Platelet activating factor (PAF-acether) promotes an early degradation of phosphatidylinositol-4,5-biphosphate in rabbit platelets

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^{32}P-Labelled washed rabbit platelets were incubated with 0.6 nM platelet activating factor (PAF-acether), giving a full aggregation and release response within 30–60 s. The major phospholipid changes observed under these conditions were: (1) An increased labelling of phosphatidic acid (PA) within 10 s and of phosphatidylinositol (MPI) at 30 s, reflecting the activation of the MPI cycle via the cytosolic phospholipase C; (2) an enhancement of phosphatidylinositol-4-phosphate (DPI) and phosphatidylinositol-4,5-*bis*phosphate (TPI) labelling at later incubation times; (3) an early degradation of TPI with a counterbalancing formation of DPI. The latter changes suggest a receptor-mediated stimulation of TPI-phosphomonoesterase, the role of which in the mechanism of platelet activation is discussed.

Platelets *Platelet activating factor* *(poly)Phosphoinositides* *phosphatidic acid* *Phosphomonoesterase*

1. INTRODUCTION

Phospholipid metabolism in stimulated platelets has received much attention. One of the earliest changes subsequent to platelet stimulation by specific agonists like ADP, thrombin, collagen or platelet activating factor (PAF-acether) seems to be the activation of a phosphatidylinositol (MPI) specific phospholipase C [1,2] leading to the production of diglycerides, phosphatidic acid (PA), lyso PA and arachidonate [1,3–13]. This so-called MPI effect was described in numerous tissues under specific conditions of stimulation [14–17].

Other metabolic changes observed in stimulated cells involve the polyphosphoinositides (poly(PI)): phosphatidylinositol-4-phosphate (DPI) and phosphatidylinositol-4,5-*bis*phosphate (TPI). An increased labelling of poly(PI) was revealed in platelets stimulated by various agonists [3,4,13,18–20]. In [21] thrombin promoted a net synthesis of platelet DPI and TPI, which occurs as a late event and parallels the release of lysosomal enzymes. In [22] a specific degradation of TPI was observed in the first seconds of platelet activation by thrombin: TPI hydrolysis was not dependent on calcium mobilization – it was suggested to participate in the release of membrane-bound calcium [23].

This study was undertaken to investigate the mechanism of action of PAF-acether, a powerful phospholipid mediator able to promote platelet aggregation and secretion [24–27]: besides promoting an early acitvation of MPI-specific phospholipase C and a secondary phosphorylation of DPI and TPI, PAF-acether also induces an early degradation of TPI.

Abbreviations: PAF-acether, platelet-activating factor or 1-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; PA, phosphatidic acid; MPI, phosphatidylinositol; DPI, phosphatidylinositol-4-phosphate; TPI, phosphatidylinositol-4,5-*bis*phosphate; poly (PI), polyphosphoinositides

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2. MATERIALS AND METHODS

$HP^{32}PO_4^{2-}$, di-sodium salt, carrier-free, was obtained from the Commissariat à l'Energie Atomique (Saclay). 5-Hydroxy [G-^{3}H]-tryptamine, creatinine sulfate (10–20 Ci/mmol) was from NEN (Boston, MA). 1-Hexadecyl (*rac*) glycerophosphorylcholine was purchased from Medmark (Heidelberg). Liquid scintillation counting was performed using Picofluor 30 (Packard, Palo Alto CA) and an LS 3000 spectrophotometer (Kontron, Basel).

2.1. *Preparation of washed rabbit blood platelets*

Blood was withdrawn by intracardiac puncture of male New Zealand rabbits using 8 mM EDTA (pH 7.4) as an anticoagulant. The platelets were prepared essentially as in [28]. Labelling was performed by incubating either platelet-rich plasma with [^{3}H]serotonin (100 nCi/ml, 30 min, 20°C) or the first washing fluid (5×10^8 cells/ml) with [^{32}P]phosphate (50 μCi/ml, 90 min, 20°C). Final suspension (5×10^8 cells/ml) was in Tyrode buffer (pH 7.4) containing 1 mM $MgCL_2$, 1 mM $CaCl_2$, 5.5 mM glucose and 3.5 g/l of bovine serum albumin.

2.2. *Synthesis of 1-hexadecyl-2-acetyl (rac)-glycerophosphorylcholine (PAF-acether)*

PAF-acether was obtained according to [25] and purified by thin-layer chromatography on silica gel [29]. It was stored in ethanol at −20°C until use.

