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# The novel inositol lipid phosphatidylinositol 3,4-bisphosphate is produced by human blood platelets upon thrombin stimulation

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Radioactive PtdIns(3)*P* was detected in human platelets incubated with [$^{32}$P]$P_i$, but remained unaffected by thrombin treatment. In contrast, [$^{32}$P]PtdIns(3,4)$P_2$ was absent from resting platelets, but was produced by thrombin-activated platelets in a dose- and time-dependent manner. [$^{32}$P]PtdIns$P_3$ was never found under these conditions. These changes are similar to those elicited in other cells by platelet-derived growth factor or the oncogene product pp60$^{c\text{-}src}$.

## INTRODUCTION

The concept of receptor-operated phosphoinositide breakdown has deserved a great deal of research since its discovery by Hokin & Hokin (1955). However, the model is still evolving, even though the linkage to $Ca^{2+}$ movements, suggested by Michell (1975), is now well accepted, although it is more complex than was believed previously (Berridge, 1989; Rana & Hokin, 1990).

Recently much evidence demonstrated the existence of novel inositol lipids, phosphorylated on the 3-position of the inositol ring (Whitman *et al.*, 1988). This represents a parallel pathway which could also be a means of compartmentation of the roles of inositol lipids. It has been demonstrated that fibroblasts possess two types of PtdIns kinases, leading to two pathways of phosphorylation. Type I PtdIns kinase leads to the synthesis of PtdIns(3)*P* (Whitman *et al.*, 1988) and PtdIns(3,4)$P_2$ (Auger *et al.*, 1989), type II PtdIns kinase being the source of the more abundant PtdIns(4)*P*. Interestingly, the type I kinase has been demonstrated to be physically associated to a complex formed by the polyoma virus middle-T antigen and the protein pp60$^{c\text{-}src}$ (Courtneidge & Heber, 1987; Kaplan *et al.*, 1987; Whitman *et al.*, 1987). The same physical association of the type I PtdIns kinase (or of a protein necessary to its activation) with a receptor-associated tyrosine kinase, i.e. PDGF receptor, has been demonstrated in smooth muscle cells (Auger *et al.*, 1989).

This association of type I PtdIns kinase with the protein pp60$^{c\text{-}src}$ makes platelets likely to possess the novel inositol lipids, owing to the fact that pp60$^{c\text{-}src}$ is present in large amounts in platelets and peripheral-blood lymphocytes (Golden *et al.*, 1986). This protein could be linked to the secretory processes, since it has been found to be enriched in the secretory granules of resting platelets (Rendu *et al.*, 1989) and of chromaffin cells (Parsons & Creutz, 1986).

We demonstrate here that human $^{32}$P-labelled platelets indeed contain basal low levels of [$^{32}$P]PtdIns(3)*P*, which are not modified upon thrombin addition. At the resting stage, they lack [$^{32}$P]PtdIns(3,4)$P_2$, which increases significantly upon thrombin stimulation. The precise role of this novel lipid still remains obscure.

## EXPERIMENTAL

Platelets were prepared as previously described (Ardlie *et al.*, 1970), the starting material being platelet concentrates, anti-coagulated as described by Aster & Jandl (1964), and obtained from the local blood bank (Centre Régional de Transfusion Sanguine, Toulouse, France). Platelet suspensions in a $Ca^{2+}$-free Tyrode's buffer (pH 6.5, with 0.2 mM-EGTA) were labelled during 90 min at room temperature with 185 MBq of sodium [$^{32}$P]orthophosphate (Amersham International, Amersham, Bucks., U.K.), then washed with the same buffer lacking EGTA, and finally suspended at a final concentration of $5 \times 10^8$ cells/ml in Tyrode's buffer, pH 7.4, containing 0.34% BSA.

Platelets were incubated with human thrombin (Sigma, St. Louis, MO, U.S.A.) at the concentrations shown in the Figures for adequate periods of time, at 37 °C in a shaking water bath.

