

## Platelet-activating factor stimulates phosphatidylinositol turnover in human platelets

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Platelet-activating factor stimulates phosphatidylinositol turnover in human platelets as indicated by [<sup>32</sup>P]phosphatidate accumulation in platelets pre-labelled with [<sup>32</sup>P]P<sub>i</sub>, and by [<sup>3</sup>H]phosphatidate accumulation and [<sup>3</sup>H]phosphatidylinositol loss in platelets pre-labelled with [<sup>3</sup>H]arachidonate. These effects of platelet-activating factor are direct and are independent of the production and/or release of endogenous platelet agonists such as ADP, 5-hydroxytryptamine and thromboxane A<sub>2</sub>.

2-Acetyl-1-alkyl-*sn*-glycero-3-phosphocholine, also known as platelet-activating factor, was originally reported to be a basophil-derived mediator of inflammation and anaphylaxis (Benveniste *et al.*, 1972). It is now known to be produced by other cells, including human platelets activated by thrombin or ionophore A23187 (Chignard *et al.*, 1979). Platelet-activating factor induces aggregation of platelets of several species (Benveniste *et al.*, 1972), including man (MacIntyre & Westwick, 1981). As it is produced by human platelets and is a potent inducer of human platelet activation, platelet-activating factor may function as an endogenous mediator of aggregation distinct from ADP and arachidonate metabolites (Vargaftig *et al.*, 1981).

The initial stage in the activation of platelets by agonists, such as ADP, collagen and thrombin, is combination of the agonist with a specific surface-membrane receptor (Mills & Macfarlane, 1976). Desensitization studies (Satouchi *et al.*, 1981) indicate that there are receptors for platelet-activating factor on human platelets. In platelets, the major biochemical intermediaries that translate agonist-receptor combination into a cellular response are cyclic AMP and Ca<sup>2+</sup>. An elevated intracellular cyclic AMP concentration is associated with inhibition of platelet function (Haslam *et al.*, 1978), whereas platelet activation is associated with an increase in cytosolic free [Ca<sup>2+</sup>] (Rink *et al.*, 1981). Michell (1975) has suggested that an increase in cytosolic free [Ca<sup>2+</sup>] is a consequence of agonist-induced turnover of membrane phosphatidylinositol. There is evidence that ADP and thrombin stimulate phosphatidylinositol turnover in platelets (Lloyd *et al.*, 1972; Rittenhouse-Simmons, 1981), and the mechanisms of the thrombin effect have been analysed in detail (Lapetina, 1982). It is proposed that agonist-receptor combination activ-

ates a phosphatidylinositol-specific phospholipase C, which hydrolyses phosphatidylinositol to diacylglycerol, which in turn is phosphorylated to phosphatidate. Phosphatidate may then be converted into phosphatidylinositol to complete the cycle, or acted upon by a phosphatidate-specific phospholipase A<sub>2</sub> to yield lysophosphatidate (Billah *et al.*, 1981). Phosphatidate and lysophosphatidate are calcium ionophores (Tyson *et al.*, 1976; Gerrard *et al.*, 1979) and may directly translocate calcium to the cytosol. In the present study we examined the effects of purified platelet-activating factor on phosphatidylinositol turnover in human platelets. Our results indicate that human platelet activation induced by platelet-activating factor is mediated, at least in part, by augmenting the turnover of phosphatidylinositol.

### Experimental

#### *Preparation and labelling of platelets*

Blood was obtained from the antecubital vein of healthy human volunteers, predominantly those who denied taking drugs known to affect platelet function. In some instances, we used donors who had ingested 600 mg of aspirin 2 days previously. Blood was collected in 10% (v/v) trisodium citrate (0.13 M) and centrifuged (1000 g for 4 min at 15–20°C). The supernatant platelet-rich plasma was aspirated, pelleted by centrifugation (800 g for 10 min at 15–20°C) in the presence of prostaglandin I<sub>2</sub> (0.3 μM) (Moncada *et al.*, 1982) and resuspended in a calcium-free phosphate-free buffer [150 mM-NaCl/4 mM-KCl/1 mM-MgCl<sub>2</sub>/10 mM-dextrose/5 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (pH 7.4)/0.3% bovine serum albumin] at 5 × 10<sup>9</sup>–10<sup>10</sup> cells/ml. The platelet suspension was then incubated at 37°C for 90 min with carrier-free [<sup>32</sup>P]P<sub>i</sub> (30 μCi/ml) or [<sup>3</sup>H]arachidonate (2 μCi/ml);

16 nM). Platelets were then pelleted by centrifugation (800g for 10 min at 15–20°C) in the presence of prostaglandin I<sub>2</sub> (0.3 μM) and resuspended in 1.5 times the labelling volume of fresh buffer.

