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Platelet-activating factor stimulates metabolism of phosphoinositides in horse platelets: Possible relationship to Ca^{2+} mobilization during stimulation

(phospholipase C/protein kinase C/phosphatidic acid/lysophosphatidylinositol/prostacyclin)

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ABSTRACT **Stimulation of horse platelets with platelet-activating factor (PAF) induces a rapid degradation of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. Addition of 0.1 μM PAF for 5 sec to platelets prelabeled with ^{32}P induces a 50% loss of [^{32}P]PtdIns(4,5)P_2. ^{32}P-Labeled phosphatidylinositol 4-monophosphate (PtdIns4P) and [^{32}P]phosphatidylinositol (PtdIns) also are decreased, albeit at a slower rate. Loss of ^{32}P radioactivity correlates with a net loss of fatty acids from both polyphosphoinositides. Stimulation of platelets with PAF also produces formation of [^{32}P]phosphatidic acid and [^{32}P]lysophosphatidylinositol. The initial disappearance of inositol lipids is subsequently followed by resynthesis, as evidenced by increased incorporation of ^{32}P into PtdIns(4,5)P_2, PtdIns4P, and PtdIns. The resynthesis of the inositides increases with time and is proportional to the concentration of PAF. Prostacyclin (1 μM) inhibits (*i*) the formation of phosphatidic acid and lysophosphatidylinositol and (*ii*) the resynthesis of polyphosphoinositides induced by 0.03 μM PAF without affecting the initial loss of PtdIns(4,5)P_2. The loss of inositol lipids appears to be a primary event of platelet activation. The initial loss of polyphosphoinositides might be linked to the initiation of cellular activation by mobilizing membrane-bound Ca^{2+}, whereas the subsequent formation of these lipids might be involved in mechanisms to prevent overstimulation of the cell.**

Platelet-activating factor (PAF), a phospholipid mediator of anaphylaxis (1), is released from leucocytes after immunologic and nonimmunologic stimulation (2). The structure of this compound has been elucidated as 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (3, 4). PAF induces hypotension (5, 6), neutropenia, and thrombocytopenia *in vivo* (7, 8); *in vitro*, PAF promotes chemotaxis and degranulation in neutrophils (9–11) and induces shape change, release of granule content, and aggregation in platelets (12–16).

The biochemical mechanisms by which PAF elicits physiological responses are not fully understood. In platelets, PAF has been shown to stimulate the phosphatidylinositol (PtdIns) cycle (16, 17), release of arachidonic acid from membrane phospholipids (16, 18), and the phosphorylation of specific proteins (10). These biochemical responses are induced also by other platelet stimuli such as ADP, thrombin, or ionophore A23187 (19–21) and are thought to be the consequence of the release of Ca^{2+} from a cellular pool inaccessible to chelating agents (20, 22, 23). Direct studies suggest that PAF induces a rapid mobilization of Ca^{2+} in platelets (24).

Recently, we have observed a rapid loss of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] in thrombin-stimulated horse platelets (25). Because PtdIns(4,5)P_2 might provide Ca^{2+}-binding sites in the plasma membrane (26–28), it has been suggested that the rapid loss of this phospholipid might be a mechanism by which the initial release of Ca^{2+} could be achieved (25). We now show that PAF also causes a rapid but transient decrease in PtdIns(4,5)P_2, phosphatidylinositol 4-monophosphate (PtdIns4P), and PtdIns in the absence of extracellular Ca^{2+}. This initial decrease is followed by resynthesis of the inositides.

MATERIALS AND METHODS

Platelet-activating factor, 1-O-alkyl(C_{16}+C_{18})-2-acetyl-3-phosphorylcholine glycerol (PAF), was obtained from Calbiochem–Behring; lyso-PAF (lPAF), 1-O-alkyl-3-phosphorylcholine glycerol, was kindly provided by J. Benveniste, Clamart, France. All other reagents were obtained as described (16, 25).

