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Rapid and transient thrombin stimulation of phosphatidylinositol 4,5-bisphosphate synthesis but not of phosphatidylinositol 3,4-bisphosphate independent of phospholipase C activation in platelets

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When platelets are stimulated by thrombin they immediately undergo inositol lipid hydrolysis via phospholipase C activation. However, subsequently an increased production of phosphatidylinositol 4,5-bisphosphate is observed. Phospholipases C were inhibited by lowering the cytoplasmic free calcium concentration by preincubation with Quin-2-tetra(acetoxymethyl) ester. Aggregation and secretion were also totally suppressed. Under these conditions we observed an increased labeling of phosphatidylinositol 4,5-bisphosphate, indicating a stimulation of inositol lipid kinases, independent of lipid hydrolysis by phospholipase C. Conversely the production of phosphatidylinositol 3,4-bisphosphate was totally abolished. These results suggest a different regulation of the kinases/phosphatases responsible for the production of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4-bisphosphate.

Phosphoinositide kinase; Phosphoinositide turnover; Calcium chelator

1. INTRODUCTION

It has been shown that when platelets, like many other cells, are stimulated, they undergo a hydrolysis of inositol lipids by one or more phospholipases C, and display a stimulation of inositol lipid phosphorylation, leading to an increase of PtdIns4*P* and PtdIns4,5P_2 [1,2] and to the production of PtdIns3,4P_2 [3–5]. The action of the phospholipases C is well known to produce increases of calcium via inositol *tris*phosphate release and protein kinase C activation due to the production of diacylglycerol, but little is known about the role of the increases of PtdInsP_2. However, some data indicate that the amounts of PtdIns4,5P_2 are susceptible to regulating actin polymerisation [6,7], therefore modulating the shape change and/or thrombus retraction. It has been postulated that the reported increases of PtdInsP_2 (and to some extent of PtdIns*P*) are due to a 'withdrawal effect' [8] elicited by the hydrolysis of the end-product of a chain of kinases and phosphatases. Such a hydrolysis was then supposed to be responsible for the compensating activation of these kinases and inhibition of phosphatases. Another possibility is that thrombin binding is, by itself, able to stimulate the kinases (or inhibit the phosphatases). Indeed in some cells the stimulation of receptors by their agonists has been described to directly affect the inositol lipid kinases as well as other kinases [9]. Such a mechanism encompasses the binding of inositol lipid kinases to a complex formed by the receptor itself or some other proteins, such as $pp60^{v\text{-}src}$ or polyoma virus middle T antigen, and other regulating proteins. So far this kind of association has been described for receptors with intrinsic tyrosine kinase activity, i.e. the platelet-derived growth factor [9]. When stimulated by thrombin, platelets indeed display an increase in tyrosine phosphorylation of a full set of proteins [10,11]. One part of these phosphorylations appears to be dependent upon aggregation [12,13] like the major part of the PtdIns3,4P_2 production [14]. Until now, no data were available on the regulation of PtdIns 4-kinase nor PtdIns4*P* 5-kinase in platelets. Despite the possible importance of tyrosine phosphorylation, the link between thrombin receptor occupancy and this regulating event is totally missing, even if platelets contain exceptionally large amounts of a putative tyrosine kinase $pp60^{c\text{-}src}$, as well as other non-receptor tyrosine kinases. However, the structure of the thrombin receptor casts some doubt on its ability to be an intrinsic protein tyrosine kinase [15]. It would be of interest to determine if thrombin is, nevertheless, able to induce an activation of the phosphorylation of inositol lipids, in-

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Abbreviations: PtdIns, phosphatidylinositol; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; Quin-2/AM, Quin-2-tetra(acetoxymethyl) ester; PtdIns4*P*5-kinase, phosphatidylinositol 4-monophosphate 5-kinase; PtdOH, phosphatidic acid; DMSO, dimethyl-sulfoxyde.

dependently of any other sign of platelet activation, i.e. phospholipase C activation, secretion or aggregation.

