Phosphatidylinositol 3,4,5-trisphosphate is formed from phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets

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Platelets accumulate PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 in response to thrombin and thrombin-receptor-directed peptide in a GTP-dependent manner. These phosphoinositides are considered to be mediators of signalling events in a variety of cells. We have examined the metabolic route by which PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 are synthesized by briefly (10 min) incubating platelets with high activities of [32 P]P $_i$, followed by 20 or 60 s exposure to thrombin, and analysing the relative radioactivities of the individual phosphate groups in the resulting labelled PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 . The phosphate group possessing the highest specific activity under such non-equilibrium labelling conditions indicates the last one added in a metabolic sequence. The thrombin-stimulated rate of labelling

of PtdIns(3,4) P_2 was significantly slower than that of PtdIns(3,4,5) P_3 . Increased labelled PtdIns3P was not detected within 60 s. The measured relative radioactivities decreased in the order $3 > 5 > 4 \gg 1$ for PtdIns(3,4,5) P_3 and $3 > 4 \gg 1$ for PtdIns(3,4) P_2 . On the basis of the results of both rate-of-labelling and specific radioactivity analyses we conclude that PtdIns(3,4,5) P_3 is formed by 3-OH phosphorylation of PtdIns(4,5) P_2 , whereas PtdIns(3,4) P_2 may be formed by 3-OH phosphorylation of PtdIns4P and/or dephosphorylation of PtdIns(3,4,5) P_3 . These findings point to the activation of phosphoinositide 3-kinase as a critical receptor-regulated step in thrombin-stimulated platelets.

INTRODUCTION

Phosphoinositide 3-kinase phosphorylates inositol phospholipids on the 3-hydroxy position of the inositol ring. Evidence is accumulating which suggests that this enzyme is acutely activated by a wide range of growth factors and cell activators and that its 3-phosphorylated inositol phospholipid products may function as early signals in mitogenic, trophic and inflammatory cell-signalling pathways [1,2]. For example, phosphoinositide 3-kinase activity specifically associates with anti-phosphotyrosine and/or anti-receptor immunoprecipitates following stimulation of appropriate cells with a range of growth factors. In addition, PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 , putative products of phosphoinositide 3-kinase, but usually not PtdIns3P, accumulate rapidly in activated cells.

Analysis of the metabolic pathways accounting for the formation of PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 in stimulated cells has produced conflicting results. Initial reports [3,4] favoured a pathway involving sequential phosphorylation of PtdIns to PtdIns3P to PtdIns(3,4) P_2 to PtdIns(3,4,5) P_3 in both thrombin-stimulated platelets and platelet-derived-growth-factor (PDGF)-stimulated fibroblasts and implied co-regulation of phosphoinositide 3-kinase together with the additional steps proposed to be required for the synthesis of PtdIns(3,4,5) P_3 . By contrast, a simpler scheme was proposed to account for the pattern of accumulation of 3-phosphorylated inositol phospholipids which occurred in chemotactic peptide (fMet-Leu-Phe)-stimulated human neutrophils, with PtdIns(4,5) P_2 being the principal substrate of an agonist-stimulated 3-kinase [5]. The analysis of the pattern of metabolites formed when neutrophil extracts were incubated with radiolabelled PtdIns(3,4,5) P_3 suggested that both

PtdIns(3,4) P_2 and PtdIns3P could be hydrolysis products of initially formed PtdIns(3,4,5) P_3 . Further experiments by the same authors extended these conclusions to PDGF-stimulated Swiss 3T3 fibroblasts [6], conflicting with the earlier findings noted above. In view of these contradictions, we have reassessed the pathway of 3-phosphorylated inositol phospholipid biosynthesis in thrombin-stimulated platelets using cells labelled briefly (10 min, rather than 60 min) with [32 P]P $_i$ to enhance differences in the specific radioactivities of the γ -phosphate of ATP and the monoester phosphates of phosphoinositides on which the analyses depend. The results suggest that PtdIns(3,4,5) P_3 is synthesized from PtdIns(4,5) P_2 in thrombin-stimulated human platelets.