2.3. *Stimulation of the platelets and analytical procedures*

PAF-acether was added under a minimal volume of ethanol (12 μl/ml) to prewarmed platelets suspension (1 min, 37°C) and incubation was performed at 37°C under stirring. The same volume of ethanol was added to the controls. In alle the experiments considered, a full aggregation response was obtained with 6×10^{-10} M PAF-acether.

Phospholipid analysis:

Incubation was blocked by adding 3 vol. $CHCl_3/CH_3OH$ (1/2, v/v) and EDTA (5 mM, final conc.). Lipid extraction was performed as in [30]. The procedure was repeated twice and the organic layers were washed by 4 vol. CH_3OH/1.2 N HCl (1/1, v/v) [30]. Phospholipids were separated on oxalate-impregnated silica gel plates, using the bidimensional system in [3]. After autoradiography, the radioactive spots were scraped off and submitted to liquid scintillation counting.

2.4. *Determination of the release reaction*

[^{3}H]Serotonin was detected for radioactivity in supernatants obtained by centrifugation ($1500 \times g$, 5 min, +4°C) after platelet fixation by formalin [12]. *N*-Acetyl-β-D-glucosaminidase was measured in supernatants obtained after addition of 20 mM EDTA according to [31].

3. RESULTS

3.1. *Labelling pattern of platelet phospholipids*

Fig. 1 shows an autoradiogram of phospholipids extracted from ^{32}P-prelabelled platelets. As described in [3–4,6,13,18], only TPI, DPI, MPI and PA incorporated significant radioactivity (table 1). Another unidentified compound X represented 6% of the total radioactivity. Special care was taken to identify lyso MPI. A pure standard was clearly separated from DPI (fig. 1).

3.2. *Time course of phospholipid changes during platelet stimulation by PAF-acether*

Various changes occurred during platelet aggregation induced by PAF-acether (fig. 2) PA radioactivity was increased by 60% after 10 s stimulation and levelled off at 30 s, whereas an enhanced labelling of MPI appeared secondarily.

A biphasic response was observed for poly (PI): after 10 s, TPI was significantly reduced by 12%, whereas DPI displayed a concomitant increase. At

Table 1

Radioactive labelling of phospholipid classes in rabbit platelets

Phospholipids	Percentages
TPI	54.9 ± 7.5
DPI	24.3 ± 5.5
PI	9.1 ± 3
PA	5.1 ± 1
X	6.1 ± 1

Results are mean ± SD of the percentage of the label in each phospholipid class *vs* the whole platelet lipid radioactivity

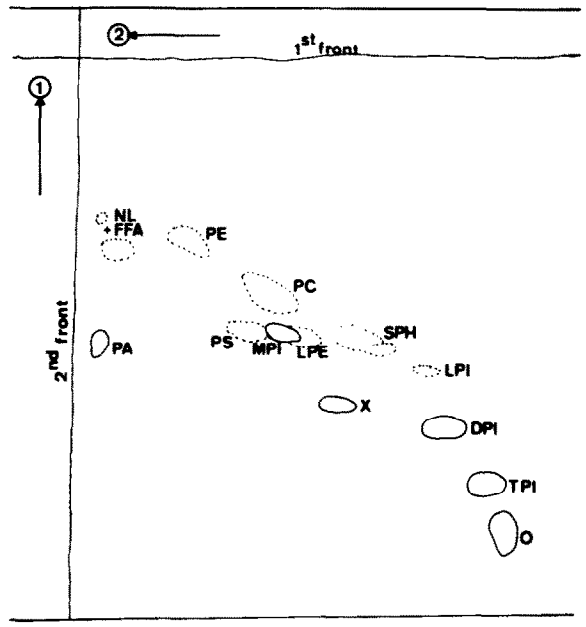

Fig. 1. Chromatogram of phospholipids extracted from pre-labelled platelets. For extraction conditions see section 2. (1) Solvent 1: $CHCl_3/CH_3OH/4$ N ammonia (9/4/2, by vol.); (2) Solvent 2: $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (60/30/12/5, by vol.); (– – –) non-radioactive iodine-stained lipids; (——) radioactive phospholipids detected by autoradiography; (O) origin; LPI, lysophosphatidylinositol (pure standard added to the lipid extract); SPH, sphingomyeline; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; NL, neutral lipids; FFA, free fatty acids; X, unkown; for other abbreviations, see the footnote.

