Materials and Methods). Descriptions of symbols are identical to those in Fig. 1.

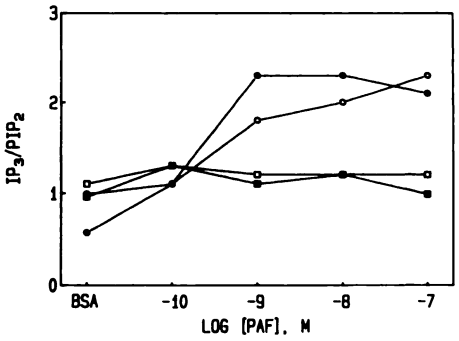

Fig. 4. PAF-stimulated [^{3}H]IP_3 production in control and pretreated cells after washing. Platelets (3×10^8/ml) were pretreated with buffer (control) or 10 nM PAF for 10 min at 37°. Half of the suspension was then washed with Tyrode's-gelatin buffer, pH 7.2 (see Materials and Methods for details) and rechallenged with different concentrations of PAF. ●, 5-sec response to PAF without washing; ○, 5-sec response to PAF after washing; ■, 5-sec response to PAF after platelets had been pretreated with 10 nM PAF for 10 min at 37°; □, 5-sec response to PAF after platelets had been pretreated with 10 nM PAF for 10 min at 37° and then washed at 25°.

buffer containing 0.5% BSA, a procedure that is believed to remove bound PAF (22) (data not shown). [^{3}H]IP_3 levels were normalized to the amount of [^{3}H]PIP_2 present in the membranes for an accurate comparison between the washed and unwashed cells. In both the washed and unwashed treatments, the stimulated PLC activity was completely abolished following 10-min pretreatments with 10 nM PAF (see Fig. 4).

In contrast to the above situation, PLC activity of the PAF-pretreated platelet was fully responsive to thrombin (2 units/ml for 2 min). Thrombin raised [^{3}H]IP_3 levels about 600% both before and after the 10-min PAF pretreatments (see Fig. 1). A similar pattern was observed for [^{3}H]IP_2 production. Thrombin (2 units/ml, 2 min) alone raised [^{3}H]IP_2 levels 800% and increased the level to 900% after the PAF pretreatments described above. The levels of [^{3}H]IP_1 were raised the most by thrombin, whereas only thrombin appeared to have any affect at all on [^{3}H]IP_4 production.

Thrombin elevated the levels of all the [^{3}H]phosphoinositides (see Fig. 3). Thrombin (2 units/ml for 2 min) alone raised the [^{3}H]PIP_2 levels 100% over control. The labeling increased another 100% as the PAF pretreatment concentration was increased to 10 nM and then decreased by 40% following pretreatment with 100 nM PAF for 10 min. PIP labeling produced by thrombin, after PAF pretreatment, was similar to that produced by PAF after the pretreatment. A small increase was seen in PI labeling when cells were rechallenged with thrombin after the PAF pretreatment. In each case, the increased radioactivity in phosphoinositides after pretreatments is probably due to the accelerated biosynthesis associated with the turnover of these lipids.

The results thus far showed that platelets pretreated with PAF became refractory (desensitized) to PAF stimulation of PLC but not to that by thrombin. They further showed that this desensitization had a threshold PAF concentration that was less than 0.5 nM for 10 min. As expected, the initial 5-sec stimulation of PLC by PAF caused [^{3}H]phosphoinositide levels to decrease, whereas longer stimulation of PLC, by PAF or thrombin, caused extensive [^{3}H]phosphoinositide turnover and increased phosphoinositide labeling.

Pretreatment with thrombin. Increasing concentrations of thrombin caused hydrolysis of [^{3}H]PIP_2 and a dose-dependent production of [^{3}H]IP_3 (Fig. 5A). [^{3}H]IP_3 levels increased 225% after being challenged with 0.15 unit/ml thrombin for 2 min. With higher concentrations of thrombin, 0.3 unit/ml for 2 min, the level of [^{3}H]IP_3 approaches 300% over control and does not increase thereafter. This response was abolished in cells pretreated with thrombin at 0.3 unit/ml for 20 min (Fig. 5A). Cells pretreated with 0.15 unit/ml thrombin for 20 min were just as refractory to rechallenge with thrombin as those cells pretreated with 2 units/ml thrombin for 20 min, and cells pretreated with 1 unit/ml thrombin for 5 min were just as

