Ca²⁺-stimulatable and protein kinase C-inhibitable accumulation of inositol 1,3,4,6-tetrakisphosphate in human platelets

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Thrombin-stimulated (10 s) human platelets produce Ins(1,4,5)P₃ and an additional inositol trisphosphate (InsP₃), in approximately a 1:20 ratio. The major InsP₃ co-migrates with Ins(1,3,4)P₃ on strong-anion-exchange h.p.l.c. To identify this species unequivocally, we treated putative Ins(1,3,4)P₃ obtained from thrombin-stimulated *myo*-[³H]inositol-labelled platelets with NaIO₄/NaBH₄ or 4-phosphomonoesterase. The products indicate that the major InsP₃ is at least 90% D-Ins(1,3,4)P₃. D-[³H]Ins(1,3,4)P₃ added to saponin-permeabilized platelets is hydrolysed to an InsP₂ (7.8%) and phosphorylated by a kinase to yield an inositol polyphosphate (0.9%) in 5 min. The phosphorylation product co-migrates with Ins(1,3,4,6)P₄ on Partisphere WAX h.p.l.c. Under similar conditions, L-[³H]Ins(1,3,4)P₃ is dephosphorylated but not phosphorylated. Relative phosphatase:kinase ratios are 8.7:1 (*V*_{max} values) and 0.86:1 (*K*_m values) with respect to D-Ins(1,3,4)P₃. The kinase activity is predominantly cytosolic (96.8% of total activity) in freeze-thaw-disrupted platelets, and the accumulation of its product is Ca²⁺-dependent. The activity is identified as a 6-kinase on the basis of its product's insensitivity to 5-phosphomonoesterase, resistance to periodate oxidation and co-migration with standard Ins(1,3,4,6)P₄ on h.p.l.c. Incubation of platelets with β-phorbol dibutyrate (β-PDBu, 76 nM), causing activation of protein kinase C, results in a 57.5% inhibition (reversible by the protein kinase C inhibitor staurosporine) of Ins(1,3,4,6)P₄ accumulation. α-PDBu, which does not stimulate protein kinase C, has no effect. Stimulation of intact platelets with thrombin results in the production of Ins(1,3,4,6)P₄ (1.4-fold rise in 30 s) and Ins(1,3,4,5)P₄, with the latter being the major InsP₄ species. Accumulation of Ins(1,3,4,6)P₄ is slightly delayed in comparison with Ins(1,3,4)P₃ and is relatively small. We propose that the major route of Ins(1,3,4)P₃ metabolism in stimulated human platelets is via phosphatase action.

INTRODUCTION

Activation of human platelets by thrombin results in the rapid (within 10 s) formation of Ins(1,4,5)P₃ and of another InsP₃ that co-migrates with standard Ins(1,3,4)P₃ on anion-exchange h.p.l.c. [1]. Most of the InsP₃ (95%) is the latter form. Ins(1,4,5)P₃ is a well-described second messenger which causes the release of Ca²⁺ from intracellular stores in numerous tissues, including platelets [2,3]. Ins(1,4,5)P₃ is phosphorylated by an Ins(1,4,5)P₃ 3-kinase yielding Ins(1,3,4,5)P₄ [4], which is dephosphorylated by a 5-phosphomonoesterase to yield Ins(1,3,4)P₃ [5]. Ins(1,3,4)P₃ has been demonstrated to release Ca²⁺ poorly, if at all [6], and its direct function, if any, has not yet been determined. The 5-phosphomonoesterase also catalyses the dephosphorylation of Ins(1,4,5)P₃ to Ins(1,4)P₂, which cannot mobilize stored Ca²⁺. We have shown that activation of protein kinase C by thrombin promotes increases in the activities of the Ins(1,4,5)P₃ 3-kinase and 5-phosphomonoesterase, resulting in removal of the Ca²⁺-mobilizing second messenger Ins(1,4,5)P₃ [7].

Ins(1,3,4)P₃ itself is dephosphorylated in brain and liver by an inositol polyphosphate 1-phosphatase to yield Ins(3,4)P₂ [8,9]. The activity in brain is inhibitable by Ca²⁺ [9], and that present in hepatic tissue is inhibited by ATP [8]. In addition, Ins(1,3,4)P₃ is dephosphorylated by a Li⁺-insensitive 4-phosphatase to yield Ins(1,3)P₂ [10,11]. Ins(1,3,4)P₃ has also been demonstrated to be a substrate for an ATP-dependent kinase in several cell types, including liver [8,12], brain [13], rat parotid acinar cells [14] and adrenal glomerulosa cells [15]. The InsP₄ formed has been identified as Ins(1,3,4,6)P₄, and thus the enzyme is an Ins(1,3,4)P₃ 6-kinase [13]. The potential function of this InsP₄ has not been

established. The phosphorylation of Ins(1,3,4)P₃ has not yet been described in platelets and is examined here.

Given that platelets produce large amounts of an inositol trisphosphate that co-migrates with standard Ins(1,3,4)P₃ on strong-anion-exchange h.p.l.c., we have undertaken a more definitive identification of this compound. In addition, we have explored the phosphorylation of Ins(1,3,4)P₃ in permeabilized and intact platelets and present evidence for the regulation of this metabolism by protein kinase C and Ca²⁺. We propose, however, that the major pathway of Ins(1,3,4)P₃ metabolism in permeabilized or intact stimulated platelets is via phosphatase action.

