Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay

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Abstract Phosphatidylinositol 5-phosphate (PtdIns5P), a novel inositol lipid, has been shown to be the major substrate for the type II PtdInsP kinases (PIPkins) [Rameh et al. (1997) Nature 390, 192–196]. A PtdInsP fraction was prepared from cell extracts by neomycin chromatography, using a protocol devised to eliminate the interaction of acidic solvents with plasticware, since this was found to inhibit the enzyme. The PtdIns5P in this fraction was measured by incubating with [γ-^{32}P]ATP and recombinant PIPkin IIα, and quantifying the radiolabelled $PtdInsP_2$ formed. This assay was used on platelets to show that during 10 min stimulation with thrombin, the mass level of PtdIns5P increases, implying the existence of an agonist-stimulated synthetic mechanism.

Key words: Phosphatidylinositol 5-phosphate; Platelet; Thrombin; Mass assay; Type II phosphatidylinositol phosphate kinase; Acid inhibition

1. Introduction

Phosphatidylinositol phosphate (PtdInsP) kinases (PIPkins) are a growing family of enzymes, which phosphorylate PtdInsPs to form $PtdInsP_2$s (for review, see [1]). The type II PIPkins were first recognised as being distinct in their substrate specificity by Rameh et al. [2], who showed that they phosphorylate not PtdIns4P as do the type I isozymes, but PtdIns5P instead, offering an alternative pathway for the synthesis of PtdIns(4,5)P_2. Evidence was also presented for the natural occurrence of PtdIns5P in cells [2].

The principal product of both type I and type II PIPkins, PtdIns(4,5)P_2, is a crucial molecule involved in many signalling pathways [1]. It is the precursor for PtdIns(3,4,5)P_3, Ins(1,4,5)P_3 and diacylglycerol, and also has many cellular functions of its own, its principal roles being actin cytoskeleton assembly regulation [3], Golgi function [4], vesicle formation and endocytosis [5], potassium channel regulation (for review, see [6]) and exocytosis [7]. Thus the 4-OH phosphorylation of PtdIns5P could represent an alternative pathway to PtdIns(4,5)P_2, perhaps localised for a specific individual function of that lipid [1].

However, it may also be that PtdIns5P is a signalling molecule in its own right, and thus that the function of type II PIPkins is to remove it. We currently know little about the production or the regulation of metabolism of PtdIns5P. As a means of gaining insight into the function of this lipid, and also to provide a basis for further investigation into the type II PIPkins, we have developed a mass assay capable of determining accurately the levels of PtdIns5P in platelets. We have followed quantitatively the changes in PtdIns5P elicited by thrombin, an activator of platelets that is known to affect lipid levels, including PtdIns(4,5)P_2 [8–10]. This assay will also be suitable for application in other cell systems.

2. Materials and methods

2.1. Materials

Carrier-free, bovine serum albumin (BSA)-free human thrombin, controlled-pore glass beads and triethylamine were from Sigma Chemical Co. (St. Louis, MO, USA). Pure synthetic PtdIns5P was from Echelon Research Laboratories (Salt Lake City, UT, USA). PtdIns was from Doosan Serdary (Englewood Cliffs, NJ, USA). [γ-^{32}P]ATP was from New England Nuclear (Boston, MA, USA). [^{3}H]Ins(1,4,5)P_3 was from Amersham (Amersham, UK). Silica-coated thin-layer chromatography (TLC) plates were from Merck KGaA (Darmstadt, Germany). PI-cellulose plates were from Mackery-Nagel GmbH and Co. KG (Düren, Germany). Whatman 3MM paper and the Partisil-10 SAX high performance liquid chromatography (HPLC) column were from Whatman International (Torrance, CA, USA). Folch lipids were prepared in-house according to the method of Folch [11].

2.2. Platelet lipid extraction

Platelets were extracted from fresh whole human blood from healthy donors as described previously [12]. The platelets were incubated at 37°C for 5 min, then $CaCl_2$ was added to 2 mM and they were incubated for a further 5 min prior to experimental use.