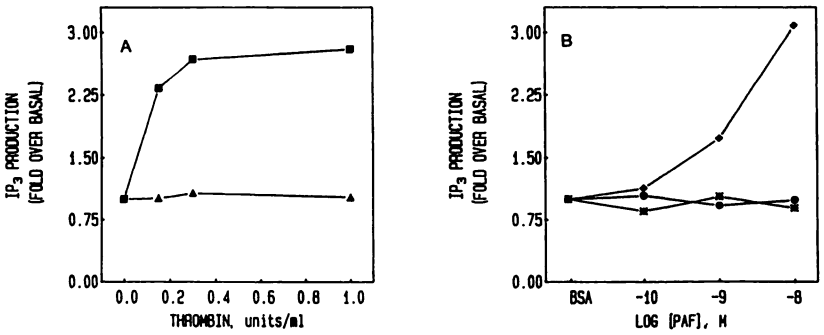

Fig. 5. [^{3}H]IP_3 production in cells pretreated with thrombin. A. [^{3}H]Inositol-labeled platelets (3×10^8/ml) were challenged with increasing concentrations of thrombin (■) for 2 min or with 0.3 unit/ml thrombin for 20 min and then rechallenged with increasing concentrations of thrombin for 2 min (▲). B. [^{3}H]Inositol-labeled platelets (3×10^8/ml) were challenged with increasing concentrations of PAF (◆) for 5 sec. In another set of incubations, platelets were pretreated with 0.3 unit/ml thrombin for 20 min (●) or 40 min (*) and then rechallenged with increasing concentrations of PAF for 5 sec.

refractory as cells pretreated with 1 unit/ml for 40 min (data not shown). The labeling of phosphoinositides remained high after all the thrombin pretreatments and increased with concentration of thrombin used (data not shown).

Fig. 5B shows that cells pretreated with thrombin, 0.3 unit/ml for 20 or 40 min, were refractory to rechallenge with increasing concentrations of PAF for 5 sec. The same results were seen after preincubations for 40 min with both higher (1 or 2 units/ml) and lower (0.15 unit/ml) concentrations of thrombin (data not shown). It should be pointed out that basal levels of [^{3}H]IP_3 were slightly elevated, over control, after all pretreatments and perhaps represented the residual IP_3 undergoing slow degradation after the peak rise. The elevated basal level of [^{3}H]IP_3 was once again dependent on the concentration of thrombin and on the length of time used (data not shown). These results demonstrated that the cell's PLC become desensitized to further stimulation by thrombin or PAF after it had been pretreated with various concentrations of thrombin (0.15–2 units/ml) for different times (5–40 min).

Discussion

Our results demonstrated that the activation of phosphoinositide-specific PLC in rabbit platelets becomes refractory to further stimulation after pretreatments with either PAF or thrombin. The thrombin-mediated pathway was affected by thrombin but not by PAF pretreatment and indicates homologous desensitization of mechanistic inputs associated with a single receptor-mediated pathway. The PAF-mediated pathway was affected by pretreatments with both PAF and thrombin, thus indicating heterologous desensitization where the distal effector system (PLC) becomes refractory following stimulation through two distinct receptor-activated mechanisms (see Ref. 14 for review).

Receptor down-regulation of receptor numbers could have occurred during homologous desensitization but probably not during thrombin's heterologous desensitization of PAF-stimulated PLC activation. Recent studies have shown that three specific PAF receptor antagonists had no influence on thrombin-stimulated aggregation in platelets that had been made refractory to PAF-stimulated aggregation (23). This suggests that thrombin receptors do not accommodate the same chemical structure recognized by the PAF receptor and thereby implies that thrombin does not interact with the PAF receptor. Thrombin could have indirectly altered PAF receptor recognition and binding by changing the physicochemical organization of the PAF receptor on (or in) the platelet's plasma membrane as well as other receptor-coupled events.