Platelet suspensions were prepared and labeled with [^{32}P]-orthophosphate as described (25). Platelet-rich plasma from 500 ml of horse blood collected with anticoagulant buffer containing 1.3% citric acid, 2.5% sodium citrate, and 2% dextrose was prepared by centrifugation at 300 × *g* for 15 min. Platelets were then separated by centrifugation at 3,000 × *g* for 20 min and resuspended in 20 ml of Hepes/saline buffer (25 mM Hepes, pH 7.2/125 mM NaCl/10 mM glucose/1 mM EGTA, pH 7.2). After addition of 2 mCi (1 Ci = 3.7×10^{10} Bq) of carrier-free [^{32}P]orthophosphate (100 μCi/ml), the platelet suspension was incubated at 37°C for 90 min. Platelets labeled with ^{32}P were then washed twice with 100 ml of Hepes/saline and resuspended in the same buffer at a platelet concentration of 10^9 cells per ml. It should be noted that the Hepes/saline buffer used throughout these experiments always contained 1 mM EGTA and no added Ca^{2+}. Under these conditions (and without stirring), addition of PAF did not cause platelet aggregation. PAF was dissolved in Hepes/saline containing 0.3% bovine serum albumin. Triplicate samples of platelet suspension (0.5 ml; 5×10^8 cells) were incubated with various concentrations of PAF for 10 sec or with 0.1 μM PAF for different times; then reactions were stopped and lipids were extracted and separated as before (16, 25). In some experiments, cells were pretreated with prostacyclin (1 μM) for 2–4 min before the addition of PAF (0.1 or 0.03 μM). All experiments, unless otherwise mentioned, represent one of at least four that gave closely similar results.

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Abbreviations: PAF, platelet-activating factor; lPAF, lyso-PAF; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid; lPtdIns, lysophosphatidylinositol.

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The fatty acid composition of PtdIns4*P* and PtdIns(4,5)P_2 was determined by gas–liquid chromatography. PtdIns4*P* and PtdIns(4,5)P_2 were extracted from ^{32}P-labeled platelets, separated by one-dimensional thin-layer chromatography (25), localized by a very brief period (2–3 min) of autoradiography, and hydrolyzed overnight at 40°C with 1.6 ml of methanol and 0.4 ml of 1 M NaOH. Then the pH was brought to 2 by adding formic acid, and the fatty acids were extracted three times with 6 ml of hexane. The hexane was evaporated under N_2, and the methyl esters of fatty acids were prepared by adding diazomethane in ether. Samples were analyzed with a 560 Tracor gas chromatograph (Tracor Analytical Instruments, Austin, TX) equipped with a column (1,800 × 2 mm) packed with SP2330 on 100/120 Supelcoport. N_2 flow rate was 30 or 20 ml/min, and the temperature was either at 166°C or 200°C, respectively.

RESULTS

Fig. 1 shows the changes in different [^{32}P]phospholipids upon stimulation of platelets for 10 sec with 0.1 μM PAF. PAF stimulation induced a decrease of labeling from PtdIns, PtdIns4*P*, and PtdIns(4,5)P_2. The percentage decreases (mean ± SD of five experiments) of ^{32}P radioactivity from PtdIns, PtdIns4*P*, and PtdIns(4,5)P_2 were 29.3 ± 8.8, 31.0 ± 5.6, and 36.8 ± 6.6, respectively. [^{32}P]Phosphatidic acid (PtdOH) (a product of the combined activities of PtdIns-specific phospholipase C and phosphorylation of the resultant 1,2-diacylglycerol by 1,2-diacylglycerol kinase; ref. 29) and [^{32}P]lysophosphatidylinositol (lPtdIns; formed by phospholipase A_2 degradation of PtdIns; ref. 30) also were produced. This indicates that PAF induces activation of both phospholipases C and A_2. lPAF (0.1 μM), which lacks the acetate group in the 2-position and the biological potency, did not induce lipid changes (Fig. 1).