MATERIALS AND METHODS

Materials

[2-³H]Ins(1,3,4,5)P₄, [³²P]P_i and *myo*-[2-³H]inositol (20 Ci/mmol) were purchased from Du Pont–New England Nuclear. Standard [³H]Ins(1,3,4,6)P₄ was generously provided by Dr. S. B. Shears (National Institute of Environmental Health Sciences, Research Triangle Park, NC, U.S.A.). ¹⁴C-labelled inositol and sorbitol were purchased from Amersham International. [¹⁴C]Altritol and [¹⁴C]adonitol (ribitol) were prepared according to Stephens *et al.* [16]. Prostaglandin E₁ was purchased from Cayman Chemical Company. Saponin, aspirin, polyol dehydrogenase (from *Candida utilis*), alkaline phosphatase (Type VII-NL from bovine intestinal mucosa) and 4-β-phorbol 12,13-dibutyrate (β-PDBu) were obtained from Sigma. α-PDBu was purchased from LC Services Corp., and α-thrombin (3000 units/mg) was

Abbreviation used: PDBu, phorbol 12,13-dibutyrate.

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kindly provided by Dr. K. G. Mann (University of Vermont). Staurosporine was obtained from Kamiya Biomedical Company. The 4-phosphomonoesterase was generously provided by Dr. P. W. Majerus (Washington University, St. Louis, MO, U.S.A.) and 5-phosphomonoesterase was kindly provided by Dr. T. Connolly (Merck Pharmaceuticals, West Point, PA, U.S.A.).

Synthesis of ^3H -labelled and unlabelled enantiomers of $\text{Ins}(1,3,4)\text{P}_3$

D- and L-*myo*- $\text{Ins}(1,3,4)\text{P}_3$ (^3H -labelled and unlabelled) were each synthesized as described by Boehm & Prestwich [17]. Their concentrations were confirmed periodically after separation on Bio-Rad formate columns and quantification of *myo*-inositol [18].

Platelet preparations

Human platelets were isolated as described previously [19] following exposure to $0.5\ \mu\text{M}$ -prostaglandin E_1 and $1.0\ \text{mM}$ -aspirin. Platelets used in experiments examining $\text{Ins}(1,3,4)\text{P}_3$ kinase activity were washed twice by centrifugation at $2420\ g$ for 2 min and resuspension of the pellet in gel-filtration buffer, pH 7.1 [19] to a final cell concentration of $5 \times 10^9/\text{ml}$. In experiments to generate $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ for structural determination, platelets were suspended in Pipes- Mn^{2+} , pH 6.5, to a cell concentration of $2 \times 10^9/\text{ml}$ and labelled with $1\ \text{mCi}$ of *myo*- $[\text{H}]\text{inositol}/3 \times 10^{10}$ platelets as described by Culty *et al.* [20]. In some experiments, platelets were labelled with $[\text{P}]\text{P}_i$ ($0.75\ \text{mCi}/\text{ml}$) as described before [19,21]. These platelets were incubated with α -thrombin ($0.6\ \text{unit}/\text{ml}$) for up to 5 min at 37°C . Incubations were terminated and processed for h.p.l.c. as described previously [1,7].

Preparation of platelet homogenates and subfractions

Unlabelled platelets ($5 \times 10^9/\text{ml}$) were isolated in a lysis buffer of gel filtration buffer, pH 7.1, containing antipain, pepstatin, leupeptin (each $1\ \mu\text{g}/\text{ml}$), phenylmethanesulphonyl fluoride ($100\ \mu\text{M}$) and $2\ \text{mM}$ -dithiothreitol. The platelet suspension was subjected to a rapid freeze (ethanol/solid CO_2)-thaw procedure ($\times 3$) to disrupt the cells, and centrifuged at $1500\ g$ for 90 s at 4°C to sediment any unbroken platelets. The soluble fraction was prepared by centrifugation of the lysate at $100\,000\ g$ for 30 min at 4°C . The resulting membrane pellet was washed once with lysis buffer ($100\,000\ g$, 30 min, 4°C) and resuspended in the same buffer. Protein was assayed by standard procedures (BCA Protein Assay; Pierce Chemical Co.).