To test the hypothesis of a direct action of thrombin on the phosphorylation of inositol lipids we took advantage of the ability of Quin-2 to chelate, with high affinity, cytosolic free calcium. Under these conditions we demonstrated that thrombin was still able to induce a slight but reproducible increase in the phosphorylation cascade producing PtdInP_2 but did no longer stimulate PtdInsP_2 hydrolysis as judged from the inhibition of PtdOH production. Interestingly, PtdIns3,4P_2 was not produced at all upon thrombin addition, in contrast to our previous data showing only a 60% reduction when aggregation was inhibited [14].

2. MATERIALS AND METHODS

2.1. *Platelet preparation*

Platelet preparation was as described previously [16] as modified [5] for the labeling of cells with [^{32}P]orthophosphate (100 μCi/ml in the first washing fluid, for 2 h at room temperature) Blood donors were informed volunteers, stating that they were free of drugs for the last ten days (Centre Régional de Transfusion Sanguine, Toulouse, France).

2.2. *Quin-2/AM incorporation*

Quin-2/AM incorporation was as described by Simon et al. [17]. Quin-2/AM (up to 200 μM) in DMSO or DMSO alone was added to the already ^{32}P-labeled platelet suspensions in calcium-free Tyrode's buffer (135 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 0.34% bovine serum albumin, pH 6.5). Incubations were for 30 min at room temperature. The final amount of DMSO was 1% of the total volume.

2.3. *Thrombin activation*

Thrombin activation of the platelets was obtained by adding 1 NIH unit of human thrombin (Sigma, Saint-Louis, MO, USA) per ml of suspension (7×10^8 platelets/ml in Tyrode's buffer, without added calcium except where it is mentioned, pH 7.4), already warmed at 37°C for 2 min Incubation was carried on for the indicated periods of time, under shaking.

2.4. *Lipid extraction*

Lipid extraction, which served also to quench the reactions, was according to a modification of the method of Bligh and Dyer [18] as described previously [5]. The analysis of the extracts was performed on silicagel G plates (Merck, Darmstadt, Germany) previously sprayed with 1% potassium oxalate. The elution solvent was chloroform/methanol/acetone/acetic acid/water, 80:26:24:16 (v/v) as described by Jolles et al. [19]. Determination of radioactivity of the different spots was performed by liquid scintillation counting after autoradiography. In some instances the analysis was performed after methylamine hydrolysis of the lipids. Deacylated extracts were then analysed by HPLC on a Partisphere SAX column 150 × 4.6 mm (Whatman, Maiden Head, England) with a 0-1 M ammonium phosphate buffer gradient [5,20].

2.5. *Miscellaneous*

In some experiments platelet lysis was evaluated by lactate dehydrogenase liberation [21] and $[Ca^{2+}]_i$ measured by spectrofluorimetry [17]. The platelet release reaction was checked by *N*-acetyl-β-D-glucosaminidase [22] and [^{14}C]serotonin appearance in the supernatants. Aggregation was checked by turbidimetry [23].

3. RESULTS

3.1. *Effect of Quin-2/AM on thrombin-induced release*

We checked that 30 min preincubation with 100 or 200 μM Quin-2/AM did not lyse the platelets (Table I). This treatment inhibited the aggregation induced by 1 NIH unit thrombin/ml, in a calcium-free Tyrode's buffer, by almost 100% at all the times tested (0–300 s). This treatment also inhibited the dense granule secretion and lysosomal release by more than 80% as judged by [^{14}C]serotonin or *N*-acetyl-β-D-glucosaminidase secretion (Table I). Addition of calcium in the extracellular medium partially reversed this inhibitory effect on platelet secretion at 30 s of thrombin stimulation. More-

Table I

Consequences of a platelet loading by high concentrations of Quin-2/AM on intracellular calcium level, cell lysis, serotonin release and PtdOH production: reversibility of these effects by addition of calcium to the extracellular medium