The fatty acid analysis of PtdIns4*P* and PtdIns(4,5)P_2 showed that 0.1 μM PAF for 10 sec induced a net loss of all fatty acids of these lipids (Table 1). This observation supports our previous conclusion (25) that the loss of ^{32}P radioactivity from PtdIns4*P* and PtdIns(4,5)P_2 is a reflection of a net loss of these lipids. PtdIns4*P* and PtdIns(4,5)P_2 had identical patterns of fatty acid composition (Table 1). PAF stimulation caused a net loss (25–40%) of fatty acids of the polyphosphoinositides, but the percentage composition remained unchanged after stimulation. In both phospholipids, about 70% of the total fatty acid content corresponded to stearic acid plus arachidonic acid, each contributing ≈35%. The other fatty acids were palmitate (9%), oleate (14%), and linoleate (4%) (Table 1).

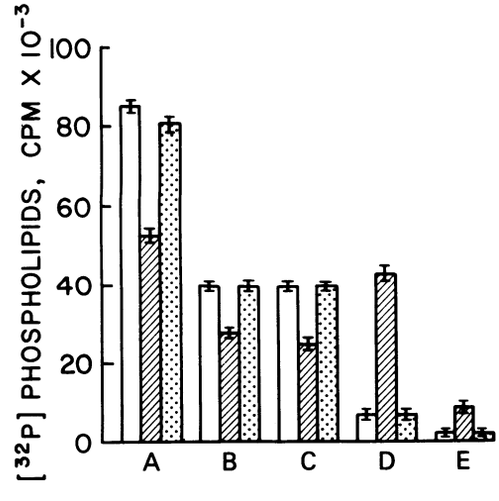

FIG. 1. Effects of PAF and lPAF on [^{32}P]phospholipids of horse platelets. Horse platelets were prelabeled with $^{32}P_i$, and triplicate samples (0.5 ml; 0.5 × 10^9 cells) were incubated with either PAF (0.1 μM) (▨) or lPAF (0.1 μM) (⊡) for 10 sec. Appropriate controls (□) containing only buffer were also included. The lipids were extracted and analyzed as described. Bar, mean ± SD of three replicate assays. A, PtdIns(4,5)P_2; B, PtdIns4*P*; C, PtdIns; D, PtdOH; E, lPtdIns.

Table 1. Changes in fatty acid content of PtdIns4*P* and PtdIns(4,5)P_2 in PAF-stimulated platelets

	Fatty acid composition, nmol per 10^{10} cells					
	PtdIns4*P*			PtdIns(4,5)P_2		
Fatty acid	Control	PAF	% loss	Control	PAF	% loss
Palmitic	5.2	3.5	32.7	4.9	3.4	30.6
Stearic	22.3	13.4	40.0	18.0	13.3	26.2
Oleic	8.2	5.3	35.4	8.0	5.4	32.5
Linoleic	2.6	3.9	—	1.9	1.4	26.4
Arachidonic	21.7	15.4	29.1	17.2	13.2	23.3
Total fatty acids/2	30.0	20.8	31.0	27.5	18.4	33.0

Samples (10 ml) of horse platelets (10^{10} cells) labeled with ^{32}P were incubated with buffer or with PAF (0.1 μM) for 10 sec. The lipids were extracted and separated by one-dimensional thin-layer chromatography (25). The silica gel areas containing PtdIns4*P* and PtdIns(4,5)P_2 were scraped, and heptadecanoic acid (Supelco, Bellefonte, PA) was added as an internal standard in appropriate amounts to each of the samples. Samples were then further processed for gas chromatographic analysis as described. The fatty acid composition of the inositides was calculated against the internal standard. Each result is the mean of two experiments. The amount of PtdIns4*P* or PtdIns(4,5)P_2 was calculated by assuming two fatty acids per molecule of PtdIns4*P* or PtdIns(4,5)P_2: the quantity "total fatty acids/2" is the amount of nanomoles of PtdIns4*P* or PtdIns(4,5)P_2 per 10^{10} cells.

PtdIns(4,5)P_2 loss was maximally induced 5 sec after addition of 0.1 μM PAF, whereas maximal loss of PtdIns4*P* and PtdIns occurred at 10 sec (Fig. 2). Furthermore, the initial losses of inositol lipids were subsequently followed by their resynthesis, as evidenced by increased incorporation of ^{32}P into these lipids. Resynthesis of PtdIns(4,5)P_2 preceded that of PtdIns or PtdIns4*P*.