$\text{Ins}(1,3,4)\text{P}_3$ kinase and phosphatase assays

$\text{Ins}(1,3,4)\text{P}_3$ kinase and phosphatase activities in permeabilized platelets were assayed by adding $20\ \mu\text{l}$ aliquots of platelets ($5 \times 10^9/\text{ml}$) to $85\ \mu\text{l}$ of buffer (pH 7.1) at 37°C containing $120\ \text{mM}$ -KCl, $4\ \text{mM}$ - MgCl_2 , $15\ \text{mM}$ -Hepes, $25\ \text{mM}$ -NaCl, $1.3\ \text{mM}$ -EGTA, $1\ \text{mM}$ - NaH_2PO_4 , $1.04\ \text{mM}$ - CaCl_2 ($[\text{Ca}^{2+}]_{\text{free}} = 1.0\ \mu\text{M}$), $1.3\ \text{mM}$ -ATP, $33\ \mu\text{g}$ of saponin/ml and $250\,000\ \text{d.p.m.}$ of D- $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ or L- $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ ($16\ \text{Ci}/\text{mmol}$), and various concentrations of D- or L- $\text{Ins}(1,3,4)\text{P}_3$. Saponin permeabilization rapidly permits entry of substrate into the platelet while allowing the cell to retain responsiveness to agonists. In addition, such platelets are more competent in their responses to effectors of protein kinase C than are platelet lysates. In studies examining Ca^{2+} dependence, different concentrations of buffered Ca^{2+} were employed [22]. In some experiments, platelets were exposed to staurosporine ($10\ \mu\text{M}$), α - or β -PDBu ($400\ \text{nM}$) or $0.1\ \%$ dimethyl sulphoxide for 2 min at 37°C before addition to permeabilization buffer. After up to 15 min, an equal volume of ice-cold $15\ \%$ (w/v) trichloroacetic acid was added and samples were centrifuged at $16\,000\ g$ for 5 min. Supernatants were neutralized

using the trioctylamine/Freon method [16]. Following neutralization, samples were analysed on anion-exchange h.p.l.c. In studies to determine subcellular localization of the kinase activity, $20\ \mu\text{l}$ aliquots of platelet fractions (obtained from freeze-thaw-disrupted platelets at $5 \times 10^9/\text{ml}$, see above) were used in the incubations. Enzyme activities were determined to be linear for at least 15 min.

High-performance anion-exchange chromatography of inositol phosphates

The Partisil 10 SAX system of Irvine *et al.* [23] was used for most separations of inositol phosphates produced in $\text{Ins}(1,3,4)\text{P}_3$ kinase/phosphatase assays. Isomers of InsP_4 were separated on a Partisphere WAX column, and InsP_2 produced by 4-phosphomonoesterase incubations of putative $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ was chromatographed on a Partisphere SAX column as described by Stephens *et al.* [13].

Digestion of InsP_4 products

InsP_4 incubation with 5-phosphomonoesterase. An aliquot of platelet soluble fraction was incubated in the presence of $2.7\ \mu\text{M}$ -D- $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ as above and the InsP_4 products were purified on Partisil 10 SAX. The InsP_4 peak was collected and desalted according to Stephens *et al.* [13] and lyophilized. The desalted material ($5000\ \text{d.p.m.}$) was incubated with purified 5-phosphomonoesterase according to Downes *et al.* [24]. A parallel incubation with standard $[\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ was also performed. Incubations were quenched with an equal volume of cold $15\ \%$ (w/v) trichloroacetic acid and samples were neutralized using the trioctylamine/Freon method [16]. The extent of hydrolysis by 5-phosphomonoesterase was determined by chromatography of the neutralized samples on Partisphere WAX h.p.l.c.

Periodate oxidation. Human platelets were prepared and labelled with $[\text{P}]\text{P}_i$ as described earlier. Aliquots of labelled platelets (2.5×10^9 platelets) were incubated with α -thrombin ($1.0\ \text{unit}/\text{ml}$) for 30 s and processed for h.p.l.c. as described previously [1,7]. Desalted $[\text{P}]\text{inositol}$ phosphates were incubated at 25°C for 48 h in the dark with $100\ \text{mM}$ -sodium periodate. Oxidized samples were reduced with $500\ \text{mM}$ -sodium borohydride in an open vessel for 12 h at 25°C and subsequently desalted on Bio-Rad AG-50W cation exchange columns [16]. Remaining borohydride was removed by addition of methanol to dried samples followed by lyophilization. Control samples were processed in parallel, except that water was substituted for periodate/borohydride treatment.

The extent of oxidation of $[\text{P}]\text{InsP}_3$ and $[\text{P}]\text{InsP}_4$ was determined by chromatography on Partisphere WAX h.p.l.c.

Preparation of putative $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$

Platelets labelled with *myo*- $[\text{H}]\text{inositol}$ ($6 \times 10^9/\text{incubation}$) were stimulated with 5 units of α -thrombin/ml for 30 s at 37°C . Samples were quenched with $3.75\ \text{vol.}$ of chloroform/methanol/ $1\ \text{M}$ HCl ($4:10:1$, by vol) and phases were split with $1\ \text{vol.}$ of chloroform and $0.5\ \text{vol.}$ of $1\ \text{M}$ -HCl. The upper phases were processed for h.p.l.c. as previously described [1], except that, following chromatography on DEAE-Sephacel, samples were combined. Desalted inositol phosphates were run on Partisil 10 SAX and $5\ \%$ of the fraction volume ($0.4\ \text{min}$ fractions) was counted for radioactivity to determine the location of the putative $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ peak. Fractions corresponding to $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ were combined and desalted as described by Stephens *et al.* [13].