Duplicate 90 μl platelet samples were added to 1 NIH-unit thrombin (carrier- and BSA-free) in plastic microtubes, and incubated at 37°C as required. Thrombin was omitted from control samples. To halt the stimulation, samples were transferred to glass microtubes containing 12.5 μl 70% (v/v) perchloric acid. Lipid extraction was carried out according to Bligh and Dyer [13] by adding 500 μl 1:1 (v/v) $CHCl_3$:CH_3OH to each sample, followed by pure PtdIns5P to some tubes as a routine check on recovery, and 1 NIH-unit of thrombin to control samples. Phases were separated by the addition of 125 μl of 2.4 N HCl, and samples were vortexed thoroughly and centrifuged at 10 000×g for 2 min at room temperature. Most (400 μl) of the upper phase was removed and discarded and replaced with 400 μl theoretical upper phase (3:48:47 (v/v) $CHCl_3$:CH_3OH:1 M HCl). This washing was repeated twice further. The final lower phase was removed and saved, and replaced with 175 μl theoretical lower phase (86:14:1 (v/v) $CHCl_3$:CH_3OH:1 M HCl) which was likewise vortexed, centrifuged and removed, and pooled with the first lower phase extraction. Duplicate samples were then pooled and dried by vacuum centrifugation.

2.3. Lipid purification

Neomycin-linked glass beads were prepared as described by Schacht

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[14]. Beads were aliquoted into glass microtubes to a packed volume of 25 μl, and storage solvent decanted from the pellet. Beads were pre-equilibrated with 120 μl 1:1 (v/v) $CHCl_3$:CH_3OH by mixing, incubating for 30 min at room temperature, mixing, centrifuging (10 000×*g*, 1 min) and pouring away. This was repeated with 120 μl $CHCl_3$.

Samples were loaded onto beads in 20 μl 1:1 (v/v) $CHCl_3$:CH_3OH, then the empty sample tubes were rinsed with a further 20 μl $CHCl_3$, which was also transferred to the beads. Carrier lipid (5 nmol PtdIns) was added to the beads in a volume of 10 μl 1:1 (v/v) $CHCl_3$:CH_3OH.

Loaded beads were washed first with 250 μl of a formate solvent (5:10:2 (v/v) $CHCl_3$:CH_3OH:H_2O, 50 mM ammonium formate final) according to Palmer [15] to remove all non-inositide lipids, vortexed, incubated at room temperature for 1 h, vortexing after 30 min and at the end of incubation, and then centrifuged as earlier and the solvent poured away. This was repeated. PtdInsP lipids were eluted finally by washing the beads using a solvent composed of 2:6:3 (v/v) $CHCl_3$:CH_3OH:2 M triethylamine bicarbonate (TEAB) (final TEAB concentration 0.55 M) using the working protocol as for the earlier ammonium formate solvent, firstly with 250 μl then 100 μl solvent, pooling the eluates in plastic microtubes. Eluates were dried down overnight. The 2 M TEAB was prepared fresh each time by bubbling CO_2 through a 2 M solution of triethylamine on ice until its pH was 4.5.

2.4. Lipid phosphorylation

Carrier lipid (20 nmol of PtdIns) was added to all samples and dried under nitrogen. The dried lipid samples were resuspended in 50 μl PIPkin buffer (Tris–HCl pH 7.4 50 mM, KCl 80 mM, Mg-acetate 10 mM, EGTA 2 mM) containing 0.1 mg/ml disodium deoxycholate by vigorous vortexing. Bacterially expressed human recombinant PIPkin IIα enzyme (0.1 μg) [16] was added to each sample. Phosphorylation reactions were initiated by the addition of radiolabelled ATP to a final concentration of 5 μM, 5 μCi [γ-^{32}P]ATP per sample. After incubation at 30°C for 1 h, reactions were halted by addition of 500 μl 1:1 (v/v) $CHCl_3$:CH_3OH followed by addition of 125 μl 2.4 N HCl and 20 μl Folch lipids from porcine brain as carrier [11]. Phosphorylated lipids were then extracted as described previously [12]. Phosphorylated lipids were resolved by TLC on silica-coated glass plates (activated for >30 min at 110°C after dipping in a solution of 50% CH_3OH, 1% potassium oxalate, 2 mM EGTA) in a Whatman 3MM paper-lined tank pre-equilibrated at room temperature (20–23°C) for 2 h. Plates were developed in a solvent of 28:40:10:6 (v/v) $CHCl_3$:CH_3OH:H_2O:NH_4OH, for 100–120 min, and radioactive lipids visualised by autoradiography.