Quin-2/AM (μM)	$CaCl_2$ (mM)	$[Ca^{2+}]_i$ (nM)	Lysis (%)	Release (%)	[^{32}P]PtdOH (%)
0	0		1.8 ± 0.1	72.0 ± 4.4	100
0	2.5			68.0 ± 1.0	112
DMSO 1%	0		0.6 ± 0.6	67.0 ± 3.6	84.6 ± 11.5
DMSO 1%	2.5		1.8 ± 0.1	73.0 ± 2.3	93.8 ± 25 6
100	0	37	1.2 ± 0.6	10.0 ± 1.9	2.0 ± 2.0
100	2.5	269	1.1 ± 0.6	36.0 ± 1.2	79.0 ± 27.5
200	0	50	2.4 ± 0.7	11.0 ± 0.9	7.7 ± 6.3
200	2.5	173	1 8 ± 0.1	30.0 ± 4.7	99.0 ± 27.7

Platelets were incubated with 100 or 200 μM of Quin-2/AM (DMSO as carrier) then resuspended in the final buffer with or without 2.5 mM of calcium as indicated in section 2. $[Ca^{2+}]_i$ and cell lysis were estimated in resting platelets while serotonin release and PtdOH production were measured after 30 s of thrombin stimulation (1 NIH U/ml) as described in section 2. Results are means ± S.E.M. of three independent experiments except for $[Ca^{2+}]_i$ data which are representative of two independent experiments and for PtdOH level in the absence of Quin-2/AM but with 2.5 mM of calcium in the extracellular medium (n = 1).

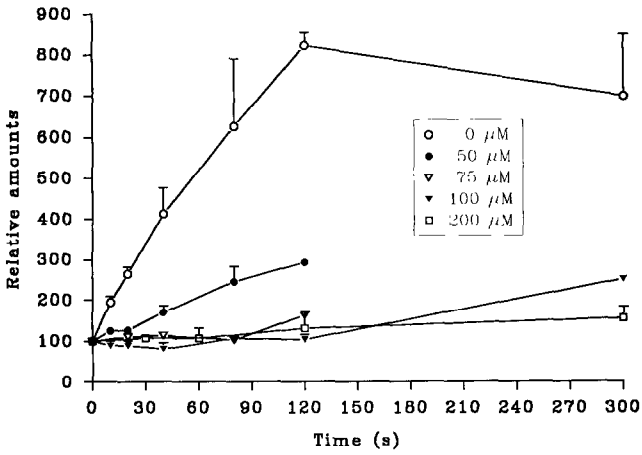

Fig. 1. Effect of increasing Quin-2/AM concentration on the production of PtdOH in thrombin-stimulated platelets. ^{32}P-labeled platelets were incubated with amounts of Quin-2/AM as indicated. After stimulation by 1 NIH unit thrombin/ml for the indicated periods, lipids were extracted and separated by thin-layer chromatography as described in section 2. Results are representative of 1 (Quin-2/AM = 75 μM) or 2 independent experiments (mean ± S.D.) and are the relative amounts of radioactivity present in the PtdOH spot, radioactivity at time 0 being taken as 100.

over, the presence of calcium in the extracellular medium provoked an increase of the intracellular calcium level in resting Quin-loaded platelets (Table I). These results argue in favor of the viability of cells preloaded by 100 or 200 μM of Quin-2/AM.

3.2. *Blockade of the activation of the phospholipase C*

When platelets were incubated with increasing amounts of Quin-2/AM, a progressive inhibition of the production of PtdOH elicited by 1 NIH unit thrombin/ml was obtained (Fig. 1). Concentrations as high as 100 μM were needed for a total inhibition of the PtdOH production during the time of experimentation (40 s). Preincubations with 100 μM Quin-2/AM were therefore chosen for the following experiments since it is generally believed that PtdOH production is a good indicator of the activity of phospholipase C. However, 200 μM Quin were used when evaluating the effect of calcium depletion on thrombin-induced PtdIns3,4P_2 production, since the incubation time was then 300 s. In some experiments, 100 μM Quin-2/AM were uneffective in totally blocking the production of PtdOH at 300 s. The effect of Quin-2/AM on the phospholipase C activity could be reversed by addition of calcium in the activation buffer (Table I). This suggests that inhibition of PtdOH production was not due to non specific effects of the calcium chelator or to byproducts, such as formaldehyde, released upon hydrolysis of Quin-2/AM.