Structural confirmation of platelet-derived $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$

The structure of the putative $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$ was determined

according to Stephens *et al.* [16], with a few modifications. Briefly, one half of the desalted sample (14000 d.p.m.) was incubated at 25 °C for 48 h in the dark with 100 mM-sodium periodate. The other half of the sample was incubated with 4-phosphomonoesterase (0.4 units/ml) for 10 min at 37 °C in 50 mM-Mes/25 mM-EDTA, pH 6.5. Incubations were quenched with cold 5% HClO_4 and extracted with trioctylamine/Freon (1:1, v/v). The dephosphorylated sample was run on Partisphere SAX h.p.l.c. and 0.4 min fractions were collected. Then, 5% of each fraction was counted for radioactivity by scintillation spectrophotometry in 5 ml of Instagel (Packard) to determine the location of the $[\text{^3H}]\text{InsP}_2$ peak. Fractions containing the $[\text{^3H}]\text{InsP}_2$ peak were desalted [13] and lyophilized. Desalted InsP_2 was then incubated with sodium periodate as outlined above. ^{14}C -labelled standards of adonitol, sorbitol and inositol were added to both inositol phosphate samples following oxidation. Oxidized samples were reduced with 500 mM-sodium borohydride in an open vessel for 12 h at 25 °C and subsequently desalted on Bio-Rad AG-50W cation-exchange columns [16]. Remaining boric acid was removed by addition of methanol to dried samples followed by lyophilization. Samples were dephosphorylated with alkaline phosphatase and desalted on Amberlite MB-3 (Sigma Chemical Co.) mixed-bed ion-exchange resin [16].

The polyols produced were identified by h.p.l.c. on a Polypore-Pb column (4.6 mm $\times$ 220 mm; Brownlee) with an injection volume of 15 μl and water flow rate of 0.2 ml/min. Fractions (0.2 ml) were collected and 10% of their volume was counted for radioactivity from putative $\text{Ins}(1,3,4)\text{P}_3$ -derived polyols and 90% of their volume counted for radioactivity from InsP_2 -derived polyols in order to localize polyol peaks. Polyols derived from samples were identified by co-migration with known standards. As erythritol and adonitol co-migrate on this h.p.l.c. system, the remaining 10% of the polyol $[\text{^3H}]\text{adonitol}$ peak obtained from the InsP_2 sample was run on a second system to separate adonitol from erythritol. The column used was a Cyclobond III (4.6 mm $\times$ 250 mm; Astec) with a mobile phase of acetonitrile/water (9:1, v/v) and a flow rate of 2.0 ml/min. Fractions (0.5 min) were collected and counted for radioactivity in 5 ml of Instagel. Identity of the polyols was determined by co-migration with known standards.

The D or L configuration of the $[\text{^3H}]\text{altritol}$ peak resulting from the putative $\text{Ins}(1,3,4)\text{P}_3$ sample was determined by incubation with L-polyol dehydrogenase. The $[\text{^3H}]\text{altritol}$ peak (with 700 d.p.m. of L- $[\text{^14C}]\text{altritol}$ as standard) was incubated with 20 mM- $\beta\text{-NAD}^+$ /100 mM-Tris/HCl, pH 8.3 and 1.2 units of L-polyol dehydrogenase for 2.5 h at 25 °C. The reaction was quenched by a 5 min incubation in boiling water, desalted on Amberlite MB-3 columns and chromatographed on Polypore-Pb h.p.l.c. as outlined above.

RESULTS

Structural determination of putative $\text{Ins}(1,3,4)\text{P}_3$

The intramolecular location of the phosphate groups in the major InsP_3 obtained from thrombin-stimulated human platelets was determined using the methods of Stephens *et al.* [16]. This method involves periodate oxidation of the $[\text{^3H}]\text{inositol}$ phosphate followed by borohydride reduction and alkaline phosphatase dephosphorylation to obtain a specific polyol which retains the enantiomeric configuration of the starting material. The identity of this polyol is determined by the location of free vicinal hydroxyl groups (and thus phosphate groups) in the parent compound.

Periodate oxidation/borohydride reduction and subsequent dephosphorylation of the putative $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ yielded a

Fig. 1. Polypore-Pb h.p.l.c. separation of putative D- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ -derived polyols

Putative D- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ from α -thrombin-stimulated platelets was subjected to periodate oxidation/borohydride reduction and alkaline phosphatase dephosphorylation. The resulting polyols were chromatographed on a Polypore Pb h.p.l.c. column. The solid line represents ^{14}C -labelled standards of inositol, adonitol and sorbitol, and the broken line represents ^3H -labelled polyol (altritol) derived from putative D- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$. The data are representative of duplicate experiments.

$[\text{^3H}]\text{polyol}$ (89.6% of total recovered polyols) that co-migrated with D/L-altritol on Polypore Pb h.p.l.c. (Fig. 1). In addition, a second minor $[\text{^3H}]\text{polyol}$ peak corresponding to inositol/adonitol (10.4% of total) was also detected. The presence of adonitol indicates D- $\text{Ins}(1,2,3)\text{P}_3$ and that of inositol indicates either D- $\text{Ins}(1,3,5)\text{P}_3$ or D- $\text{Ins}(2,4,6)\text{P}_3$ as the starting material. $[\text{^3H}]\text{Altritol}$ can be obtained only from D- $\text{Ins}(1,3,4)\text{P}_3$, its enantiomer L- $\text{Ins}(1,3,4)\text{P}_3$ [same as D- $\text{Ins}(1,3,6)\text{P}_3$], and also D- and L- $\text{Ins}(1,2,4)\text{P}_3$ [same as D- $\text{Ins}(2,3,6)\text{P}_3$].

