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 $^{[32]P}$phosphatidate accumulation in platelets pre-labelled with $^{[32]P}$P$_1$ and by $^{[3]H}$phosphatidate accumulation and $^{[3]H}$phosphatidylinositol loss in platelets pre-labelled with $^{[3]H}$arachidone. 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$_2$.

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$^{2+}$. 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$^{2+}$] (Rink et al., 1981). Michell (1975) has suggested that an increase in cytosolic free [Ca$^{2+}$] 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 activates 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$_2$ 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$_2$ (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$_2$/10 mM-dextrose/5 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (pH 7.4)/0.3% bovine serum albumin] at 5×10$^9$–10$^{10}$ cells/ml. The platelet suspension was then incubated at 37°C for 90 min with carrier-free $^{[32]P}$P$_1$ (30 μCi/ml) or $^{[3]H}$arachidonate (2 μCi/ml;
16 nm). Platelets were then pelleted by centrifuga-
tion (800 g for 10 min at 15–20°C) in the presence
of prostaglandin I₂ (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/10 mM 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 N₂ 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 I₂ 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 constitu-
tuents (ADP, 5-hydroxytryptamine) was monitored
by using an isotope (5-hydroxy[14C]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-phospho-
choline, a gift from Dr. R. N. Saunders (Sandoz),
was dissolved in iso-osmotic NaCl containing 0.25%
bovine serum albumin. Prostaglandin I₂, donated by
Dr. J. E. Pike (Upjohn), was prepared as described
previously (MacIntyre, 1979). Bovine serum al-
bumin 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 [32P]Pi was supplied
by the Regional Isotope Dispensary, Western
Infirmary, Glasgow, Scotland, U.K. 5-Hydroxy-
side chain-2,14C]tryptamine creatinine sulphate (sp.
radioactivity 53 Ci/mol) and [5,6,8,9,11,12,14,15-
3H]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
of agonist, the levels of [32P]phosphatidate and
[32P]phosphatidylinositol did not change during the

![Fig. 1. Platelet-activating-factor-induced human platelet aggregation](image-url)
course of the experiment. Platelet-activating factor induced a rapid increase in [\(^{32}\)P]phosphatidate, which was maximal (350% of zero-time control in this experiment) within 30s of agonist addition. Thereafter the levels of [\(^{32}\)P]phosphatidate declined towards control values. In contrast, significant increases in [\(^{32}\)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 phosphatic acid) are labelled directly via ATP (Rittenhouse-Simmons & Deykin, 1981). Consequently the pattern of [\(^{32}\)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 [\(^{32}\)P]phosphatidate closely follows phosphatidylinositol degradation. The labelling in phosphatidylinositol only becomes significant as the small, transient pool of [\(^{32}\)P]phosphatidate is reconverted into, and accumulates as, [\(^{32}\)P]phosphatidylinositol.

The concentration–response relationship for platelet-activating-factor-induced changes in [\(^{32}\)P]-phosphatidate and [\(^{32}\)P]phosphatidylinositol were investigated using a duration of agonist exposure of 15s and 30s (Fig. 3). At both time points, stimulation of [\(^{32}\)P]phosphatidate formation was observed at a threshold concentration of 1–10nM, with maximum stimulation at 180nM–1.8\(\mu\)M. Mean (±s.d., \(n=5\)) \(\text{EC}_{50}\) (the concentration of agonist that elicits 50% of the maximum response) values were 31±6nM at 15s and 35±5nM at 30s. At both 15s and 30s duration of agonist exposure, no statistically significant concentration-dependent changes in [\(^{32}\)P]phosphatidylinositol were evident. As our studies were performed over short incu-
bation periods and under conditions where agonist-induced uptake of $[^{32}P]$P, by platelets could not occur, such divergence in the labelling patterns of $[^{32}P]$phosphatidate and $[^{32}P]$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.

$[^{3}H]$Arachidonate, when incubated with platelets, is predominantly incorporated into the $sn$-2 position 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 $[^{3}H]$arachidonate-prelabelled platelets and monitored the effects of platelet-activating factor on $[^{3}H]$phosphatidate and $[^{3}H]$-phosphatidylinositol (Table 1). To prevent bioconversion of $[^{3}H]$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 $[^{3}H]$phosphatidate accompanied by a decrease in $[^{3}H]$phosphatidylinositol. Shaw et al. (1981) have reported that platelet-activating factor stimulates phosphatidate formation by rabbit platelets. Clearly, platelet-activating-factor-induced formation of $[^{3}P]$- and $[^{3}H]$-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 $[^{3}H]$arachidonylphosphatidate and decrease in $[^{3}H]$arachidonylphosphatidylinositol 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$_2$, 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

![Fig. 3. Concentration–response curves of platelet-activating-factor-induced $[^{3}P]$phosphatidate formation.](image)

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphatidic acid (c.p.m./mg of protein)</th>
<th>% change</th>
<th>Phosphatidylinositol (c.p.m./mg of protein)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>296 ± 16</td>
<td></td>
<td>2831 ± 105</td>
<td></td>
</tr>
<tr>
<td>Platelet-activating</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>factor 1.8 nM</td>
<td>329 ± 57</td>
<td>+11.1</td>
<td>2583 ± 301</td>
<td>−8.8</td>
</tr>
<tr>
<td>18 nM</td>
<td>419 ± 91</td>
<td>+41.5</td>
<td>2962 ± 27</td>
<td>+4.6</td>
</tr>
<tr>
<td>180 nM</td>
<td>571 ± 78</td>
<td>+93*</td>
<td>2451 ± 37</td>
<td>−13.4*</td>
</tr>
<tr>
<td>1.8 μM</td>
<td>480 ± 49</td>
<td>+63*</td>
<td>2348 ± 101</td>
<td>−15.8*</td>
</tr>
</tbody>
</table>

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preclude an obligatory role for thromboxane A₂ in the effects of platelet-activating factor, as [³H]-phosphatidate accumulation and [³H]phosphatidyl-
ninositol depletion occur in aspirin-treated platelets (Table 1). Moreover, the extent of [³²P]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 phosphatidyl-
ninositol turnover, only the highest concentration (1.8 μM) caused significant release (9.3% of total) of the platelet dense-granule marker 5-hydroxy[¹⁴C]-
tryptamine. Hence the effects of platelet-activating factor are direct and are independent of released ADP, 5-hydroxytryptamine and thromboxane A₂.

Platelet activation is dependent on an increase in cytosolic free [Ca²⁺] (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 ⁴⁴Ca²⁺ (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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References