2.5. PtdInsP analysis

$PtdInsP_2$ spots were excised from the silica plates, and either counted immediately by scintillation counting (for quantitative assessment) or processed further for analysis of individual phosphate moieties. The latter was carried out first by deacylating [17] and then deglycerating [18] the samples as described previously [19], though deglycerating by sodium periodate was for 20 min as opposed to the 90 min described, as this was found to be sufficient to convert all the $GroPIns(4,5)P_2$ to $Ins(1,4,5)P_3$. Most $InsP_3$ samples thus generated were dephosphorylated by recombinant type I inositol polyphosphate 5-phosphatase enzyme [20] for 60 min at 37°C to yield $InsP_2$ and inorganic phosphate. Samples were separated by HPLC on a Whatman Partisil-10 SAX column using a NaH_2PO_4 (pH 3.75) elution gradient from 0 to 0.75 M over 65 min. Samples were collected at 45 s intervals for scintillation counting.

For some samples, deglyceration was not carried out and the $GroPInsP_2$s were analysed by PI-cellulose TLC. Samples were dissolved in 2 μl 10 mM KH_2PO_4/20 mM HCl, centrifuged (10 000×*g*, 1 min) and loaded onto a PI-cellulose plate in small (~0.4 μl) aliquots, drying between aliquots. This was repeated, to ensure complete loading, and plates were allowed to dry fully. Loaded plates were placed in a TLC tank containing 50 ml 0.48 M HCl and left to resolve for up to 45 min. Plates were removed and, after thorough drying, exposed to X-ray film.

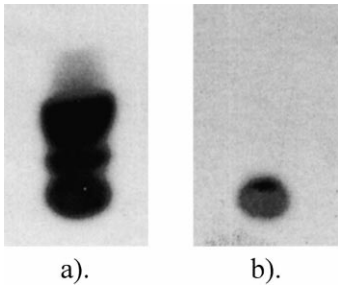

Fig. 1. Solvents were dried in plastic microtubes prior to their use in a standard PIPkin assay on 1 nmol PtdIns5P. Figures show autoradiographs of TLC plates to demonstrate PIP_2 generation from substrate by PIPkin IIα. Solvents were (a) $CHCl_3$ and (b) theoretical lower phase (see Section 2). The three radioactive spots in (a) are (from the bottom): origin, lyso-$PtdIns(4,5)P_2$ and $PtdIns(4,5)P_2$. In (b) only the origin is radiolabelled.