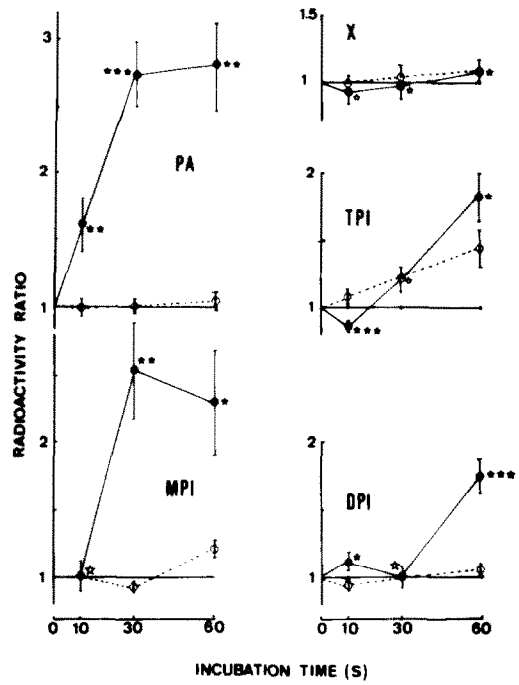

Fig. 2. Effect of PAF-acether on phospholipid metabolism of pre-labelled rabbit platelets. Results are expressed as the ratio of the radioactivity of a given phospholipid *vs* the radioactivity of the same phospholipid at time zero. Mean ± SD of 6 (10 s) or 10 (30 s and 60 s) independent experiments: assays (●——●); controls (○---○); $p < 0.05$, according to Student's *t*-test (★); $p < 0.02$, (★★); $p < 0.01$, (★★★); non-significant (☆).

later incubation times, radioactivity of TPI and DPI was enhanced over controls, the difference being significant for DPI at 60 s.

In contrast, phospholipid X did not display any significant modification during the whole incubation time. Also, no radioactive spot was detected at the position of lyso MPI.

Fig. 3 gives for comparison the time course of 3 platelet responses. At the [PAF-acether] used, serotonin release appeared as the earliest event and reached 30% of total serotonin at 10 s, when aggregation and *N*-acetyl-β-D-glucosaminidase release were still hardly detectable. However, the two secretory responses were completed within 30 s, whereas a full aggregation was obtained at 60 s.

4. DISCUSSION

This study reports 3 modifications of inositol–phospholipid metabolism occurring in rabbit platelets stimulated with PAF-acether. One is the activation of the MPI-specific phospholipase C, as evidenced by the production of PA, followed by an increased labelling of MPI confirming [9,13]. As discussed in [9], PA produced in the first seconds of stimulation might play a central role in the mechanism of platelet activation. This would fit with the observation that lyso PA are able to promote platelet aggregation [32,33].

As to the increased labelling of DPI and TPI happening at later times, similar results were reported in [13] by measuring the incorporation of exogenous-[^{32}P]phosphate into platelet phospho-

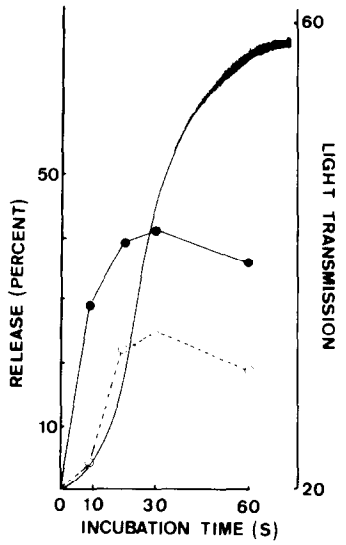

Fig. 3. Time course of aggregation and release reaction of rabbit platelets upon stimulation with PAF-acether. Results are the percentages of [^{3}H]serotonin (●—●) or *N*-acetyl-β-D-glucosaminidase (○---○) in supernatants *vs* the whole platelet content of these markers.

lipids during PAF-acether challenge. However, those changes could be due to an increased membrane permeability and might not reflect intrinsic changes of phospholipid metabolism. Such a possibility can be ruled out here, where platelets had incorporated the radioactive precursor which was only present on the cell interior. These modifications are similar to those induced by thrombin [18,20,21] and might correspond to an increased phosphorylation process. In the later case, poly(PI) increase was more closely parallel to aggregation and *N*-acetyl-β-D-glucosaminidase release [21]. It still remains difficult to understand the possible relationship between these biochemical modifications and the process of platelet activation.

The last change we found was a decrease of TPI occurring in the first seconds of platelet stimulation by PAF-acether. Such a modification was described in thrombin-treated horse platelets [22]. The authors gave evidence that TPI hydrolysis is a calcium-independent process and they suggested that it migh be due to a phosphomonoesterase [23]. Such an hypothesis could be reinforced from our present results, since we found a concomitant accumulation of DPI. However, one must notice that this counterbalancing increase of DPI is not stoichiometric to the decrease of TPI since relative changes were taken in account, whereas TPI radioactivity is twice higher than DPI radioactivity.