The enantiomeric configuration of the $[\text{^3H}]\text{altritol}$ obtained from this preparation of $[\text{^3H}]\text{InsP}_3$ was determined by incubating it with L-polyol dehydrogenase. The substrate requirement of this enzyme is a polyol in the L-configuration, and thus D-altritol will not serve as an effective substrate. Both the $[\text{^3H}]\text{altritol}$ derived from the platelet $[\text{^3H}]\text{InsP}_3$ and an L- $[\text{^14C}]\text{altritol}$ internal standard were converted into tagatose (81% conversion of ^3H -labelled unknown and ^{14}C -labelled standard). This is consistent with the starting material having been either D- $\text{Ins}(1,3,4)\text{P}_3$ or D- $\text{Ins}(1,2,4)\text{P}_3$; however, identification of the former was indicated by digestion with 4-phosphomonoesterase and characterization of the product. We obtained $[\text{^3H}]\text{InsP}_2$ {57.8% of $[\text{^3H}]\text{InsP}_3$ was recovered as a $[\text{^3H}]\text{InsP}_2$, consistent with that recovered as $[\text{^3H}]\text{InsP}_2$ from a known $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ standard under similar conditions}, which was subjected to periodate oxidation/borohydride reduction and dephosphorylation as before. The polyols obtained were run on Polypore-Pb h.p.l.c. which yielded a single peak (99% purity) corresponding to inositol or adonitol. This h.p.l.c. system does not resolve inositol from adonitol, and therefore the peak of $[\text{^3H}]\text{inositol/adonitol}$ obtained was run on a more definitive h.p.l.c. system (Cyclobond III) for separation of these polyols. A single peak that co-migrated with $[\text{^14C}]\text{adonitol}$ standard was obtained (99% purity), which is consistent with the starting material having been D- or L- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$.

These results indicate that the major InsP_3 produced in thrombin-stimulated platelets is D- $\text{Ins}(1,3,4)\text{P}_3$ (at least 90% of major InsP_3). These data thus confirm our earlier finding based on co-migration with known $\text{Ins}(1,3,4)\text{P}_3$ on strong-anion-exchange h.p.l.c. [1].

Metabolism of D- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ in saponin-permeabilized platelets and platelet homogenates

Incubation of 136 nM-D- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ with saponin-

Fig. 2. D-Ins(1,3,4) P_3 kinase (●) and phosphatase (○) activities

Human platelets (1×10^8) were permeabilized with 25 μ g of saponin/ml in the presence of various concentrations of D-Ins(1,3,4) P_3 and D-[3 H]Ins(1,3,4) P_3 . Generation of Ins P_4 and Ins P_2 was linear for up to 15 min. Incubations were terminated after 10 min with cold 7.5% (w/v) trichloroacetic acid and samples were processed for Partisil 10 SAX h.p.l.c. resolution of inositol phosphates. Data are averages $\pm$ range of duplicate determinations and are representative of two experiments. A Hanes-Woolf plot of $[S]/v$ versus $[S]$ [where $S = \text{Ins}(1,3,4)P_3$] yields, for phosphatase, $V_{\max} = 65 \times 10^{-8}$ μ mol/min per 10^8 platelets and $K_m = 0.43$ μ M, and for kinase, $V_{\max} = 7.5 \times 10^{-8}$ μ mol/min per 10^8 platelets and $K_m = 0.5$ μ M.

permeabilized platelets resulted in both dephosphorylation and phosphorylation of the substrate; these reactions were both linear for up to 15 min. The dephosphorylation product migrated near standard Ins(1,4) P_2 (isomer undetermined) on Partisil 10 SAX h.p.l.c. and represented $7.8 \pm 1.0\%$ (mean $\pm$ S.E.M., $n = 15$) of the original substrate. The phosphorylation product co-migrated with standard [3 H]Ins(1,3,4,5) P_4 (indicating only that the product is an Ins P_4 , as this h.p.l.c. system cannot resolve Ins P_4 isomers) and represented $0.9 \pm 0.1\%$ conversion of the original substrate. The majority of the substrate (91.3%) was unmetabolized. We identify the phosphorylation product as an isomer of Ins P_4 , and thus the enzymic activity as an Ins(1,3,4) P_3 kinase.

As also seen in Fig. 2, in which kinetic data are plotted for the same platelet experiments, the phosphatase is significantly more active than the kinase. For such permeabilized platelets, the phosphatase $K_m = 0.43$ μ M and the $V_{\max} = 65 \times 10^{-8}$ μ mol/min per 10^8 platelets, whereas the kinase $K_m = 0.5$ μ M and the $V_{\max} = 7.5 \times 10^{-8}$ μ mol/min per 10^8 platelets. Although definitive kinetic data await purification of the respective enzymes, the relative activities are clearly predictive of predominant phosphatase activity in intact platelets.