Radiolabelled platelets (0.4 ml, containing 0.5–1 mg of protein) were dispensed into plastic tubes at 37°C. Reactions were initiated by addition of platelet-activating factor (or vehicle in control samples) and terminated by transferring the entire platelet sample into a test tube containing 2 ml of chloroform/methanol/10M-HCl (25:50:4, by vol.) at room temperature (Lloyd *et al.*, 1972).

#### Lipid extraction and t.l.c.

Platelet lipids were extracted essentially by method B of Lloyd *et al.* (1972), dried at 40°C under N<sub>2</sub> and stored at –20°C until use. The lipids were redissolved in 0.15 ml of chloroform/methanol (9:1, v/v) and spotted on silica-gel t.l.c. plates for two-dimensional separation of phospholipids (Yavin & Zutra, 1977). In some cases 5 μg of carrier phosphatidate was used. Individual spots were visually detected by exposure to I<sub>2</sub> vapour and/or autoradiography on Ilford 25 EP X-ray film (15–20 h). Spots corresponding to phosphatidylinositol and phosphatidate were scraped into vials and counted for radioactivity in a liquid-scintillation spectrometer.

#### Assays

Platelet aggregation was measured photometrically and release of platelet-dense granule constituents (ADP, 5-hydroxytryptamine) was monitored by using an isotope (5-hydroxy[<sup>14</sup>C]tryptamine) pre-labelling technique (MacIntyre *et al.*, 1978). Protein was determined by the method of Lowry *et al.* (1951).

#### Materials

2-Acetyl-1-heptadecyl-*sn*-glycero-3-phosphocholine, a gift from Dr. R. N. Saunders (Sandoz), was dissolved in iso-osmotic NaCl containing 0.25% bovine serum albumin. Prostaglandin I<sub>2</sub>, donated by Dr. J. E. Pike (Upjohn), was prepared as described previously (MacIntyre, 1979). Bovine serum albumin and phospholipid carrier and standards were purchased from Sigma. T.l.c. plates (Macherey-Nagel; Polygram Sil G pre-coated plastic sheets) were purchased from Camlab, Cambridge, U.K. All organic solvents and other laboratory reagents were analytical-grade. Carrier-free [<sup>32</sup>P]P<sub>1</sub> was supplied by the Regional Isotope Dispensary, Western Infirmary, Glasgow, Scotland, U.K. 5-Hydroxy-[*side chain-2-<sup>14</sup>C*]tryptamine creatinine sulphate (sp. radioactivity 53 Ci/mol) and [5,6,8,9,11,12,14,15-<sup>3</sup>H]arachidonic acid (sp. radioactivity 127 Ci/mmol) were from Amersham International.

## Results and discussion

Platelet responsiveness (aggregation) induced by platelet-activating factor gradually declines with time after platelet preparation (McManus *et al.*, 1981). In the present study, agonist additions to platelets were routinely performed between 3 and 4 h after blood sampling. Platelets prepared under radiolabelling conditions aggregated when exposed to platelet-activating factor (11 nM–1.1 μM) (Fig. 1).

Fig. 2 depicts the time course of the effects of platelet-activating factor on platelet [<sup>32</sup>P]phosphatidate and [<sup>32</sup>P]phosphatidylinositol. In the absence of agonist, the levels of [<sup>32</sup>P]phosphatidate and [<sup>32</sup>P]phosphatidylinositol did not change during the