Fig. 3 shows the changes in [^{32}P]PtdIns(4,5)P_2, [^{32}P]PtdIns4*P*,

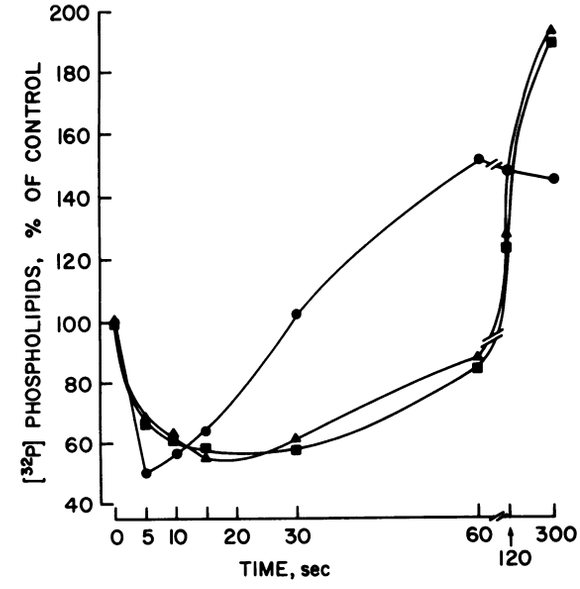

FIG. 2. Time-dependent changes induced by PAF on ^{32}P-labeled inositides of horse platelets. Triplicate samples (0.5 ml) of platelets prelabeled with $^{32}P_i$ (0.5 × 10^9 cells) were stimulated with PAF (0.1 μM) for different periods of time. All the other conditions are as in Fig. 1. ●, PtdIns(4,5)P_2; ▲, PtdIns4*P*; ■, PtdIns. Each data point is the mean of three replicate assays, and the SD is within ±3% of the mean.

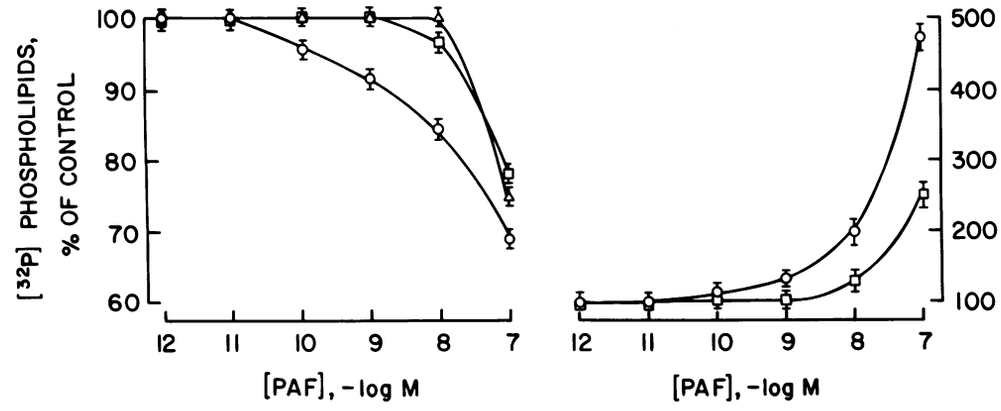

FIG. 3. The effect of various concentrations of PAF on [^{32}P]phospholipids of horse platelets. Platelets prelabeled with $^{32}P_i$ were incubated for 10 sec with different concentrations of PAF as indicated. All the other conditions were as in Fig. 1. Bars, mean ± SD of three replicate assays. (*Left*) △, PtdIns4*P*; □, PtdIns; ○, PtdIns(4,5)P_2. (*Right*) ○, PtdOH; □, lPtdIns.

[^{32}P]PtdIns, [^{32}P]lPtdIns, and [^{32}P]PtdOH induced by different concentrations of PAF. Up to a concentration of 0.01 μM PAF, there was a dose-dependent decrease of [^{32}P]PtdIns(4,5)P_2 without major changes in PtdIns4*P* or PtdIns. However, higher concentrations of PAF (e.g., 0.1 mM) induced loss of ^{32}P radioactivity from all inositol lipids. PtdOH and lPtdIns also were produced in a concentration-dependent manner (Fig. 3).