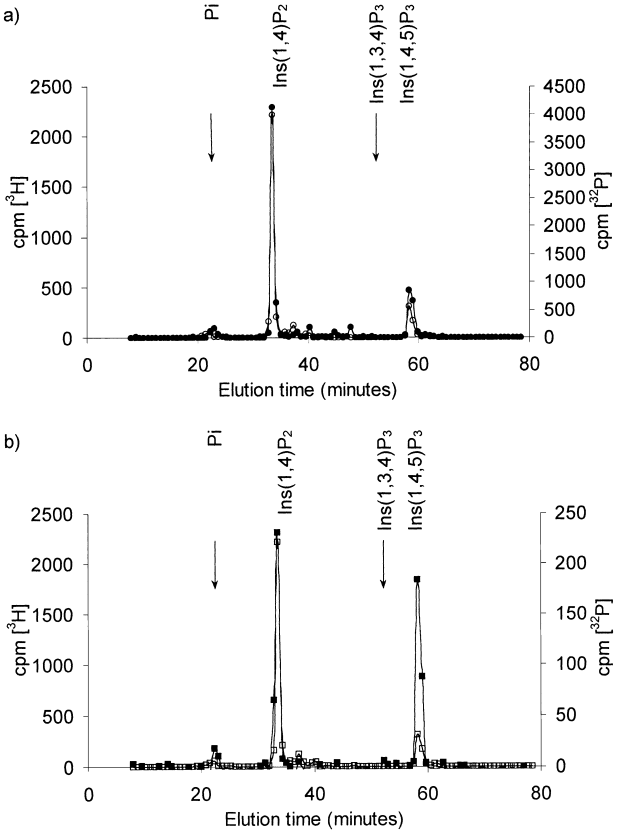

Fig. 2. Qualitative HPLC analysis of lipids and standards by scintillation counting of collected fractions. (a) 5-Dephosphorylated [^{3}H]$Ins(1,4,5)P_3$ (open circles) and 5-dephosphorylated $InsP_3$ generated from [γ-^{32}P]-phosphorylated pure PtdIns5P (closed circles). (b) 5-Dephosphorylated [^{3}H]$Ins(1,4,5)P_3$ (open squares) and 5-dephosphorylated $InsP_3$ generated from [γ-^{32}P]-phosphorylated PtdInsP extracted from unstimulated platelets (closed squares).

3. Results and discussion

3.1. Development of the PtdIns5P assay

Preliminary attempts to quantify platelet PtdIns5P were hindered by inhibition of the type IIα PIPkin enzyme, which was eventually traced to two sources. The first was due to lipids present in the platelets that were inhibitory to the final assay with recombinant PIPkin, and this problem was easily eliminated by neomycin bead chromatography of the extracted lipids to purify the PtdIns5P. The second problem was more variable, and was eventually tracked down to interactions between some batches of the plasticware in common use in our laboratory with acidic chloroform-based solvents.

For example, Fig. 1 shows autoradiographs from a standard PIPkin assay performed in dry tubes that had either been prerinsed with pure chloroform (Fig. 1a) or with acidic lower phase (from Bligh and Dyer [13], Fig. 1b). Clearly, plasticware previously in contact with acidic lower phases causes inhibition of the enzyme assay. We did investigate carrying out all procedures in glass, but the final enzyme assay proved too variable under these conditions, probably due to adsorption of the ATP, and possibly also of the PtdIns5P. However, we put the neomycin bead chromatography ultimately to a further purpose, as purification of the PtdInsP fractions on neomycin beads removed any inhibitory substances derived from the previous acidic lipid extraction procedures. The final elution solvent containing TEAB (see Section 2) was designed to be sufficiently volatile that it could be removed by vacuum centrifugation without any need for phase separations, thus avoiding the need for any acids to be used after the neomycin chromatography.

Prior to quantification of PtdIns5P in platelets, it was necessary to establish firmly that the only lipid to be phosphorylated would be PtdIns5P. We confirmed the observations of Rameh et al. [2] that in vitro the type II PIPkin phosphorylates PtdIns5P more than 100-fold faster than PtdIns4P, and about 15 times faster than PtdIns3P if pure lipid substrates are used at 10 μM (not shown). However, other lipids in the extract (not removed by neomycin chromatography) may affect this specificity. Any complication with PtdIns3P was easily eliminated, as analysis of the GroPtdInsP_2 by PI-cellulose chromatography or InsP_3 by HPLC derived from radiolabelled PtdInsP_2 formed during the assays revealed that it was entirely the 4,5 and not the 3,4 isomer (see for example Fig. 2). A more serious concern was that as there is likely to be much more PtdIns4P present in platelets than PtdIns5P [2], even the greater than 100-fold preference of the type II PIPkin for PtdIns5P may not be sufficient to guarantee specificity of the assay. However, the data (Fig. 2) show that with either pure PtdIns5P or platelet lipid PtdInsP fraction as a substrate, all the detectable ^{32}P label generated by 5-dephosphorylation of the Ins(1,4,5)P_3 derived from the PtdInsP_2 was recovered in the Ins(1,4,)P_2, and not as inorganic phosphate. These results confirm that the PtdIns(4,5)P_2 formed during the assay was labelled in the 4, not the 5, position of the inositol ring. Thus overall these experiments show that, under these conditions, incorporation of radiolabel into a PtdInsP_2 fraction truly allows accurate quantification of PtdIns5P in the lipid extract being assayed.