Lipids were extracted as described by Schacht (1981) as modified (Mauco *et al.*, 1978, 1987). Briefly, extraction was performed in the presence of 20 mM-EDTA and 0.6 M-HCl, at 4 °C in order to extract acidic lipids with minimal damage to ester links. Analysis of the deacylated lipids was performed exactly as described by Auger *et al.* (1989), either on the discrete spots obtained after t.l.c. (Gonzalez-Sastre & Folch-Pi, 1968) or on the total lipid extract. Authentic tritiated PtdIns, PtdIns(4)*P* and PtdIns(4,5)$P_2$ (Amersham) as well as [$^{32}$P]phosphatidic acid and [$^{32}$P]$P_i$ were used to confirm the positive identification of the lipids formed in platelets. Furthermore, the elution profile of the most common inositol phosphates was checked, and these were found to be separated from the deacylated phospholipids. Finally, each assay mixture of deacylated lipids was added to a tracer amount of non-labelled ATP and ADP. The $A_{260}$ of the nucleotides allowed standardization of the elution times, which were not significantly different from one run to the other. Radioactivity eluted from the 4.6 mm × 100 mm Partisphere SAX column (Whatman International, Maidstone, Kent, U.K.) was monitored and quantified with a Berthold LB506C detector (Munich, Germany), by using the Čerenkov effect for $^{32}$P or after admixture of Liquiscint 303 (Zinsser, Maidenhead, Berks., U.K.) for tritiated samples.

## RESULTS

### $^{32}$P labelling of PtdIns(3)*P* and PtdIns(3,4)$P_2$ in resting and stimulated platelets

Fig. 1 shows the elution profile obtained by h.p.l.c. of deacylated lipids from $^{32}$P-labelled resting and activated platelets.

Abbreviation used: PDGF, platelet-derived growth factor.

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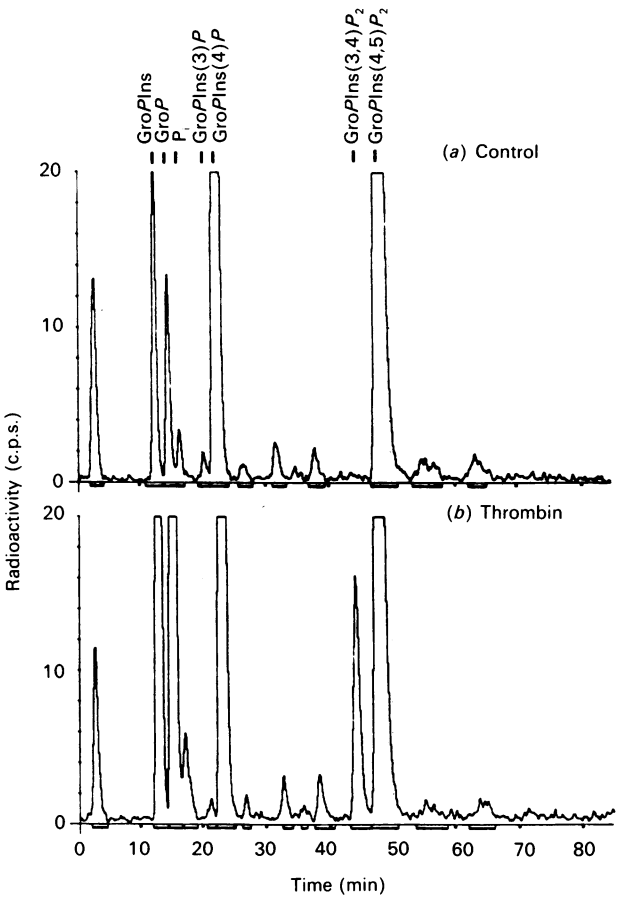


**Fig. 1. H.p.l.c. profiles of the deacylated inositol phospholipids from total lipid extracts**

$^{32}P$ labelled platelets ($1.5 \times 10^9$ cells) were incubated for 5 min at 37 °C in the absence (Control) or in the presence of 1 IU of thrombin/ml. Lipids were extracted, submitted to alkaline hydrolysis, and their deacylated derivatives were separated by h.p.l.c. as described in the Experimental section. Abbreviations: Gro*P*Ins, *sn*-glycero-3-phosphoinositol; Gro*P*Ins(3)*P*, *sn*-glycero-3-phosphoinositol 3′-phosphate; Gro*P*Ins(4)*P*, *sn*-glycero-3-phosphoinositol 4′-phosphate; Gro*P*Ins(3,4)$P_2$, *sn*-glycero-3-phosphoinositol 3′,4′-bisphosphate; Gro(4,5)*P*Ins$P_2$, *sn*-glycero-3-phosphoinositol 4′,5′-bisphosphate.