Prostacyclin increases intraplatelet cyclic AMP levels which, in turn, inhibits platelet responses by blocking Ca^{2+} mobilization (19, 23). Both prostacyclin and cyclic AMP have been shown to inhibit PAF-induced platelet responses (2, 16). Prostacyclin (1 μM) exerted little or no inhibitory action on the loss and synthesis of inositol lipids or on the formation of PtdOH and lPtdIns when platelets were stimulated with 0.1 μM PAF (not shown). However, at a relatively lower concentration of PAF (0.03 μM), the resynthesis of PtdIns(4,5)P_2 was effectively blocked by prostacyclin, whereas the loss of PtdIns(4,5)P_2 remained unaffected

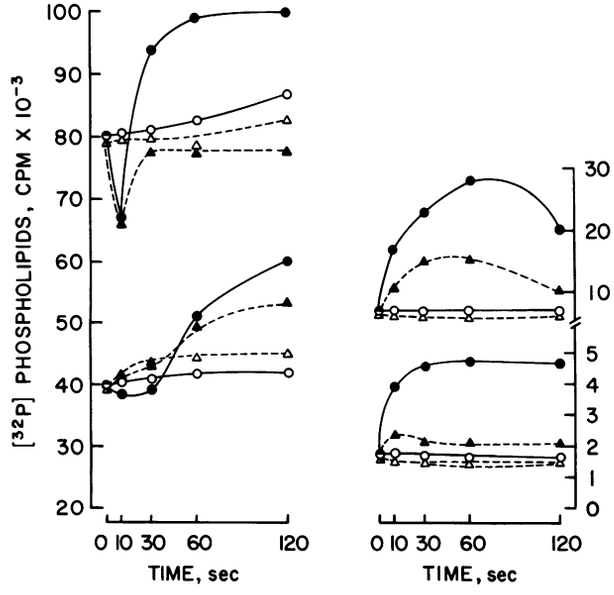

FIG. 4. Time course of changes in [^{32}P]phospholipids induced by PAF in the absence or in the presence of prostacyclin. All of the conditions were as in Fig. 2 except that the PAF concentration was 0.03 μM and the concentration of prostacyclin was 1 μM. Additions: ○, none (control) ●, PAF; △, prostacyclin; ▲, prostacyclin/PAF. Each data point is the mean of three replicate assays, and the SD is within ±3% of the mean. (*Left*) PtdIns(4,5)P_2 (*Upper*) and PtdIns4*P* (*Lower*). (*Right*) PtdOH (*Upper*) and lPtdIns (*Lower*).

(Fig. 4). Under these conditions, the Ca^{2+}-dependent formation of PtdOH and lPtdIns (21, 29, 30) also was substantially reduced (Fig. 4). Thus, it appears that the initial loss of PtdIns(4,5)P_2 is independent of intracellular mobilization of Ca^{2+}, whereas its resynthesis is a Ca^{2+}-regulated process. This interpretation is further supported by the observation that the Ca^{2+} ionophore A23187, which mobilizes Ca^{2+}, induced the synthesis of PtdIns(4,5)P_2 without producing the initial loss (25).

DISCUSSION

Our present results indicate that PAF induces two distinct changes in inositol lipid metabolism: an initial decrease and subsequent resynthesis. The changes in PtdIns(4,5)P_2 precede those in PtdIns4*P* and PtdIns. The loss of PtdIns(4,5)P_2 occurs very early during stimulation and is sensitive to relatively low concentrations of PAF. Furthermore, this early loss of PtdIns(4,5)P_2, induced by low concentrations of PAF, is the only lipid change not affected by prostacyclin. These observations suggest that the loss of PtdIns(4,5)P_2 is a primary event during platelet stimulation and independent of mobilization of cytoplasmic Ca^{2+}. The PtdIns(4,5)P_2 resynthesis, on the other hand, appears later on, and is secondary to the mobilization of internal platelet Ca^{2+}. PAF also generates PtdOH and lPtdIns, indicating that PtdIns is degraded by both phospholipase C and phospholipase A_2 activities. These metabolic changes induced by PAF are qualitatively similar to those observed in thrombin-stimulated platelets (25). The enzymatic basis for the stimulus-coupled loss of polyphosphoinositides is not yet clear; the relative importance of PtdIns and PtdIns4*P* kinases, polyphosphoinositide phosphatases, and phosphodiesterases has been discussed (25). However, recent information (31) indicates that PtdIns(4,5)P_2 is degraded by phosphodiesteratic cleavage in thrombin-stimulated platelets.