According to [22,23], activation of TPI phosphomonoesterase might be linked to occupancy of the thrombin receptor and could be involved in the release of membrane-bound calcium, leading to further activation of various calcium-dependent events. Some evidence is available for the existence of a platelet membrane receptor of PAF-acether [34,35], which would suggest that both PAF-acether and thrombin might act by a common mechanism. However, further studies are still necessary to better know the platelet enzymes responsible for poly(PI) metabolism as well as the factors involved in their regulation.

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REFERENCES

[1] Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587.
[2] Mauco, G., Chap, H. and Douste-Blazy, L. (1979) FEBS Lett. 100, 367–370.
[3] Lloyd, J.V., Nishizawa, E.E., Haldar, J. and Mustard, J.F. (1972) Brit. J. Haematol. 23, 571–586.
[4] Lloyd, J.V. and Mustard, J.F. (1974) Brit. J. Haematol. 26, 243–253.
[5] Bills, T.K., Smith, J.B. and Silver, M.J. (1976) Biochim. Biophys. Acta 424, 303–314.
[6] Mauco, G., Chap, H., Simon, M.F. and Douste-Blazy, L. (1978) Biochimie 60, 653–661.
[7] Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Proc. Natl. Acad. Sci. USA 76, 3238–3241.
[8] Lapetina, E.G., Billah, M.M. and Cuatrecasas, P. (1981) Nature 292, 367–369.
[9] Lapetina, E.G. (1982) J. Biol. Chem. 257, 7314–7317.
[10] Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 11856–11859.
[11] Broekman, M.J., Ward, J.W. and Marcus, A.J. (1980) J. Clin.Invest. 66, 275–283.
[12] Holmsen, H., Dangelmeier, C.A. and Holmsen, H.K. (1981) J. Biol. Chem. 256, 9393–9396.

[13] Shukla, S.D. and Hanahan, D.J. (1982) Biochem. Biophys. Res. Commun. 106, 697–703.
[14] Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–147.
[15] Hawthorne, J.N. and Pinckard, M.R. (1979) J. Neurochem. 32, 5–14.
[16] Putney, J.W. jr (1981) Life Sci. 29, 1183–1194.
[17] Berridge, M.J. (1981) Mol. Cell. Endocrinol. 24, 115–140.
[18] Kaulen, H.D.R. and Gross, R. (1976) Thromb. Haemost. 35, 364–376.
[19] Best, L.C., Bone, E.A. and Russel, R.G.G. (1981) FEBS Lett. 134, 88–90.
[20] Best, L.C., Bone, E.A. and Russel, R.G.G. (1982) Thromb. Res. 26, 241–247.
[21] Perret, B., Plantavid, M., Chap, H. and Douste-Blazy, L. (1983) Biochem. Biophys. Res. Commun. in press.
[22] Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 12705–12708.
[23] Billah, M.M. and Lapetina, E.G. (1982) Biochem. Biophys. Res. Commun. 109, 217–222.
[24] Demopoulos, C., Pinckard, R.N. and Hanahan, D.J. (1979) J. Biol.Chem. 254, 9355–9358.
[25] Benveniste, J., Tencé, M., Varenne, P., Bidault, J., Boullet, C. and Polonsky, J. (1979) CR Acad. Sci. (ser. D) Paris 289, 1037–1040.
[26] Blank, M.L., Snyder, F., Byers, L.W., Brooks, B. and Muirhead, E.E. (1979) Biochem. Biophys. Res. Commun. 90, 1194–1200.
[27] Hanahan, D.J., Demopoulos, C.A., Liehr, J. and Pinckard, R.N. (1980) J. Biol. Chem. 255, 5514–5516.
[28] Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970) Brit. J. Haematol. 19, 7–17.
[29] Chap, H., Mauco, G., Simon, M.F., Benveniste, J. and Douste-Blazy, L. (1981) Nature 289, 312–314.
[30] Schacht, J. (1981) Methods Enzymol. 72, 626–631.
[31] Day, H.J., Holmsen, H. and Hovig, T. (1969) Scand. J. Haematol. suppl. 7, 3–35.
[32] Benton, A.M., Gerrard, J.M., Michiel, T. and Kindom, S.E. (1982) Blood 60, 642–649.
[33] Simon, M.F., Chap, H. and Douste-Blazy, L. (1982) Biochem. Biophys. Res. Commun. 108, 1743–1750.
[34] Lalau-Keraly, C. and Benveniste, J. (1982) Brit. J. Haematol. 51, 313–322.
[35] Valone, F.H., Coles, E., Reinhold, V.R. and Goetz, J. (1982) J. Immunol. 129, 1637–1641.