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ARE POLYPHOSPHOINOSITIDES INVOLVED IN PLATELET ACTIVATION ?

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SUMMARY : In human platelets, the amounts of triphosphoinositides (TPI) and diphosphoinositides (DPI) increase after 30 sec and level off after 120 sec of thrombin stimulation. After 180 sec of thrombin challenge, TPI and DPI increase accounts for 66 and 80 %, respectively. Polyphosphoinositide changes roughly parallel the release of N-acetyl-β-D-glucosaminidase and appear as a later event compared to aggregation and serotonin secretion.

It is concluded that an increased phosphorylation of polyphosphoinositides might participate in platelets to the process of stimulus-activation coupling and might be linked to thrombin receptor occupancy. A role of DPI in platelet activation is suggested by the observation that DPI promote platelet aggregation, the mechanism of which is discussed.

An increased turnover of inositol phosphatides upon specific cell stimulation has been recognized for a long time as a widely distributed event (1,2). During the most recent years, platelets revealed an interesting and convenient model to study the so-called "phosphatidylinositol effect". Extending previous data from Lloyd and Mustard (3), the activation of a phosphatidylinositol-specific phospholipase C was found as one of the earliest biochemical changes occurring during platelet activation (4-9). The suddenly produced diglycerides might serve several purposes, including release of arachidonic acid by a diglyceride lipase (10-14), stimulation of a phospholipid- and calcium-dependent protein kinase C (15) and production of phosphatidic and lysophosphatidic acids (7-10). These are able to promote cell activation owing to their calcium ionophoric properties (16-19), as well as to participate in arachidonic acid release (20).

However, relatively little attention has been brought to the platelet di- and tri-phosphoinositides (DPI and TPI). A few studies were devoted to

Abbreviations : TPI, triphosphoinositides ; DPI, diphosphoinositides.

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the labelling pattern of these compounds in resting and activated platelets and some of them led to the conclusion of an increased turnover of the polar moiety of DPI and TPI in activated platelets (3,21,22). In the present work, we show that thrombin stimulation induces a net synthesis of polyphosphoinositides. Furthermore, dispersions of DPI were found to promote platelet aggregation, suggesting a possible role of these phospholipids in transmembrane signal transmission.

MATERIALS AND METHODS

Materials

L-α-phosphatidylinositol 4-monophosphate (DPI), L-α-phosphatidylinositol 4,5-diphosphate (TPI) and human thrombin (3000 NIH units/mg of protein) were purchased from Sigma, Saint Louis, MO, USA. 5-[1,2-^{3}H(N)]-Hydroxytryptamine, creatinine sulfate (serotonin) (28 Ci/mmole) was from New England Nuclear, Boston, MA, USA.

Determination of TPI and DPI in thrombin-stimulated platelets

Platelet concentrates prepared from healthy human volunteer donors were obtained from the Blood Transfusion Centre and used within 18 hours after blood collection. Platelets were isolated and washed according to Ardlie et al. (23). The final suspension (5.10^8 cells ml^{-1}) was in Tyrode's buffer (pH 7.35) containing 5.5 mM glucose, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 0.35 % (w/v) bovine serum albumin. After equilibration for 30 sec at 37°C human thrombin (Sigma, final concentration 2 U/ml) or control buffer was added and platelets were incubated under stirring at 37°C for various times. The reaction was blocked by addition of EDTA (final concentration 12.5 mM), followed by 5 volumes of chloroform/methanol/10 N HCl (125/250/20, v/v) and lipids were extracted as previoulsy described (10). Separation of inositol phosphatides was carried out by thin-layer chromatography on silicagel F 254 precoated plates (Merck) using two successive solvent runs according to Farese et al. (24). After revelation with Dittmer reagent (25) and identification by comparison with pure standards, each spot was scrapped off and phosphorus was determined according to (26).

Determination of platelet aggregation and secretion induced by thrombin

Platelet suspensions were prepared as above except that they were incubated in the presence of [^{3}H] serotonin as in (9). Platelet aggregation was monitored by the method of Born (27) and secretion of serotonin and N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) was determined according to Holmsen et al. (9).

Determination of platelet aggregation induced by polyphosphoinositides

Platelet suspensions were prepared according to Lagarde et al. (28) from fresh human blood collected from normal volunteers having not taken any medication for at least 10 days. Aggregation was determined at 37°C according to Born (27). Cuvettes contained 250 µl of platelet suspensions ($3.5 . 10^8$ cells ml^{-1}) to which 2.5 µl of an ethanolic solution of pure DPI or TPI was added at the time indicated by the arrow.