D-[3 H]Ins(1,3,4) P_3 was incubated with platelet freeze-thaw lysates, and with soluble and particulate fractions derived from them. The D-[3 H]Ins(1,3,4) P_3 was phosphorylated by all preparations to yield an [3 H]Ins P_4 that co-migrated with [3 H]Ins(1,3,4,5) P_4 on Partisil 10 SAX h.p.l.c. The kinase activity in the total soluble and particulate fractions was greater than that in the total lysate fraction (146% of lysate activity), with 96.8% of the activity in the subfractions being cytosolic (Table 1). In addition, lysates and soluble fractions exhibited phosphatase activity that catalysed the formation of a product that had chromatographic properties of an Ins P_2 (Table 1), with 98% of the activity in the subfractions being cytosolic (98% of total activity in lysate fraction) (Table 1).

The ratio of kinase to phosphatase activity was lower in lysate fractions than in saponin-permeabilized platelets. This was a reflection of elevated phosphatase activity in lysed fractions compared with saponin-treated platelets. Saponin-treated platelets, in which only the plasma membrane is permeabilized,

Table 1. D-[3 H]Ins(1,3,4) P_3 metabolism by platelet lysate, soluble and particulate fractions

Aliquots (20 μ l) of fractions obtained from freeze-thaw-disrupted platelets (5×10^9 /ml) were incubated in the presence of 136 nM-D-[3 H]Ins(1,3,4) P_3 for 5 min at 37 $^{\circ}$ C. Reactions were terminated with cold 5% (w/v) trichloroacetic acid and processed for Partisil 10 SAX h.p.l.c. as outlined in the Materials and methods section. Phosphatase (Ins P_2) and kinase (Ins P_4) activities are expressed as the means $\pm$ ranges of duplicate determinations. The % of lysate activity represents $100 \times (\text{fraction activity} \times \text{fraction protein})/(\text{lysate activity} \times \text{lysate protein})$.

	Phosphatase		Kinase	
	(d.p.m./5 min per mg of protein)	(% of lysate activity)	(d.p.m./5 min per mg of protein)	(% of lysate activity)
Lysate	50 294 $\pm$ 5726	100.0	3696 $\pm$ 217	100.0
Soluble	66 297 $\pm$ 2719	80.3	8594 $\pm$ 88	141.5
Particulate	5404 $\pm$ 4871	1.5	1186 $\pm$ 192	4.7

more closely approximate intact cells in their responsiveness to agonists than do freeze-thaw lysates; furthermore, freeze-thaw lysates may include phosphatase activities (as yet undefined) not ordinarily accessible to Ins(1,3,4) P_3 in intact cells.

Metabolism of L-[3 H]Ins(1,3,4) P_3 in platelet soluble fractions

Soluble fractions of platelets were incubated in the presence of 238 nM-L-[3 H]Ins(1,3,4) P_3 and the aqueous phases were run on Partisil 10 SAX h.p.l.c. for resolution of inositol phosphates produced. The substrate was not phosphorylated under these conditions (results not shown); however, significant phosphatase activity [$52.1 \pm 4.1\%$ (range) conversion of substrate] was detected which resulted in the formation of products with chromatographic properties of Ins P_2 and Ins P .

Identification of the Ins(1,3,4) P_3 phosphorylation product

Since isomers of Ins P_4 are not resolved on Partisil 10 SAX, we desalted and combined the [3 H]Ins P_4 -containing fractions obtained in incubations of D-[3 H]Ins(1,3,4) P_3 with saponin-permeabilized platelets and rechromatographed the resulting material on Partisphere WAX h.p.l.c. This system, which resolves the isomers of Ins P_4 [13], yielded a single peak that co-migrated with [3 H]Ins(1,3,4,6) P_4 (Fig. 3). Incubation of known [3 H]Ins(1,3,4,5) P_4 with 5-phosphomonoesterase resulted in 94.2% hydrolysis of the original material, whereas incubation of the putative [3 H]Ins(1,3,4,6) P_4 from Partisil 10 SAX h.p.l.c. runs under similar conditions resulted in only 13.4% hydrolysis. This minimal level of hydrolysis could reflect the presence of a small amount of [3 H]Ins(1,3,4,5) P_4 produced via a 5-kinase acting on D-[3 H]Ins(1,3,4) P_3 , an activity that has been described in liver homogenates [12].

Putative [32 P]Ins(1,3,4,6) P_4 from thrombin-stimulated platelets was insensitive to periodate oxidation (4.0% oxidation compared with untreated control), indicating that this Ins P_4 could not be Ins(1,2,3,4) P_4 , which is sensitive to periodate oxidation and would yield alritol. [32 P]Ins(1,3,4) P_3 and [32 P]Ins(1,4,5) P_3 , which are sensitive to periodate treatment, were oxidized under these conditions (68.0% compared with untreated control), indicating that periodate treatment was effective.

We therefore identify the Ins(1,3,4) P_3 phosphorylation product that co-migrates with Ins(1,3,4,6) P_4 as more than 90% Ins(1,3,4,6) P_4 , and the enzymic activity as an Ins(1,3,4) P_3 6-kinase.