3.3. *Effect of Quin-2/AM on the basal metabolism of inositol lipids*

We wanted to explore the inositol lipid metabolism in resting platelets preincubated with 200 μM of Quin-2/AM. Analysis of the [^{32}P]phospholipids by HPLC showed no significant changes of the ^{32}P incorporation into inositol lipids, particularly in the case of the PtdIns4,5P_2 which varied from 42.5 ± 0.6 in the absence of Quin-2 to 43.4 ± 0.6 with Quin-2 (data are the percentages of the total radioactive lipids, mean ± S.D., n = 3). The total radioactivity in Quin-2/AM treated platelets was 110 ± 23% of that in control cells.

3.4. *Time course of inositol lipid labeling upon thrombin stimulation*

When control platelets were challenged with 1 NIH unit thrombin/ml, we obtained the classical appearance of PtdOH and a transient decrease of PtdInsP_2 (Fig. 2). However, this was not still true when Quin-2-loaded platelets were challenged under the same conditions. Indeed, as already mentioned, PtdOH production was abolished and the metabolism of PtdInsP_2 was very different. As it can be seen in Fig. 2, instead of a decrease of the labeling of PtdInsP_2, obtained in control cells, an increase of the ^{32}P incorporation was observed and lasted for 20 s in Quin-2-treated platelets. This significant increase accounted for 11% by comparison with the basal level. Experiments prolonged after 60 s of thrombin activation showed no variation in the radioactivity of [^{32}P]PtdIns4,5P_2 at later periods (results not shown). HPLC analysis of the deacylated lipids (Fig. 3) revealed that no PtdIns3,4P_2 was produced, at any time, up to 300 s, while a maximal labeling was obtained for control platelets, as already reported [5]. The increase of labeling of PtdInsP_2, as observed by TLC, was then exclusively due to an increase of [^{32}P]tdIns4,5P_2. During the same period the labeling of PtdIns4P was not significantly altered by Quin-2 loading (Fig. 2). [^{32}P]PtdIns did not decrease as it was observed in control platelets, which is consistent with an inhibition of the phospholipase(s) C (Fig. 2).

4. DISCUSSION

The data reported above reveal that, when the hydrolysis of inositol lipids was impaired, thrombin was able to specifically increase the labeling of [^{32}P]PtdIns4,5P_2 in platelets. This suggests an action of the thrombin receptor on the PtdIns phosphorylation equilibrium, in a manner independent of the 'withdrawal' hypothesis. The conditions used in our experiments are close to those of other authors [17,24,25] who used the buffering capacity of Quin-2 to modulate the calcium levels in the cytosol. According to Johnson et al. [26], the external Quin-2/AM concentration is directly proportional to the final cytosolic Quin-2 concentration and under our conditions, 100 μM or 200 μM external Quin-2/AM the intracellular Quin-2 concentration should be about 10 or 20 mM, respectively. Lew et al. [25] demonstrated that 100 μM of Quin-2/AM has