RESULTS

Using the chromatographic system of Farese et al. (24), TPI and DPI were clearly separated from each other (Rf 0.28 and 0.39, respectively) and

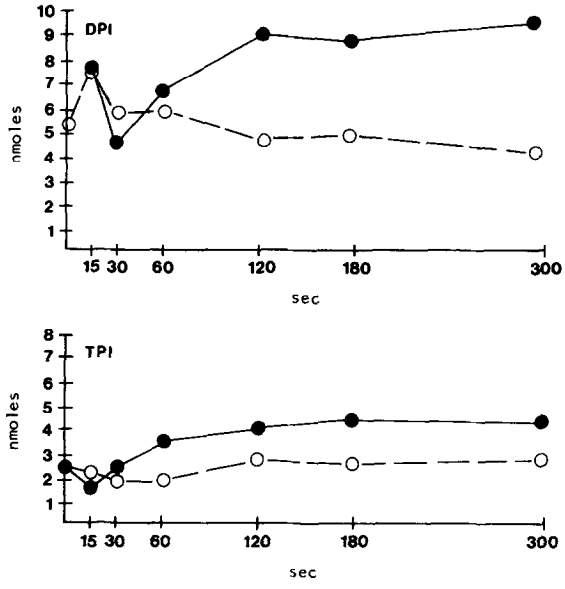

Fig. 1 : Variations of platelet polyphosphoinositides during thrombin-induced platelet aggregation. Platelet suspensions (4 ml, corresponding to 2.10^9 platelets) were incubated for the indicated times as described in the text. Dashed lines correspond to control incubations, continuous lines correspond to thrombin-treated platelets. Each point is representative of 2 to 7 experiments.

from the other phospholipids, including phosphatidic acid (Rf 0.85), lysophosphatidic acid (Rf 0.63), CDP-diglyceride (Rf 0.59), phosphatidylinositol (Rf 0.69), lysophosphatidylcholine (Rf 0.49) and lysophosphatidylinositol (Rf 0.54). Special care was taken to separate the two latter compounds, since their production through a phospholipase A_2 activated during thrombin-induced aggregation was already reported (7,29).

TABLE I : Tri- and di-phosphoinositide content of human platelets incubated in the absence or in the presence of thrombin

	nmoles/assay			nmoles/1000 nmoles of phospholipids		
	controls	thrombin	p	controls	thrombin	p
TPI	2.6 ± 0.2	4.3 ± 0.5	< 0.01	2.1 ± 0.3	3.5 ± 0.4	< 0.02
DPI	4.9 ± 0.5	8.8 ± 0.7	< 0.001	4.0 ± 0.6	7.3 ± 0.6	< 0.01

Incubations were performed for 3 min as described under Materials and Methods. Values are expressed either as nmoles/assay (corresponding to 2.10^9 platelets) or as nmoles/1000 nmoles of phospholipids. They represent means ± s.e.m. (n = 7). p : probability of significance according to Student's t-test.

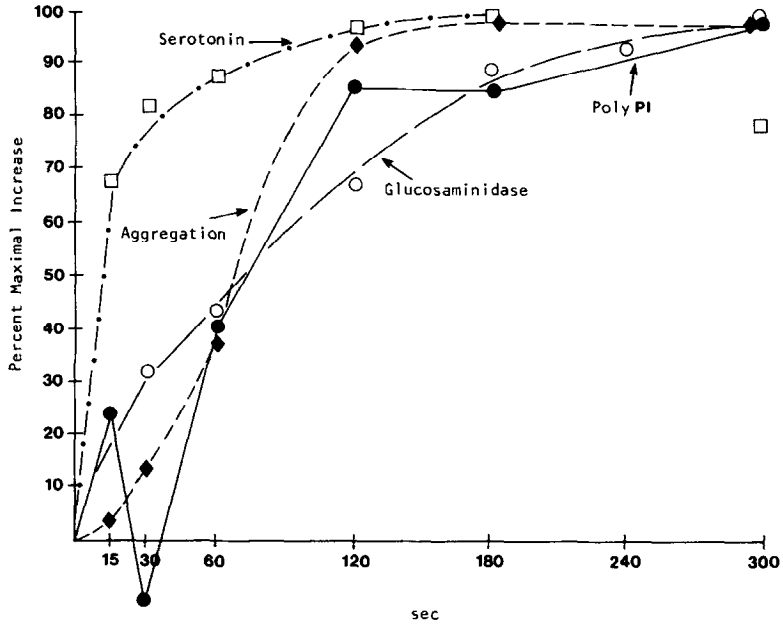

Fig. 2 : Time course of various platelet responses induced by thrombin. Platelet suspensions were prepared and incubated with thrombin as described under Materials and Methods. TPI + DPI correspond to data of Fig. 1. Results represent the percentages of maximal response.

As shown in Fig. 1, the amounts of both polyphosphoinositides remained stable in control platelets over the whole incubation procedure, whereas deep changes were observed upon thrombin stimulation. At the early stage of platelet activation, a small drop of both TPI and DPI was measured at 15 sec and 30 sec, respectively, but these variations were not statistically significant and were also observed to some extent in the controls. In the opposite, a parallel increase of both phospholipids was noted starting from that time ; it levelled off after 2 min and remained stable during at least 5 min. After 3 min of incubation, TPI and DPI displayed a 66 % and 80 % increase, respectively (Table I).