EXPERIMENTAL

[32 P]P $_i$ was purchased from du Pont–NewEngland Nuclear and α -thrombin was the gift of Dr. Ken Mann (University of Vermont, Burlington, VT, U.S.A.). Platelet-rich plasma, free from erythrocytes and leukocytes [7], was prepared freshly (in the presence of 0.5 μ M prostaglandin E $_1$ and 1 mM aspirin) from blood donated by healthy volunteers, as described in [8]. Platelets were washed by centrifugation [8] and suspended to 2×10^9 cells/ml in modified Tyrode's buffer containing 129 mM NaCl, 10.9 mM trisodium citrate, 8.9 mM NaHCO $_3$, 5.6 mM dextrose, 10 mM Tris base, 2.8 mM KCl, 2.0 mM Hepes, 10 μ M Na $_2$ HPO $_4$, and 0.8 mM MgCl $_2$, pH 7.3. For most positional labelling studies (destined for phosphatase treatment of isolated inositol phosphates), platelet suspensions at 37 °C were incubated for 10 min with 5.3 mCi of [32 P]P $_i$ /ml and 500 μ l aliquots were added immediately to 5 units/ml thrombin for 20 or 60 s in triplicate.

Abbreviations used: PtdOH, phosphatidic acid; PME, phosphomonoesterase; s.a.x., strong-anion-exchange; w.a.x., weak-anion-exchange; GroPIns, glycerophosphoinositol; PI, phosphatidylinositol; 'phosphoinositide' or 'phosphatidylinositol' refers to the inositol-containing phospholipid species; PDGF, platelet-derived growth factor.

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In some cases, incubations with [^{32}P]P_i were for 20 min in the presence of 200 μM P_i, prior to thrombin stimulation $\pm 2\text{ mM}$ Ca²⁺. For platelet samples not destined for phosphatase digestion, incubations of platelets with 0.5 mCi/ml [^{32}P]P_i proceeded for 10 or 60 min, followed by 0, 20 or 60 s incubations with thrombin in triplicate. Incubations were terminated with chloroform/methanol (1:2, v/v), followed by 0.75 ml of 3.1 M HCl and 1 ml of chloroform. Vigorously mixed samples were centrifuged, and the upper phases were extracted twice with 1.5 ml of chloroform. The pooled lower phases were evaporated to dryness, extracted lipids were resolved by t.l.c., and phosphatidic acid (PtdOH) was separated and quantified by scintillation spectrophotometry [8–10]. The phosphoinositides detected by autoradiography were deacylated [11] and subjected to h.p.l.c. as described in [8–10]. Homogeneous peaks which had the expected mobilities of glycerophosphoinositol 3,4-bisphosphate [GroPIns(3,4)P₂] and GroPIns(3,4,5)P₃ were identified by Čerenkov counting of column fractions. Retention times of ^{32}P -labelled standards prepared from nerve-growth-factor-stimulated PC12 cells [10] were determined in parallel runs on the same columns. Collected peak fractions of GroPIns(3,4)P₂ and GroPIns(3,4,5)P₃ were neutralized with triethylamine, pooled, and de-salted on Bio-Rad AG1X8 columns. P_i from the h.p.l.c. eluate was removed with 200 mM ammonium formate/0.1 M formic acid, and GroPIns(3,4)P₂ and GroPIns(3,4,5)P₃ were each eluted with 2 M ammonium formate/0.1 M formic acid, neutralized with NH₃ and freeze-dried. Deglyceration proceeded as described in [12]. Deglycerated, desalted samples were spiked with known amounts of [^3H]Ins(1,3,4)P₃ or [^3H]Ins(1,3,4,5)P₄. The inositol phosphates were then further chromatographed on Partisphere strong-anion-exchange (s.a.x.) [8–10] or weak-anion-exchange (w.a.x.) h.p.l.c. columns, the latter of which are capable of resolving different InsP₄ isomers [13,14]. Recoveries of InsPs after the h.p.l.c. steps, desalting and deglyceration were determined. Radioactivity in aliquots of the purified InsPs were counted such that the counting error for both isotopes was $< 1\%$, using a dual-label program to obtain a $^{32}\text{P}/^3\text{H}$ ratio. The remainder was subjected to dephosphorylation by enzymes present in human erythrocyte ghosts {Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ 5-phosphomonoesterase (PME); [5,15]} and rat brain high-speed supernatant in the presence of EDTA [Ins(1,3,4)P₃ 4-PME and Ins(1,3)P₂ 3-PME] as described in [16–18]. Erythrocyte ghosts were prepared as described [19], and packed ghosts were stored in 20 mM Tris/1 mM EDTA, pH 7.2 at -70°C prior to use.