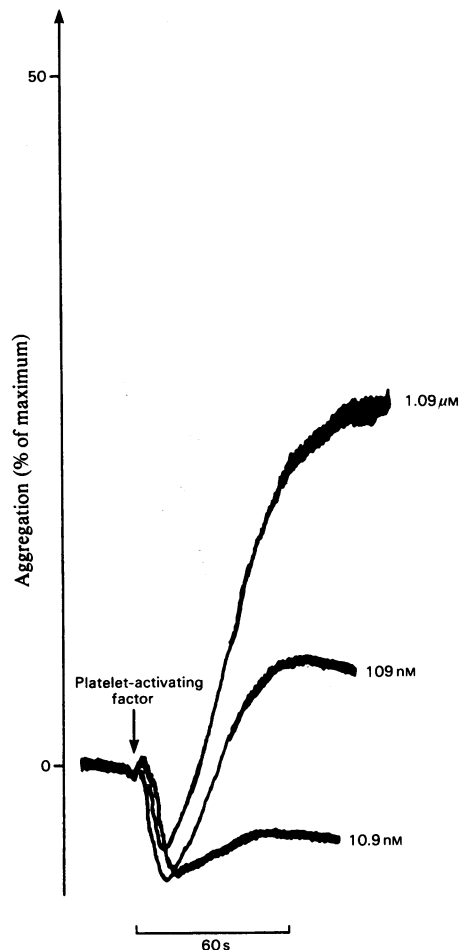


Fig. 1. Platelet-activating-factor-induced human platelet aggregation

Plasma-free suspensions of platelets (0.17 ml) prepared under radiolabelling conditions (see the Experimental section) were exposed to platelet-activating factor at the concentrations indicated, and aggregation was monitored photometrically.

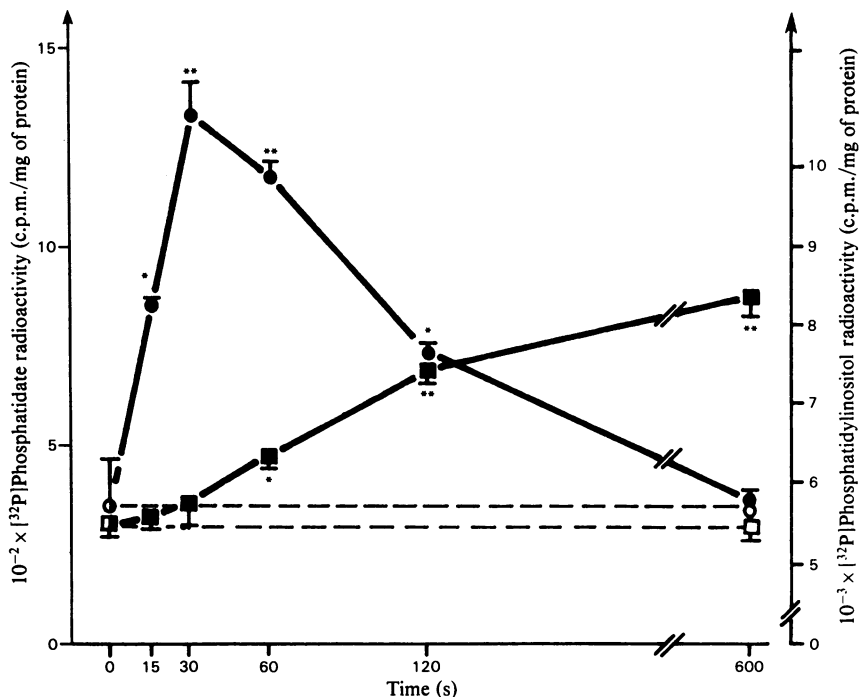


Fig. 2. Time course of  $^{32}\text{P}$ -labelled phospholipid formation induced by platelet-activating factor

Portions (0.4 ml) of  $^{32}\text{P}$ -prelabelled platelets were incubated at  $37^\circ\text{C}$  in the presence of platelet-activating factor ( $1.8\ \mu\text{M}$ ) (filled symbols) or saline in control samples (open symbols) for the time indicated. Reactions were quenched and phospholipids extracted, separated and the levels of  $^{32}\text{P}$ phosphatidate ( $\circ$  and  $\bullet$ ) and  $^{32}\text{P}$ phosphatidylinositol ( $\square$  and  $\blacksquare$ ) were measured. Results shown are means  $\pm$  s.e.m. of triplicate determinations. The experiment shown is typical of three similar experiments using platelets from different donors. Statistical analysis (control level versus exposure to platelet-activating factor) was by Student's *t* test. \* $P < 0.05$ ; \*\* $P < 0.01$ .

course of the experiment. Platelet-activating factor induced a rapid increase in  $^{32}\text{P}$ phosphatidate, which was maximal (350% of zero-time control in this experiment) within 30s of agonist addition. Thereafter the levels of  $^{32}\text{P}$ phosphatidate declined towards control values. In contrast, significant increases in  $^{32}\text{P}$ phosphatidylinositol induced by platelet-activating factor were not evident until at least 60s after agonist addition and progressively increased with time (163% of zero-time control at 600s). Unlike the other major phospholipids, phosphatidylinositol (and phosphatidic acid) are labelled directly via ATP (Rittenhouse-Simmons & Deykin, 1981). Consequently the pattern of  $^{32}\text{P}$ -labelled glycerophospholipid labelling after exposure of platelets to platelet-activating factor is consistent with an increased turnover of phosphatidylinositol. According to the scheme proposed by Lapetina (1982), exposure of platelets to certain agonists results in activation of a phosphatidylinositol-specific phospholipase C, which catalyses the formation of 1,2-diacylglycerol. 1,2-Diacylglycerol is then

rapidly phosphorylated by diacylglycerol kinase to yield phosphatidic acid. Thus the appearance of  $^{32}\text{P}$ phosphatidate closely follows phosphatidylinositol degradation. The labelling in phosphatidylinositol only becomes significant as the small, transient pool of  $^{32}\text{P}$ phosphatidate is reconverted into, and accumulates as,  $^{32}\text{P}$ phosphatidylinositol.