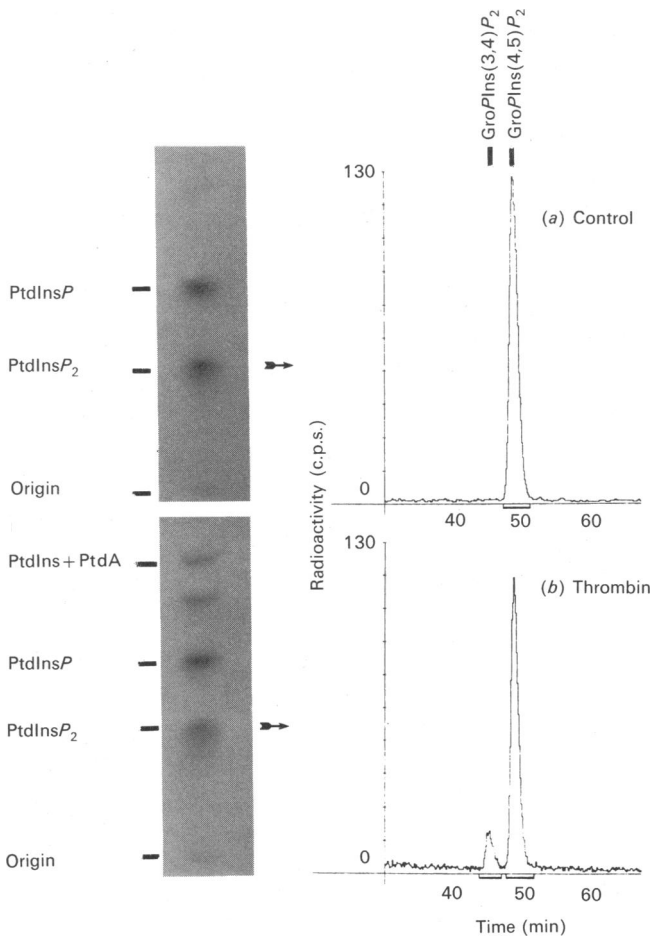


**Fig. 2. Identification of the novel inositol lipids in the spots obtained by t.l.c.**

Platelets were incubated for 5 min at 37 °C as described in the legend to Fig. 1. In this case, lipids extracted from $1.5 \times 10^9$ cells were separated by t.l.c., followed by autoradiography (left panels). The spots containing PtdIns$P_2$ (arrows) were scraped off, submitted to alkaline hydrolysis, and the deacylated derivatives were separated by h.p.l.c. (panel *b*) as described in the Experimental section. For abbreviations, see Fig. 1. Abbreviation: PtdA, phosphatidate.

The elution pattern was very similar to those described by others, and allowed for the identification of all inositol phospholipids. It was possible to detect in resting platelets a small peak of labelled PtdIns(3)*P*, as deduced from the elution time of deacylated PtdIns(3)*P* from the Partisphere SAX column. This represented only 1 % of the total radioactive PtdIns*P*s, and this proportion did not change after 5 min of stimulation by 1 IU of thrombin/ml. On the other hand, resting platelets did not contain detectable levels of [$^{32}$P]PtdIns(3,4)$P_2$ or [$^{32}$P]PtdIns$P_3$. However, [$^{32}$P]-PtdIns(3,4)$P_2$ was detected in stimulated platelets, as discussed below and as shown in Fig. 1. When lipid extracts were run on t.l.c. plates, it was not possible to discriminate between 4-phosphorylated and 3-phosphorylated inositol lipids, but alkaline hydrolysis and chromatography of the deacylated compounds on the Partisphere SAX column confirmed the identity of the products. The spot migrating on t.l.c. as PtdIns*P* was separated by h.p.l.c. into two peaks corresponding to deacylated PtdIns(3)*P* and PtdIns(4)*P* (results not shown), and the one identified as PtdIns$P_2$ appeared to contain both PtdIns(3,4)$P_2$ and PtdIns(4,5)$P_2$ in thrombin-treated platelets (Fig. 2).

## Time course of the appearance of the novel lipids in stimulated platelets

Thrombin stimulation induced the appearance of labelled PtdIns(3,4)$P_2$ after a lag time of 10 s (Fig. 3*a*), which did not parallel the classical modifications observed for the more usual radioactive PtdIns(4,5)$P_2$ or PtdIns(4)*P* (Fig. 3*b*). During the same time, the radioactivity of PtdIns(3)*P* did not vary significantly (Fig. 3*a*). The proportion of [$^{32}$P]PtdIns(3,4)$P_2$ versus the total radioactivity of PtdIns$P_2$ increased steadily up to 5 min of incubation, and then remained constant or slightly declined for the time of the experiment. At the maximum, [$^{32}$P]PtdIns(3,4)$P_2$ accounted for $11.0 \pm 0.4$ % (mean ± S.E.M. of six independent preparations) of the total [$^{32}$P]PtdIns$P_2$ after 5 min of stimulation. Conversely, the proportion of [$^{32}$P]-PtdIns(3)*P* remained constant at about 1 % of the total [$^{32}$P]PtdIns*P* (range 0.4–1.8 %) throughout the experiment.