The receptor presumably has a site that interacts directly with distal effector systems. Receptors can become uncoupled from these effectors through modification of the interaction site on the receptor or on the effector. Several studies have shown that desensitization follows receptor phosphorylation that occurs after short-term pretreatment with an agonist (14). Other studies have shown that desensitization follows changes in the guanine nucleotide-regulatory protein population ratios (G_i/G_s) (24) and that the guanine nucleotide-binding protein itself might be the site of agonist-induced phosphorylation (25, 26).

Guanine nucleotide-binding proteins have been implicated in the regulation of PLC activity (27). It has been suggested that thrombin exerts effects through both the inhibitory guanine nucleotide protein, G_i, and the putative guanine nucleotide protein, G_p (17, 20). PAF also exerts effects through G_i (15); however, platelet GTPase activity associated with PAF stimulation was only slightly attenuated by treatment with pertussis toxin (13) and indicates that G_p might be the predominant guanine nucleotide-binding protein mediating PAF's signal. Differential coupling to a heterogenous population of guanine nucleotide-binding proteins has been suggested for other cell types (25, 27), and, although this could explain thrombin's dual pathway, the possibility of separate PLCs (isozymes) also ex-

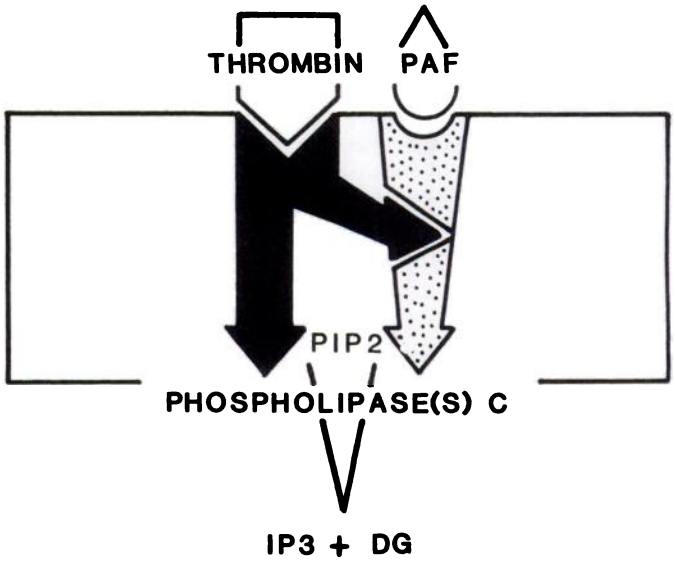

Fig. 6. Schematic representation of the transmembrane pathways used by PAF and thrombin in relation to the activation of PLC. Thrombin has pathways (*solid arrows*) that branch from its receptor to interact with both PLC and the pathway used by PAF (*dotted arrow*). According to this scheme, the transmembrane mechanism of PAF becomes desensitized following short-term stimulation by either PAF or thrombin, whereas thrombin's mechanism becomes desensitized only after short-term exposure to thrombin. *DG*, diacylglycerol.

ists. Both cytosolic and particulate PLC isozymes have been isolated from platelets and both are capable of hydrolyzing PIP_2 (28–30). The particulate species has enhanced affinity for PIP_2, whereas the cytosolic species shows a preference for PI in addition to having higher Ca^{2+} requirements (28, 30). If PAF preferentially used one of these while thrombin used both, then thrombin could continue to activate PLC while the PAF mechanism was inoperative.

Although the exact mechanism for desensitization remains to be defined at this stage, it was obvious that it was not due to substrate (PIP_2) depletion since thrombin caused the hydrolysis of [^{3}H]PIP_2 after the PAF pretreatments. Washing experiments were designed to remove any bound PAF which might have been blocking the receptor and affecting the platelets' response to subsequent challenge by PAF. The results demonstrated that washing of PAF-pretreated platelets with Tyrode's buffer (with and without 0.5% BSA) could not restore the sensitivity of platelets to PAF. However, it remains to be ascertained whether receptor blockade was responsible for PAF-induced desensitization of PAF-stimulated PLC activity.