The assay was found to be linear with PtdIns5P quantities up to 100 pmol (not shown). The radiolabelling efficiency varied between experiments, so in all platelet experiments standard amounts of PtdIns5P were added to some samples to check recovery from the starting material, and as a standardisation of each assay. Platelets were stimulated with 1 NIH-unit of carrier-free human thrombin at 37°C for 1, 2, 5 or 10 min and the mass of PtdIns5P was compared with these standards.

3.2. PtdIns5P measurement in platelets

Fig. 3 demonstrates the combined results from four experiments investigating the presence of a time-dependent change in PtdIns5P levels on thrombin stimulation. Resting platelets had a PtdIns5P level of 15.77 ± 1.65 pmol/mg platelet protein, expressed as a mean of four experiments ± S.E.M. After a significant rise in PtdIns5P levels to 358 ± 104% of control levels after 5 min thrombin stimulation, a fall is seen to 202 ± 67% control levels after a total of 10 min. This is in contrast with earlier data for PtdIns, PtdIns4P and PtdIns(4,5)P_2 which are all found to decrease on thrombin stimulation [10].

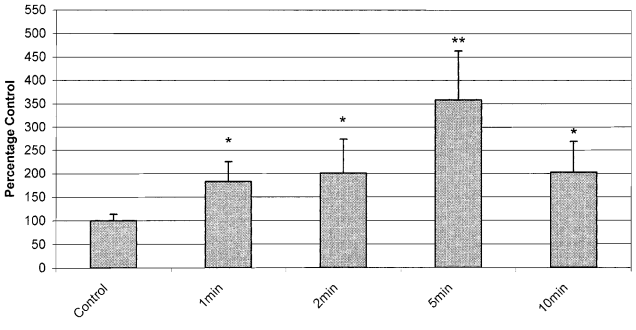

Fig. 3. Quantitative analysis of PtdIns(4,5)P_2 generated from PtdIns5P extracted from platelets either unstimulated or thrombin-stimulated for various time periods. Bars show mean percentages of control levels for four experiments (2 min: three experiments)+ S.E.M. Control levels of PtdIns5P in the four experiments were 15.77 ± 1.65 pmol/mg protein. One-tailed Student's *t*-test: $*P < 0.05$, $**P < 0.01$ compared with control.

These data advance our understanding of four aspects that relate to the physiological significance of the reaction catalysed by type II PIPkins. Firstly, we confirm the presence, in a naturally occurring cell (as opposed to cell cultures), of the novel lipid, PtdIns5P. Secondly, we have for the first time quantified the mass level of this lipid in a cell. Thirdly, the levels of this lipid change when the cells, platelets, are subject to a physiological stimulus, thrombin, which is well known to have complex effects on inositol lipid metabolism [8,9,21]. This is to our knowledge the first time agonist-stimulated changes in PtdIns5P have been demonstrated.

Fourthly, the most interesting aspect of the data is the way that the levels of PtdIns5P change. We have previously reported that the activity of type II PIPkin in human platelets changes during activation by thrombin [22]. Specifically, the enzyme is activated during the first few minutes of platelet activation, and then the activity of the type II PIPkin returns to nearer control levels during the next 5–10 min. At no time during this stimulation have we found evidence for an inhibition of type II PIPkin activity, and as the time course in Fig. 3 suggests that PtdIns5P levels actually increase during this time, it implies that the synthesis of this lipid has also been stimulated as a result of thrombin receptor activation. At present, we do not know if the route of synthesis of PtdIns5P is by a PtdIns 5-kinase, or by 4-dephosphorylation of PtdIns(4,5)P_2, but either way, the data suggest that the synthesis of PtdIns5P is tightly controlled, in turn implying that the lipid may have a distinct physiological function. The mass assay that we describe here will be a powerful tool in further studies on this novel inositol lipid.

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