## Lack of PtdIns$P_3$ production

Under all circumstances, [$^{32}$P]PtdIns$P_3$ remained undetectable, either in resting or in stimulated platelets.

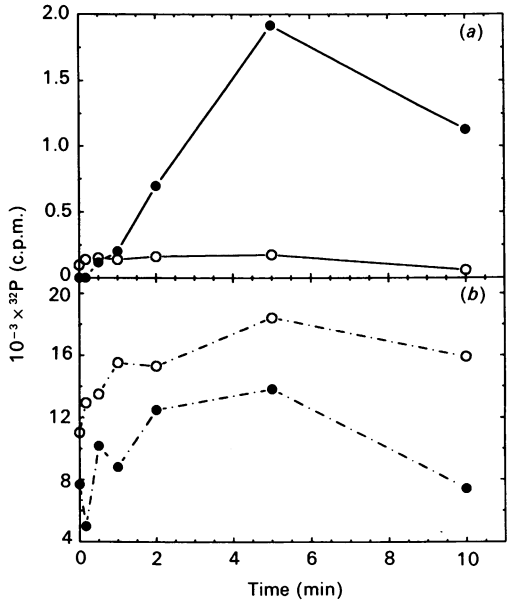


**Fig. 3. Time course of the $^{32}P$ labelling of the novel inositol lipids in stimulated platelets**

Platelets ($1.5 \times 10^9$ cells) were incubated with 1 IU of thrombin/ml at 37 °C. At the indicated times, lipids were extracted and processed as described in the Experimental section. Data are expressed as $^{32}P$ c.p.m. present in deacylated derivatives of PtdIns(3)$P$ (*a*, ○), PtdIns(3,4)$P_2$ (*a*, ●), PtdIns(4)$P$ (*b*, ○) and PtdIns(4,5)$P_2$ (*b*, ●).

### Effect of thrombin concentration

When studied at the optimal time of 5 min, the production of [$^{32}P$]PtdIns(3,4)$P_2$ followed a classical pattern of stimulation upon increasing the agonist concentration (Fig. 4). As little as 0.1 IU of thrombin/ml induced a detectable increase in the radioactivity of the novel PtdIns$P_2$, which levelled off at 1 IU of thrombin/ml.

## DISCUSSION

This study demonstrates the presence in platelets of the novel inositol phospholipids, phosphorylated at the 3-position, and the activation of their metabolism by human thrombin. All the data provided here have been obtained by radioactive quantification of the lipids. Although if it is agreed that the phosphomonoesters of the more usual inositol lipids, PtdIns4$P$ and PtdIns(4,5)$P_2$, are in isotopic equilibrium with the $\gamma$-phosphate group of [$^{32}P$]ATP (Tysnes *et al.*, 1987; Verhoeven *et al.*, 1987), this is not known at all for the novel inositol lipids phosphorylated at the 3-position of the inositol ring. In other words, it is still difficult to infer from our data whether the changes in $^{32}P$ radioactivity of PtdIns(3,4)$P_2$ reflect an increased turnover of the phosphomonoester groups, or whether there is a net synthesis of the phospholipid. However, mass determination of these compounds appears to be extremely difficult in the absence of specific means of separation (t.l.c., for instance). This was made impossible in our h.p.l.c. experiments, since the elution buffers contained ammonium phosphate. Another yet unanswered question is to know whether these changes are subsequent to modifications in the activity of PtdIns 3- and/or 4-kinases or of the corresponding phosphomonoesterases. However, a main conclusion of this study is that PtdIns 3-kinase is present in platelets and participates in the generation of novel phospholipids.