Fig. 3. Partisphere WAX h.p.l.c. separation of $[^3\text{H}]\text{Ins}P_4$ isomers

Putative $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ was prepared by incubation of 136 nM-D- $[^3\text{H}]\text{Ins}(1,3,4)P_3$ in platelet soluble fractions as described in the Materials and methods section and purified on Partisil 10 SAX h.p.l.c. The $\text{Ins}P_4$ peak was collected, desalted and rechromatographed on a Partisphere WAX column for separation of $\text{Ins}P_4$ isomers according to Stephens *et al.* [13]. The solid line represents standard $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ and $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ as indicated and the broken line represents putative $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ derived from platelets.

Inhibition of $\text{Ins}(1,3,4,6)P_4$ accumulation by β -PDBu

Platelets were incubated with the protein kinase C activator β -PDBu (76 nM final) or with its non-activating analogue α -PDBu (76 nM), followed by saponin-permeabilization in the presence of D- $[^3\text{H}]\text{Ins}(1,3,4)P_3$ (136 nM). Stimulation of protein kinase C by β -PDBu resulted in 57.5% inhibition of kinase activity, whereas α -PDBu had no effect (Fig. 4). The protein kinase C inhibitor staurosporine (10 μM) reversed the β -PDBu-induced inhibition (Fig. 4). Phosphatase activity with respect to $\text{Ins}(1,3,4)P_3$ was unaffected by protein kinase C stimulation/inhibition under these circumstances (results not shown). Platelet soluble kinase activity was not affected by β -PDBu. These results indicate a possible inhibitory role of protein kinase C activation on $\text{Ins}(1,3,4)P_3$ kinase or a stimulatory role on $\text{Ins}(1,3,4,6)P_4$ phosphatase (as yet unidentified).

Ca^{2+} dependence of $\text{Ins}(1,3,4,6)P_4$ accumulation in saponin-permeabilized platelets and platelet soluble fraction

Saponin-permeabilized platelets and platelet soluble fraction

Fig. 4. Inhibition of $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ accumulation by β -PDBu

Human platelets (1×10^8) were treated with 400 nM- β -PDBu (■), 400 nM- α -PDBu (□), 10 μM -staurosporine (□) or 10 μM -staurosporine followed by 400 nM- β -PDBu (▨) for 2–3 min at 37 °C. Aliquots of treated platelets were then incubated with 25 μg of saponin/ml and 136 nM-D- $[^3\text{H}]\text{Ins}(1,3,4)P_3$. Incubations were terminated after 10 min with cold 7.5% (w/v) trichloroacetic acid and samples were processed for h.p.l.c. resolution of inositol phosphates on a Partisil 10 SAX column as described in the Materials and methods section. Data are expressed as means $\pm$ s.d. of at least 4 determinations.

Fig. 5. Ca^{2+} -dependence of $[^3\text{H}]\text{Ins}(1,3,4,6)P_4$ accumulation

Saponin-permeabilized platelets (1×10^8) or platelet soluble fractions from freeze-thaw-disrupted platelets (equivalent to 1×10^8 platelets) were incubated with various concentrations of free Ca^{2+} in the presence of 136 nM-D- $[^3\text{H}]\text{Ins}(1,3,4)P_3$. Incubations were terminated after 10 min with cold 7.5% (w/v) trichloroacetic acid and samples were processed for h.p.l.c. analysis on a Partisil 10 SAX column as outlined in the Materials and methods section. $\circ$, Incubations with saponin-permeabilized platelets; $\bullet$, incubations with soluble fractions. Data are expressed as means $\pm$ range of duplicate determinations and are representative of two experiments.

Fig. 6. Accumulation of $[^{32}\text{P}]\text{inositol}$ polyphosphates in thrombin-stimulated platelets

Human platelet (2.5×10^6), labelled with ^{32}P as described, were incubated with α -thrombin (0.6 unit/ml) for up to 5 min at 37 °C. Incubations were terminated and processed for Partisphere WAX h.p.l.c. as described in the Materials and methods section. (a) $[^{32}\text{P}]\text{Ins}P_3$; (b) $[^{32}\text{P}]\text{Ins}(1,3,4,6)P_4$; (c) $[^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$. Data are expressed as the averages $\pm$ range of determinations performed in duplicate and are representative of three experiments.

were incubated with various concentrations of free Ca^{2+} in the presence of $\text{D-}[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$. Kinase product accumulation was found to be Ca^{2+} -dependent in the range 0–1.0 μM in both permeabilized platelets and soluble fractions (4.6-fold increase in this range) (Fig. 5). Phosphatase activity towards $\text{Ins}(1,3,4)\text{P}_3$ was unaffected over this range of Ca^{2+} concentrations in both permeabilized platelets and soluble fractions.