Ins(1,3,4,5)P₄ from PtdIns(3,4,5)P₃ was incubated with 1 mg/ml packed ghosts in 20 mM Hepes/2 mM EGTA/10 mM MgCl₂, pH 7.2 (5 ml) for 4 h, and the reaction was terminated with 500 μl of ice-cold 60% HClO₄. After vortex-mixing and 15 min on ice, the mixture was spun for 5 min, and supernatant removed and neutralized with tri-*n*-octylamine/1,1,2-trichlorotrifluoroethane [20]. Samples were subjected to s.a.x. h.p.l.c. and the Ins(1,3,4)P₃ peak identified by Čerenkov counting. Peak fractions were pooled and 10% was removed to determine the $^{32}\text{P}/^3\text{H}$ ratio. The remainder was de-salted.

Rat brain cerebral cortex from one brain was dissected on ice and homogenized in 5 ml 20 mM Hepes/2 mM EGTA/20 mM EDTA/10 mM 2-mercaptoethanol/5 $\mu\text{g}/\text{ml}$ (each) pepstatin + benzamidine + leupeptin, pH 7.2, at 4°C . Supernatant was obtained after dilution of the homogenate with buffer to 10 ml and centrifugation at 60000 *g* for 35 min. Ins(1,3,4)P₃ species from 5-PME treatment or from PtdIns(3,4)P₂ were incubated in 500 μl of 20 mM Hepes/2 mM EGTA/20 mM EDTA/0.8–1 mg/ml rat brain supernatant protein, pH 7.4, for 20 min at 37°C , and reactions were terminated with 50 μl of ice-cold 60% HClO₄. Neutralized supernatants (as above) were subjected to

h.p.l.c. as above, and 0.5 ml fractions of Ins(1,3)P₂ and InsP were counted for radioactivity to determine $^{32}\text{P}/^3\text{H}$ ratios, such that counting error was $< 1\%$.

RESULTS AND DISCUSSION

Tracer [^{32}P]P_i labelling was used initially by Brockerhoff and Ballou [21] and more recently by Müller et al. [22] and King et al. [23] to assess the metabolic interrelationships of the inositol phospholipids. Experiments using hepatocytes have revealed that the monoester phosphates of PtdIns4P and PtdIns(4,5)P₂ achieve isotopic equilibrium with the γ -phosphate of ATP quickly [19]. In hepatocytes, as well as in platelets [24], the rate of uptake of P_i appears to exert the greatest influence on the rate of labelling of the γ -phosphate of ATP and the subsequent labelling of monoester phosphates in phosphoinositides. Thus, in order to observe differences in the specific radioactivities of the individual phosphate groups of these rapidly metabolized compounds, it is essential to use brief periods of labelling to establish conditions in which the γ -phosphate of ATP has a significantly higher specific radioactivity than the groups to be analysed.

Figure 1 Effect of pre-incubation period with [^{32}P]P_i on labelling of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂.

Platelets were incubated with 0.5 mCi of [^{32}P]P_i/ml for 10 or 60 min at 37°C prior to mixing with 5 units/ml thrombin for periods indicated. Lipids were extracted and quantified as described. Data are presented as increments above $t = 0$ values and should be multiplied by 10.6 as an adjustment for 5.3 mCi/ml labelling conditions to facilitate comparisons with data in Tables. — — —, PtdIns(3,4,5)P₃; —, PtdIns(3,4)P₂. (a) Pre-incubation for 60 min. Basal values: $12.48 \pm 0.33 \times 10^5$ d.p.m. of PtdIns(4,5)P₂; 6202 ± 1052 d.p.m. of PtdIns(3,4,5)P₃; 1942 ± 208 d.p.m. of PtdIns(3,4)P₂. (b) Pre-incubation for 10 min. Basal values: 2.92 ± 10^5 d.p.m. of PtdIns(4,5)P₂; 1791 ± 195 d.p.m. of PtdIns(3,4,5)P₃; 1020 ± 268 d.p.m. of PtdIns(3,4)P₂. The 20 and 60 s values for PtdIns(3,4)P₂ were statistically different, whereas the 20 and 60 s values for PtdIns(3,4,5)P₃ were not.