The concentration-response relationship for platelet-activating-factor-induced changes in  $^{32}\text{P}$ phosphatidate and  $^{32}\text{P}$ phosphatidylinositol were investigated using a duration of agonist exposure of 15s and 30s (Fig. 3). At both time points, stimulation of  $^{32}\text{P}$ phosphatidate formation was observed at a threshold concentration of 1–10nM, with maximum stimulation at 180nM– $1.8\ \mu\text{M}$ . Mean ( $\pm$ s.d.,  $n = 5$ )  $\text{EC}_{50}$  (the concentration of agonist that elicits 50% of the maximum response) values were  $31 \pm 6\ \text{nM}$  at 15s and  $35 \pm 5\ \text{nM}$  at 30s. At both 15s and 30s duration of agonist exposure, no statistically significant concentration-dependent changes in  $^{32}\text{P}$ phosphatidylinositol were evident. As our studies were performed over short incu-

bation periods and under conditions where agonist-induced uptake of [ $^{32}\text{P}$ ]P<sub>i</sub> by platelets could not occur, such divergence in the labelling patterns of [ $^{32}\text{P}$ ]phosphatidate and [ $^{32}\text{P}$ ]phosphatidylinositol is not unexpected. Indeed, a similar pattern of labelling was observed by Lloyd *et al.* (1973) after short-term exposure of platelets to ADP.

[ $^3\text{H}$ ]Arachidonate, when incubated with platelets, is predominantly incorporated into the *sn*-2 position

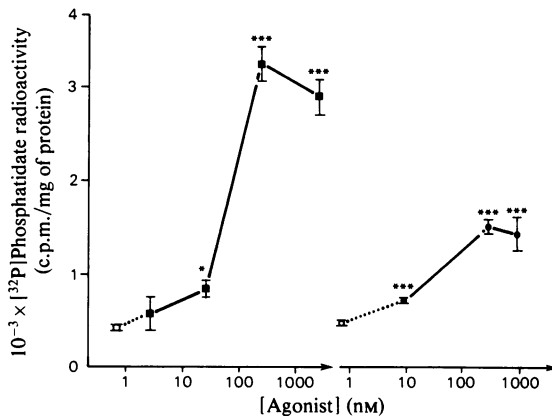


Fig. 3. Concentration-response curves of platelet-activating-factor-induced [ $^{32}\text{P}$ ]phosphatidate formation. Portions (0.4 ml) of  $^{32}\text{P}$ -prelabelled platelets were incubated at 37°C for 30 s (□ and ■) or 15 s (○ and ●) in the presence of platelet-activating factor at the concentrations indicated (● and ■) or saline in control samples (○ and □). Phospholipids were analysed as described in the legend to Fig. 2. Results shown are means  $\pm$  s.e.m. of triplicate or quadruplicate determinations. The experiments shown are typical of five similar experiments using platelets from different donors. Statistical analysis (control levels versus exposure to platelet-activating factor) was by Student's *t* test. \* $P < 0.05$ ; \*\* $P < 0.001$ .

of the glycerol backbone of phospholipids. Phosphatidate accumulation by platelets precedes deacylation of arachidonate from the phospholipids of stimulated platelets (Lapetina & Cuatrecasas, 1979). We therefore used [ $^3\text{H}$ ]arachidonate-pre-labelled platelets and monitored the effects of platelet-activating factor on [ $^3\text{H}$ ]phosphatidate and [ $^3\text{H}$ ]phosphatidylinositol (Table 1). To prevent bio-conversion of [ $^3\text{H}$ ]arachidonate into prostanoids during the incubation procedure, these studies were performed by using platelets from aspirin-treated donors. Platelet-activating factor produced a concentration-dependent increase in [ $^3\text{H}$ ]phosphatidate accompanied by a decrease in [ $^3\text{H}$ ]phosphatidylinositol. Shaw *et al.* (1981) have reported that platelet-activating factor stimulates phosphatidate formation by rabbit platelets. Clearly, platelet-activating-factor-induced formation of [ $^{32}\text{P}$ ] and [ $^3\text{H}$ ]phosphatidate could occur by mechanisms other than degradation of phosphatidylinositol, since diacylglycerol, the precursor of phosphatidate, may be generated from other phospholipids or from triacylglycerol. However, this is unlikely because platelet phospholipase C shows an absolute specificity for phosphatidylinositol (Rittenhouse-Simmons, 1979) and human platelets contain negligible triacylglycerol lipase activity (Lote & Lowery, 1979). Although synthesis *de novo* of phosphatidate from glycerol 3-phosphate may occur, the observed increase in ([ $^3\text{H}$ ]arachidonyl)phosphatidate and decrease in ([ $^3\text{H}$ ]arachidonyl)phosphatidylinositol strongly indicate that platelet-activating factor stimulates phosphatidylinositol metabolism.