PAF elicits physiological responses by increasing the intracellular concentration of Ca^{2+} (24). But, unlike PtdOH, it exhibits no ionophoretic properties in intact lipid bilayers (32). Moreover, recent binding studies indicate the presence of platelet receptors for PAF (33). All this information might suggest that Ca^{2+} mobilization in PAF-stimulated platelets is somehow linked to certain early biochemical changes occurring upon receptor activation. One such biochemical change could be the degradation of inositol phospholipids, especially that of PtdIns(4,5)P_2. These lipids located at the cytoplasmic face of the plasma membrane can provide important cellular Ca^{2+}-binding sites (25–28) and also might be required for the operation of Ca^{2+}-ATPase (34). Therefore, upon degradation of these lipids, the mem-

brane-bound Ca^{2+} can be released in the cytosol and activate various Ca^{2+}-dependent enzymes such as phospholipases (16–21) and protein kinases (19). The formation of 1,2-diacylgylcerol that might be produced by phosphodiesteratic degradation of PtdIns(4,5)P_2 also could play an important role in transmembrane signaling by stimulation of protein kinase C (35).

The possible role for polyphosphoinositides in Ca^{2+} mobilization is supported by several lines of evidence. (*i*) The loss of inositol lipids, especially that of PtdIns(4,5)P_2, is a very early event (Fig. 2) and appears to be mediated by receptor activation (25). (*ii*) This response is induced by other platelet agonists such as thrombin (25) and ADP (unpublished data), which are known to mobilize intraplatelet Ca^{2+}, and yet this effect itself appears to be insensitive to Ca^{2+}. (*iii*) A similar receptor-mediated loss of PtdIns(4,5)P_2 has been observed after hormonal stimulation of hepatocytes (36) and parotid acinar cells (37), and it appears to be independent of Ca^{2+} mobilization (36, 37).

Formation of PtdOH is a consequence of activation of PtdIns-specific phospholipase C, a Ca^{2+}-dependent enzyme (16, 20, 29). The loss of PtdIns(4,5)P_2 is independent of Ca^{2+} mobilization and might precede and trigger the degradation of PtdIns and the subsequent formation of PtdOH (16, 20, 21, 29). PtdOH can then act as an intracellular Ca^{2+} ionophore (32) and, thus, cause further release of Ca^{2+} from the dense tubular system (38).

Our results on the increased incorporation of ^{32}P into polyphosphoinositides, subsequent to their initial loss, are in agreement with those recently published (17). This response also is shared by a variety of platelet agonists such as thrombin, collagen, and ADP (25, 39), and it appears [contrarily to the initial loss of PtdIns(4,5)P_2] to be a consequence of increased intracellular Ca^{2+} concentrations. This response is also provoked by ionophore A23187 (25) and is inhibited when the increase in cytosolic Ca^{2+} concentration is prevented by prostacyclin (Fig. 3). The increased formation of polyphosphoinositides can bind divalent cations (26–28) and might enhance the efficiency of the Ca^{2+} pump (33). The resultant decrease in cytosolic levels of Ca^{2+} can then limit both the degradation of membrane components and the degree of cellular activation. An alternative role for the increased formation of polyphosphoinositides is suggested by a recent study (40) showing that increased incorporation of PtdIns(4,5)P_2 into the erythrocyte ghost membrane causes increased lateral mobility of erythrocyte glycoprotein. It might well be that the increased formation of PtdIns(4,5)P_2 in stimulated platelets is associated with similar specific changes in the platelet membrane.

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