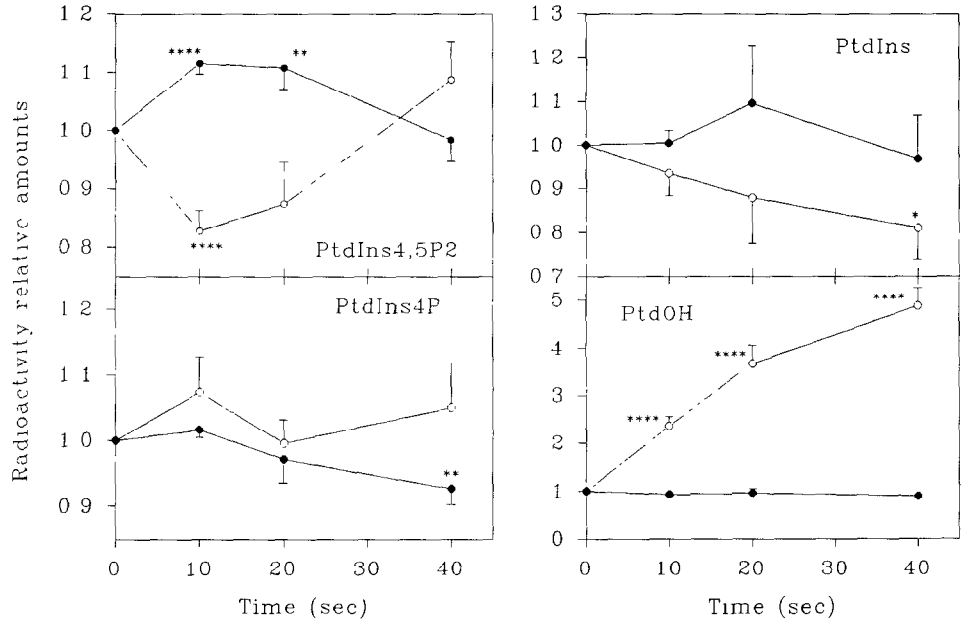

Fig. 2. Time course of radioactive inositol lipids and PtdOH production by platelets challenged with thrombin (1 NIH unit/ml) in the presence or absence of Quin-2/AM. Platelets were incubated with (closed circles) or without (open circles) Quin-2/AM as indicated in section 2 and then stimulated with thrombin for the indicated times. [^{32}P]Lipids were extracted and quantified after thin-layer chromatography. Results are means ± S.D. of 10 independent experiments. Data are expressed as relative amounts of radioactivity present in correspondent lipid species from platelets before stimulation. Student's *t*-test (unpaired) gave probabilities of the observed means to be identical as indicated. $*P < 0.05$, $**P < 0.01$, $****P < 0.001$.

a specific effect on the neutrophil metabolism, with maintenance of the ATP level and without influence of the formaldehyde formed. Under our experimental conditions, the free calcium concentration has been lowered to sub-resting levels. Under these stringent calcium-poor conditions, we succeeded in abolishing all the production of PtdOH, from which we infer that the phospholipase(s) C were inactive.

The stimulation of PtdIns4,5P_2 labeling is generally believed to reveal an increase in the mass of the lipid since 90 min preincubation of the platelets with $^{32}PO_4H_3$ has been shown to label the phosphomonoesters at equilibrium with the γ phosphate of ATP [27]. However, a precise determination of the mass is needed for the correct understanding of variations of phosphorylated lipids in living cells. Unfortunately, we were unable to measure the slight variations of mass by determination of phosphorus by colorimetry. Nevertheless we can assume, as it is classically admitted, that the equilibrium between PtdIns4*P* kinase and PtdIns4,5P_2 phosphatase as well as that between PtdIns kinase and PtdIns4*P* phosphatase are shifted in such a manner that the mass of PtdIns4,5P_2 is increased. We cannot infer from our data if the actual enzymes affected by thrombin stimulation are kinases (then stimulated) or phosphatases (inhibited). However, the indication of an increase of PtdIns4,5P_2 is an argument for a direct effect of thrombin on inositol lipid metabolism. As far as we can infer from what is known on transmembrane receptors, it seems that the thrombin receptor recently cloned [15] is of a type believed to interact directly with G proteins and not to belong to the tyrosine kinase receptor family. This would imply that a relay molecule is needed to activate inositol lipid metabolism. The increase is observed early (up to 20 s after addition of maximal doses of thrombin), therefore the machinery between the thrombin receptor and the kinase and/or phosphatase should be fast enough. It is interesting to note the result of Urumow and Wieland [28], showing the involvement of a small unidentified G protein in the regulation of rat