It is relevant to mention that desensitization of agonist-stimulated Ca^{2+} efflux in 1321 astrocytoma cells occurs after cells have been exposed to carbachol for 75 min and leaves phosphoinositide hydrolysis intact, whereas longer exposure (150 min) leads to a loss of phosphoinositide hydrolysis and muscarinic receptors (31). All of the changes mentioned leave the more distal responses refractory to further stimulation by carbachol. By analogy, thrombin's activation of PLC, which is temporally distinct from PAF, may leave the platelets' PLC refractory to other (e.g., PAF) receptor-coupled stimulation if changes were at a common convergent effector of PLC.

In summary, these studies have established that, in rabbit platelets, the receptor activation of phosphoinositide-specific PLC (which results in production of second messengers IP_3 and diacylglycerol) become refractory (desensitized) to further stimulation following short-term (min) exposure to either PAF or thrombin. Interestingly, platelets which were totally desensitized to PAF by pretreatment with these stimuli exhibited a normal stimulation of PLC by thrombin. Conversely, platelets pretreated with thrombin were desensitized to both thrombin and PAF. We therefore propose a scheme involving two pathways which branch from thrombin, with one branch overlapping the pathway used by PAF (see Fig. 6) and suggest that the platelet's PAF refractory state results from a mechanistic uncoupling between the PAF receptor and PLC.

Acknowledgments

We wish to thank Lindy Corkins for typing the manuscript. This work was supported by NIH grant 1R01 DK 35170.