Accumulation of ^{32}P inositol polyphosphates in thrombin-stimulated platelets

Human platelets labelled with ^{32}P were stimulated with α -thrombin (0.6 unit/ml) for up to 5 min at 37 °C and the desalted aqueous phases were chromatographed on Partisphere WAX h.p.l.c. InsP_3 levels [largely $\text{Ins}(1,4,5)\text{P}_3$, as $\text{Ins}(1,4,5)\text{P}_3$ is a small component of InsP_3 in platelets [1]] rose rapidly to a maximal level in 30 s and decreased dramatically in the ensuing 60 s (Fig. 6a). The rise in $\text{Ins}(1,3,4,5)\text{P}_4$ levels preceded that of $\text{Ins}(1,3,4)\text{P}_3$ (maximal in 10 s), consistent with $\text{Ins}(1,3,4,5)\text{P}_4$ being the precursor of $\text{Ins}(1,3,4)\text{P}_3$ (Fig. 6c). A second ^{32}P -labelled peak was observed on h.p.l.c. in the region of InsP_4 having chromatographic properties and periodate oxidation resistance (described earlier) consistent with this peak being $\text{Ins}(1,3,4,6)\text{P}_4$. This peak achieved a maximal level within 30 s (1.4-fold of basal) (Fig. 6b). Accumulation of this material was slightly delayed with respect to that of $\text{Ins}(1,3,4)\text{P}_3$ and was relatively small. In the 30–90 s following thrombin stimulation, the majority of $^{32}\text{P}[\text{Ins}(1,3,4)\text{P}_3]$ was not metabolized to $^{32}\text{P}[\text{InsP}_4]$ (Fig. 6) or to more phosphorylated species (results not shown), indicating that the major pathway for $\text{Ins}(1,3,4)\text{P}_3$ metabolism in intact platelets is via phosphatase action. This finding is consistent with our observations with saponin-permeabilized platelets.

DISCUSSION

We have identified the major InsP_3 produced in thrombin-stimulated platelets and a kinase activity that acts upon it. Rigorous structural analysis defines the InsP_3 as $\text{D-Ins}(1,3,4)\text{P}_3$. We have also identified the presence in platelets of $\text{Ins}(1,3,4)\text{P}_3$ 6-kinase, as has been reported for other tissues [8,12–15].

Accumulation of $\text{Ins}(1,3,4,6)\text{P}_4$ in cell preparations other than platelets has been shown to be Ca^{2+} -insensitive [12,13]; however, we have observed a distinct Ca^{2+} sensitivity in the range 0–1.0 μM . The kinase activity in permeabilized platelets is specific for $\text{D-Ins}(1,3,4)\text{P}_3$ as opposed to $\text{L-Ins}(1,3,4)\text{P}_3$.

The potent protein kinase C agonist β -PDBu inhibits $\text{Ins}(1,3,4,6)\text{P}_4$ accumulation in permeabilized platelets but not in soluble fractions. This inhibition is reversed by the protein kinase C inhibitor staurosporine, and no effect of the inactive phorbol ester α -PDBu is observed. These results indicate for the first time a possible negative regulatory role for activated protein kinase C in regulating $\text{Ins}(1,3,4)\text{P}_3$ 6-kinase activity. One would expect that activation of protein kinase C and the rise in cytosolic Ca^{2+} to micromolar levels would have opposing effects on $\text{Ins}(1,3,4,6)\text{P}_4$ concentrations, but that phosphatase, being unaffected by Ca^{2+} or protein kinase C, and being 8.7-fold as active as 6-kinase at concentrations of $\text{Ins}(1,3,4)\text{P}_3$ (approx. 20 μM [1]) achieved within 10 s of platelet stimulation would account for the bulk of $\text{Ins}(1,3,4)\text{P}_3$ metabolism.

As noted above, $\text{Ins}(1,3,4)\text{P}_3$ is predominantly dephosphorylated (maximal velocity 8.7-fold that of kinase activity) by permeabilized platelets, yielding a product with chromatographic properties of InsP_2 . In an exaggeration of these findings, thrombin stimulation of intact platelets yields a relatively small amount of $\text{Ins}(1,3,4,6)\text{P}_4$, as has been reported for other tissues [13,14,25]. Less than 1000 d.p.m. of $^{32}\text{P}[\text{Ins}(1,3,4,6)\text{P}_4]$ accumulated as a function of thrombin stimulation (15–90 s), whereas

in 30–90 s 50000 d.p.m. of $^{32}\text{P}[\text{Ins}(1,3,4)\text{P}_3]$ are metabolized. The missing products do not appear as more highly phosphorylated species and are more likely processed, via phosphatase action, through the large pool of InsP_2 found in stimulated platelets [26]. This indicates that the ratio of phosphatase to 6-kinase activities in intact stimulated platelets is greater than 50:1. Such a discrepancy in magnitude with respect to the results obtained in saponin-permeabilized platelets may be attributable to enhanced phosphatase, inhibited 6-kinase, or both, in intact thrombin-stimulated platelets. We know of no activatable systems that enhance $\text{Ins}(1,3,4)\text{P}_3$ phosphatase activity, but there is evidence for strong inhibition of the 6-kinase by $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ [12,14] in liver. $\text{Ins}(1,3,4,5)\text{P}_4$ would not be present at significant levels in lysates or permeabilized platelets. $\text{Ins}(3,4,5,6)\text{P}_4$, if present as a stable, relatively unlabelled pool, would be diluted 30-fold at the concentrations of permeabilized or lysed platelets employed here. Therefore the effects of such species, or of any other inhibitory substances, on 6-kinase would be minimized in lysed/permeabilized preparations compared with intact cells. The possible regulatory effects of InsP_4 isomers as well as effects of potential stable pools of InsP_5 and InsP_6 species on inositol phosphate metabolism in platelets are thus an interesting subject for further investigation.

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