The time course of these metabolic changes is compared to that of other platelet responses in Fig. 2. It is clear that the polyphosphoinositide increase occurred starting from 30 sec, at which time it paralleled the release of N-acetyl-β-D-glucosaminidase, whereas serotonin secretion and aggregation appeared as earlier events.

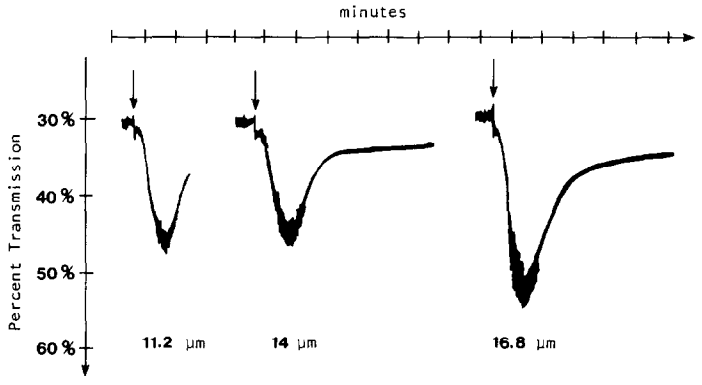

Fig. 3 : Platelet aggregation induced by diphosphoinositides. The numbers refer to the final concentrations of DPI (µM).

As shown in Fig. 3, micromolar concentrations of DPI produced a dose-dependent aggregation. This was always reversible and was never accompanied by serotonin secretion at the concentrations used. However, identical concentrations of TPI were without effect.

DISCUSSION

The present results indicate that, after a small drop occurring in the early seconds, thrombin stimulation of platelets induces a net synthesis of polyphosphoinositides. Production of TPI and DPI normally occurs via phosphorylation through specific kinases (1,2). Their catabolism involves either dephosphorylation by phosphatases or loss of the polar head group through the action of phosphodiesterases (1,2). Our data thus suggest that thrombin stimulation of human platelets greatly promotes the phosphorylation pathway.

Under similar conditions of incubation, the phosphatidylinositol content of total platelet goes down from 5 % to 3.5 % of total cell phospholipid (6,7). Around 80 % of this loss is recovered as phosphatidic acid and lysophosphatidylinositol, the sum of which thus corresponds to 1.2 % of total phospholipids (29). So the production of polyphosphoinositides might account for this incomplete recovery, as the TPI + DPI increase measured in the present study corresponds to 0.5 % of total cell phospholipids (see Table I). One can thus conclude that phosphatidylinositol displays three major pathways of

conversion in thrombin-activated platelets : degradation by phospholipase C, hydrolysis by phospholipase A_2 and phosphorylation into polyphosphoinositides.

The possible physiological significance of an increased production by platelets of polyphosphoinositides upon thrombin challenge remains presently unknown. The effects of thrombin on human platelets are rather complex and Holmsen et al. (9) recently showed that, if aggregation and release of dense bodies are the consequence of the initial action of thrombin on these cells, release of arachidonic and of lysosomal enzymes requires a continuous binding of thrombin to its membrane receptor. From the time course experiments, it might be tempting to speculate that the increased phosphorylation of phosphatidylinositol could be related to thrombin receptor occupancy. However, this is not proved in the present study and would require further investigations.

Our results would fit with those of Farese et al. (24,30), showing that adrenocorticotropin as well as parathormone stimulate polyphosphoinositide synthesis in their respective target cells. Moreover, these authors reported that DPI are able to reproduce some effects of adrenocorticotropin, like stimulation of pregnenolone synthesis and secretion, suggesting that DPI might directly participate in signal transmission (31). As shown in Fig. 3, DPI are able to produce the same kind of effects towards platelets. But activation induced by DPI remains limited to shape change and aggregation, since no evidence of release reaction could be found. As to the higher potency of DPI versus TPI, this still remains unexplained.

In short, our study shows that thrombin stimulation of human platelets induces a phosphorylation process leading to the accumulation of polyphosphoinositides. Till now, studies of phosphorylation during platelet activation have been mostly focused on diglycerides (7-10) and on proteins (32-34). In this respect, Jolles et al. (35) recently demonstrated that the ACTH-sensitive protein kinase is able to phosphorylate DPI. The hereby described phosphorylation of inositolphospholipids might thus be interesting to study in relation to protein phosphorylation occurring in stimulated platelets.

Another aspect of this finding which remains open to question concerns the mechanism by which polyphosphoinositides participate in the process of platelet activation. Anionic phospholipids have been described as calcium ionophores and might be involved in signal transmission owing to their calcium gating properties (16-18). But other possibilities are also worth being explored, since anionic phospholipids have also been shown to carry calmodulin-like properties (36). In conclusion, the production of polyphosphoinositides observed in thrombin-stimulated platelets might offer a new interesting model to study the biochemical events linked to agonist-effector cell membrane interactions.

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