References

1. Abdel-Latif, A. A. Calcium-mobilizing receptors, phosphoinositides, and the generation of second messengers. *Pharmacol. Rev.* **38**:246–272 (1986).
2. Vickers, J. D., and J. F. Mustard. The phosphoinositides exist in multiple metabolic pools in rabbit platelets. *Biochem. J.* **238**:411–417 (1986).
3. Shukla, S. D., C. G. Franklin, and M. G. Carter. Activation of phospholipase C in platelets by platelet activating factor and thrombin causes hydrolysis of a common pool of phosphatidylinositol-4,5-bisphosphate. *Biochim. Biophys. Acta* **929**:134–141 (1987).
4. Shukla, S. D. Platelet activating factor-stimulated formation of inositol triphosphate in platelets and its regulation by various agents including Ca^{2+}, indomethacin, CV-3988 and forskolin. *Arch. Biochem. Biophys.* **240**:674–681 (1985).
5. Huang, E. M., and T. C. Detwiler. Thrombin-induced phosphoinositide hydrolysis in platelets. *Biochem. J.* **242**:11–18 (1987).
6. Keraly, C. L., and J. Benveniste. Specific desensitization of rabbit platelets by platelet-activating factor (PAF-acether) and derivatives. *Br. J. Haematol.* **51**:313–322 (1982).
7. Tokumura, A., J.-I. Yoshida, N. Okasaka, K. Fukuzawa, and H. Tsukatani. Platelet aggregation induced by ether-linked phospholipids. 2. Mechanism of desensitization of rabbit platelets by platelet activating factor and reversibility of inhibitory actions of its antagonist. *Thromb. Res.* **46**:153–161 (1987).
8. O'Flaherty, N. T., C. J. Lees, C. H. Miller, C. E. McCall, J. C. Lewis, S. H. Love, and R. L. Wykle. Selective desensitization of neutrophils: further studies with 1-*O*-alkyl-*sn*-glycero-3-phosphocholine analogues. *J. Immunol.* **127**:731–737 (1981).
9. Chesney, C. M., D. D. Pifer, and K. M. Huch. Desensitization of human platelets by platelet activating factor. *Biochem. Biophys. Res. Commun.* **127**:24–30 (1985).
10. Valone, F. H., E. Coles, V. R. Reinhold, and E. J. Goetzl. Specific binding of phospholipid platelet-activating factor by human platelets. *J. Immunol.* **129**:1637–1641 (1982).
11. Detwiler, T. C., and E. B. McGowan. Platelet receptors for thrombin. *Adv. Exp. Med. Biol.* **192**:15–28 (1985).
12. McGowan, E. B., and T. C. Detwiler. Characterization of the thrombin-induced desensitization of platelet activation by thrombin. *Thromb. Res.* **31**:297–304 (1983).
13. Houslay, M. D., D. Bojanic, and A. Wilson. Platelet activating factor and U44069 stimulate a GTPase activity in human platelets which is distinct from the guanine nucleotide regulatory proteins, N_s and N_i. *Biochem. J.* **234**:737–740 (1986).
14. Sibley, D. R., P. Nambi, and R. J. Lefkowitz. Molecular mechanisms of hormone receptor desensitization, in *Molecular Mechanisms of Transmembrane Signalling*. (P. Cohen and M.D. Houslay, eds.), Vol. 4. Elsevier/North-Holland, New York, 359–374 (1985).
15. Williams, K. A., and R. J. Haslam. Effects of NaCl and GTP on the inhibition of platelet adenylate cyclase by 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycerol-3-phosphocholine (synthetic platelet-activating factor). *Biochim. Biophys. Acta* **770**:216–223 (1984).
16. Taylor, C. W., and J. E. Merritt. Receptor coupling to phosphoinositide turnover: a parallel with the adenylate cyclase system. *Trends Pharmacol. Sci.* **7**:238–242, (1983).
17. Brass, L. F., M. Laposata, H. S. Banga, and S. E. Rittenhouse. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide binding protein. *J. Biol. Chem.* **261**:16838–16847 (1986).
18. Hrbolich, J. K., M. Culty, and R. J. Haslam. Activation of phospholipase C associated with isolated rabbit platelet membranes by guanosine 5′-[γ-thio] triphosphate and by thrombin in the presence of GTP. *Biochem. J.* **243**:457–465 (1987).
19. Haslam, R. J., and M. L. Davidson. Guanine nucleotides decrease the free [Ca^{2+}] required for secretion of serotonin from premeabilized blood platelets. Evidence of a role for a GTP-binding protein in platelet activation. *FEBS Lett.* **174**:90–95 (1984).
20. Houslay, M. D., D. Bojanic, D. Gawler, and A. Wilson. Thrombin, unlike vasopressin, appears to stimulate two distinct guanine nucleotide regulatory proteins in human platelets. *Biochem. J.* **238**:109–113 (1986).
21. Shattil, S. J., and L. F. Brass. Induction of the fibrinogen receptor on human platelets by intracellular mediators. *J. Biol. Chem.* **262**:992–1000 (1987).
22. Homma, H., A. Tokumura, and D. J. Hanahan. Binding and internalization of platelet-activating factor 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine in washed rabbit platelets. *J. Biol. Chem.* **262**:10582–10587 (1987).
23. Adnot, S., D. Joseph, and B. B. Vargaftig. Antagonist of PAF-acether do not suppress thrombin-induced aggregation of ADP-deprived and aspirin-treated human platelets. *Agents Actions* **21**:195–202 (1987).
24. Parsons, W. J., and G. L. Stiles. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes. *J. Biol. Chem.* **262**:841–847 (1987).
25. Houslay, M. D. Ion channels controlled by guanine nucleotide regulatory proteins. *Trends Biochem. Sci.* **12**:167–168 (1987).
26. Murphy, G. J., V. J. Hruby, D. Trivedi, M. J. O. Wakelan, and M. D. Houslay. The rapid desensitization of glucagon-stimulated adenylate cyclase in a cyclic AMP-independent process that can be mimicked by hormones which stimulated inositol phospholipid metabolism. *Biochem. J.* **243**:39–46 (1987).
27. Cockcroft, S. Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, G_p. *Trends Biochem. Sci.* **12**:75–77 (1987).
28. Rittenhouse, S. E. Human platelets contain phospholipase C that hydrolyzes phosphoinositides. *Proc. Natl. Acad. Sci. USA* **80**:5417–5420 (1983).
29. Low, M. G., R. C. Carroll, and A. C. Cox. Characterization of multiple forms of phosphoinositide-specific phospholipase C purified from human platelets. *Biochem. J.* **237**:139–145 (1986).
30. Manne, V., and H.-F. Kung. Characterization of phosphoinositide-specific phospholipase C from human platelets. *Biochem. J.* **243**:763–771 (1987).
31. Masters, S. B., M. T. Quinn, and J. H. Brown. Agonist-induced desensitization of muscarinic receptor-mediated calcium efflux without concomitant desensitization of phosphoinositide hydrolysis. *Mol. Pharmacol.* **27**:325–332 (1985).

Send reprint requests to: Dr. Shivendra D. Shukla, Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, MO 65212.