Platelet-activating-factor-induced human platelet activation is associated with the synthesis and/or secretion of thromboxane A<sub>2</sub>, ADP and 5-hydroxytryptamine (MacIntyre & Westwick, 1981). The effects of platelet-activating factor on phosphatidylinositol turnover could be secondary to the action of these endogenous platelet agonists. We can

Table 1. Changes in the [ $^3\text{H}$ ]arachidonate-labelled phospholipids of washed human platelets stimulated with platelet-activating factor

Platelets were obtained from donors who had ingested aspirin (600 mg) 2 days previously. Portions (0.4 ml) of [ $^3\text{H}$ ]arachidonate-prelabelled platelets were incubated at 37°C for 30 s in the presence of platelet-activating factor at the concentrations indicated. Phospholipids were analysed as described in the legend to Fig. 2. Results are mean  $\pm$  s.e.m. of triplicate determinations. The experiment shown is typical of two similar experiments. Statistical analysis by Student's *t* test. \*Statistically significant difference compared with control ( $P < 0.05$ ).

Addition	Phosphatidic acid		Phosphatidylinositol	
	(c.p.m./mg of protein)	(% change)	(c.p.m./mg of protein)	(% change)
Control (saline)	296 $\pm$ 16		2831 $\pm$ 105	
Platelet-activating factor				
1.8 nM	329 $\pm$ 57	+11.1	2583 $\pm$ 301	-8.8
18 nM	419 $\pm$ 91	+41.5	2962 $\pm$ 27	+4.6
180 nM	571 $\pm$ 78	+93*	2451 $\pm$ 37	-13.4*
1.8 $\mu\text{M}$	480 $\pm$ 49	+63*	2348 $\pm$ 101	-15.8*

preclude an obligatory role for thromboxane A<sub>2</sub> in the effects of platelet-activating factor, as [<sup>3</sup>H]-phosphatidate accumulation and [<sup>3</sup>H]phosphatidylinositol depletion occur in aspirin-treated platelets (Table 1). Moreover, the extent of [<sup>32</sup>P]phosphatidate accumulation induced by platelet-activating factor is the same in control and in aspirin-treated platelets (D. E. MacIntyre & W. K. Pollock, unpublished work). Of the concentrations of platelet-activating factor that induce phosphatidylinositol turnover, only the highest concentration (1.8 μM) caused significant release (9.3% of total) of the platelet dense-granule marker 5-hydroxy[<sup>14</sup>C]-tryptamine. Hence the effects of platelet-activating factor are direct and are independent of released ADP, 5-hydroxytryptamine and thromboxane A<sub>2</sub>.

Platelet activation is dependent on an increase in cytosolic free [Ca<sup>2+</sup>] (Rink *et al.*, 1981), and stimulation of phosphatidylinositol turnover has been implicated in calcium gating (Michell, 1979) and in platelet responses to ADP, collagen and thrombin (Lloyd *et al.*, 1972; Mauco *et al.*, 1979; Rittenhouse-Simmons, 1981). The mechanisms of platelet-activating-factor-induced platelet activation have been examined mainly using rabbit platelets. In that species, platelet-activating factor has been reported to stimulate an influx of <sup>45</sup>Ca<sup>2+</sup> (Lee *et al.*, 1981) and to stimulate phosphatidylinositol metabolism (Shukla & Hanahan, 1982). Using human platelets, we have shown that platelet activation induced by platelet-activating factor is selectively inhibited by the calcium-‘antagonist’ drugs verapamil and methoxyverapamil (MacIntyre & Shaw, 1982). This could indicate that the effects of platelet-activating factor on human platelets are more dependent on increased calcium availability than are the effects of other agonists. We contend that activation of human platelets induced by platelet-activating factor could be modulated, at least in part, by stimulation of phosphatidylinositol turnover.

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