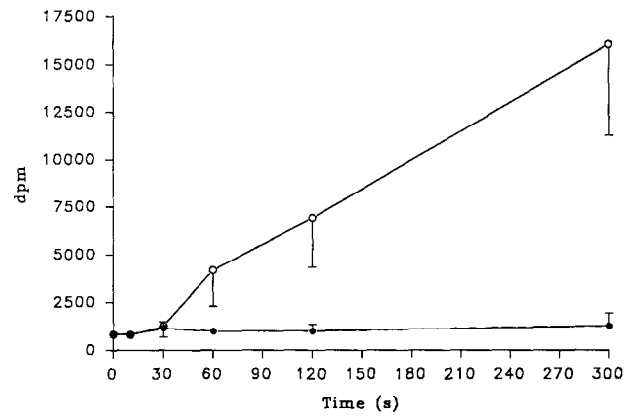

Fig. 3. Effect of Quin-2/AM on PtdIns3,4*P*2 production in thrombin-stimulated platelets. Platelets were incubated with 200 μM Quin-2/ AM, then stimulated by 1 NIH unit thrombin/ml. After extraction, radioactive phospholipids were deacylated and submitted to HPLC as described in section 2 Results are means ± S.E.M. of 2 independent experiments.

liver plasma membrane PtdIns4*P* kinase activity. On the other hand, it is also of interest to know that the PtdIns3,4P_2 production, recently described in platelets, is a late event [3–5] while the effect reported here is an early one. Usually it is believed that PtdIns3,4P_2 production is due to the activation of a PtdIns4*P* 3-kinase by a tyrosine protein kinase, which is itself activated as a consequence of aggregation [12–14]. However, recently the role of protein kinase C has also been demonstrated [29]. The total inhibition of PtdIns3,4P_2 production by Quin-2 could not be totally explained by the total lack of aggregation obtained under these circumstances. This inhibition is of a much greater amplitude than that obtained previously with the tetrapeptide RGDS, or even with Glanzmann's thrombasthenic patients who totally failed to aggregate to thrombin [14]. It is not known if any of the reported inositol lipid 3-kinases are calcium sensitive, and one should test the hypothesis of a more central mechanism for this inhibition. For instance it is quite obvious that protein kinase C is strongly inhibited by simultaneous calcium chelation and lack of diacylglycerol production.

A report by Lassing and Lindberg [30] already demonstrated that, at very early times of stimulation by small amounts of thrombin, a slight increase of PtdIns4,5P_2 could be observed, very transiently, just before the classical decrease of the lipid, due to phospholipase C action. Besides confirming this previous observation, our present data exclude the possibility that the increase of PtdIns4,5P_2 could occur in response to phospholipase C activation ('withdrawal effect' hypothesis). On the other hand, the work of Steen et al. [31] has shown a strong correlation between the ^{32}P incorporation into PtdIns4*P* and PtdIns4,5P_2 and the PtdOH production upon thrombin stimulation in platelets, even though they only explored the 90th second of thrombin stimulation. Therefore, our results and those of Lassing and Lindberg [30] on the one hand, and the results of Perret et al. [1], Mauco et al. [2] and Steen et al. [31] on the other, argue in favor of two steps in the PtdIns4,5P_2 synthesis. The first rapid step could be dependent directly on thrombin receptor activation. The second slower step (after the first min of thrombin activation) could be regulated by the phospholipase C/protein kinase C pathway. The possible existence of different pools of PtdIns4,5P_2 in platelets remain to be explored, which could be synthesized by different PtdIns4*P* 5-kinase isoforms, and have different functions. Perhaps, for the precocious pool of PtdIns4,5P_2 synthesized, a role in the regulation of the cytoskeleton organization as evoked by Lassing and Lindberg [30] could be proposed.

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