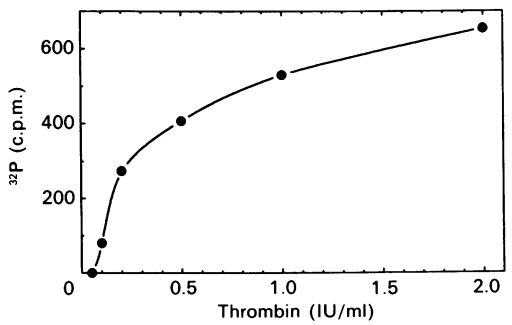


**Fig. 4. Effect of increasing doses of thrombin on the $^{32}P$ labelling of PtdIns(3,4)$P_2$**

Platelets ($1.5 \times 10^9$ cells) were incubated for 5 min with the given amounts of human thrombin, and total lipid extracts were processed as described in the Experimental section. Results are $^{32}P$ c.p.m. present in the deacylated derivative of PtdIns(3,4)$P_2$ in each assay.

So far, the presence of type I PtdIns kinase has been correlated with the mitogenic signal generated by growth factors, namely PDGF on fibroblasts (Whitman *et al.*, 1987) or BALB/3T3 cells (Kaplan *et al.*, 1987). In smooth-muscle cells, Auger *et al.* (1989) reported the occurrence of the products of this enzyme, i.e. PtdIns(3)$P$ and PtdIns(3,4)$P_2$. The association of type I PtdIns kinase with the complex polyoma virus middle-T antigen–pp60$^{c\text{-}src}$ has also been well documented (Courtneidge & Heber, 1987; Kaplan *et al.*, 1987; Whitman *et al.*, 1988). Furthermore, when transfected with PDGF receptors lacking the ability to associate with the PtdIns 3-kinase, cells show a much decreased response to the mitogenic effects of PDGF, even if they still display autophosphorylation of the PDGF receptor and phosphoinositide hydrolysis (Coughlin *et al.*, 1989). However, very little is known about inositol phospholipid 3-phosphorylations in terminally differentiated non-dividing normal cells. Platelets are somehow among the most terminally differentiated cells in mammals; therefore the observation that they are able to synthesize the novel inositol lipids is of paramount importance. Indeed, for the first time, a dose–response relationship is found between a physiological agonist and the production of PtdIns(3,4)$P_2$ in normal terminally differentiated cells. Also, the observation that the time course of labelling of this novel lipid was different from that of all other phospholipids suggests an exclusive regulation for the metabolism of this novel lipid. Furthermore, the amplitude of its variations was greater than for any other phospholipid, including phosphatidic acid. We did not find any significant amount of radioactivity eluted at a time consistent with deacylated PtdIns$P_3$ (i.e. after 70 min; see Fig. 1). However, this could have been due to inappropriate incubation times and/or amounts of radioactivity. If the latter is true, it means that [$^{32}P$]PtdIns$P_3$ is a very minor component, even after stimulation, since we were able to detect peaks containing as little as 100 c.p.m. of $^{32}P$. Thus, if present in platelets, [$^{32}P$]PtdIns$P_3$ could at most account for less than 0.5 % of the total radioactive inositol lipids.

The role of these novel lipids in non-mitogenically active cells is not yet known. However, one should notice that at least platelets and chromaffin cells contain high amounts of pp60$^{c\text{-}src}$ (Golden *et al.*, 1986; Parsons & Creutz, 1986). One common feature of these two cells is their ability to secrete the content of specialized granules. Interestingly, a very recent report by Pignataro & Ascoli (1990) describes the effect of epidermal growth factor on MA-10 Leydig cells. This factor is not a mitogen for these cells, but regulates their hormonal respon-

siveness to human choriogonadotropin and human luteinizing hormone; it makes the MA-10 Leydig cells produce PtdIns(3,4)$P_2$ in a dose-dependent manner. Altogether, these data suggest that PtdIns(3,4)$P_2$ production could be linked to the secretion properties of the cells. This hypothesis could be reinforced by the finding by Rendu *et al.* (1989) of a large enrichment of $pp60^{c\text{-}src}$ in the platelet dense granules. Another possibility is that a product secreted by platelets could be involved in the response observed after thrombin addition. Interestingly PDGF, which is secreted from α-granules, has been suggested as a ligand for platelets themselves (Bryckaert *et al.*, 1986, 1989). Such a possible role for PDGF would explain the lag phase observed in our studies, compatible with the time needed for the release of intracellular compounds. More advanced studies should elucidate this putative relationship.

During the preparation of this manuscript, a paper by Nolan & Lapetina (1990) reported essentially identical results in human platelets stimulated with 5 IU of thrombin/ml. It appears from the present data that PtdIns(3,4)$P_2$ is produced in response to more physiological concentrations of thrombin, i.e. as low as 0.1 IU/ml.

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