Scheme 1 Flow chart of analysis of PtdIns(3,4,5) P_3

Figure 1 shows the effects of labelling platelets with [^{32}P]P_i either briefly for 10 or for 60 min, and then stimulating with thrombin, on the levels of radioactive PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 . [^{32}P]PtdIns(4,5) P_2 for platelets incubated with [^{32}P]P_i for 10 or 60 min (prior to thrombin addition) was used as a monitor of ^{32}P incorporation into the γ -phosphate of metabolic ATP under basal conditions. This value increased 4.27 ± 0.54 (S.E.M.)-fold from 10 to 60 min. The thrombin-induced increases in [^{32}P]PtdOH were then used as a monitor of the relative specific radioactivity of the γ -phosphate of ATP [25] after platelet stimulation. These increments for 60 min prior labelling were 2.81 ± 0.18 -fold and 2.95 ± 0.23 -fold the increments for 10 min prior labelling, at 20 and 60 s + thrombin respectively. That these values are significantly lower than the basal (thrombin-free) ATP ratio of 4.27 probably reflects the relatively greater impact of thrombin-stimulated uptake of [^{32}P]P_i [24] upon the metabolic ATP pools at 10 or 60 min. As noted previously [8], following a

60 min prelabelling period (Figure 1a), PtdIns(3,4,5) P_3 increased to 3–4-fold basal levels within 20 s of thrombin addition, at which time it had reached the peak value. By contrast, PtdIns(3,4) P_2 increased substantially after 60 s following a delay in the first 20 s. As before, [^{32}P]PtdIns3 P was not altered significantly (results not shown). When platelets were labelled for 10 min and then stimulated (Figure 1b), the rise in PtdIns(3,4,5) P_3 again notably preceded that in PtdIns(3,4) P_2 . Further, the thrombin-induced increments in the ^{32}P content of the 3-phosphorylated inositol phospholipids relative to the ^{32}P content of control PtdIns(4,5) P_2 were greater than those following a 60 min labelling period, e.g. the ratio of thrombin-stimulated PtdIns(3,4,5) P_3 at 60 s to basal PtdIns(4,5) P_2 shifted from $(1.15 \pm 0.05) \times 10^{-2}$ for a 60 min labelling period to $(2.00 \pm 0.25) \times 10^{-2}$ for a 10 min labelling period. This was to be expected. As noted above, any differences in specific radioactivity of the relevant phosphate groups are likely to be enhanced by

Table 1 Recoveries of phosphoinositides and phosphatase digestion products

Data shown are the means $\pm$ S.D. for three experiments in triplicate for platelets exposed for 20 or 60 s to thrombin after a 10 min load with [32 P] P_i . Examples of 32 P beginning counts [GroPIns(3,4,5) P_3 and GroPIns(3,4) P_2] are shown in parentheses. 'Pre-phosphatase' includes two desalting stages, dehydration and h.p.l.c., and indicates recoveries of GroPIns(3,4,5) P_3 as Ins(1,3,4,5) P_4 and GroPIns(3,4) P_2 as Ins(1,3,4) P_3 , just prior to digestion with phosphatases. 'Post 5-PME,' all inositol products were recovered as Ins(1,3,4,5) P_4 and Ins(1,3,4) P_3 , and numbers indicate recovery as Ins(1,3,4) P_3 after desalting. All post-phosphatase recoveries are based on recoveries of 3 H carriers.

	3 H (%)			Distribution of products			
	Pre-phosphatase	Post-5-PME	Post-3- and 4-PME	Ins(1,3,4) P_3	Ins(1,3) P_2	Ins1 P	Ins
PtdIns(3,4,5) P_3							
20 s	75 $\pm$ 5 (68 720 d.p.m.)	69 $\pm$ 0.0	84 $\pm$ 2	41.6 $\pm$ 5.3	44.8 $\pm$ 3.2	13.3 $\pm$ 2.3	0.3 $\pm$ 0.2
60 s	68 $\pm$ 7 (87 142 d.p.m.)	61 $\pm$ 13	82 $\pm$ 2	42.8 $\pm$ 0.3	43.6 $\pm$ 0.9	13.4 $\pm$ 1.4	0.2 $\pm$ 0.0
PtdIns(3,4) P_2							
20 s	62 $\pm$ 5 (25 398 d.p.m.)	—	78 $\pm$ 2	1.9 $\pm$ 0.6	49.2 $\pm$ 3.4	47.9 $\pm$ 4.0	1.0 $\pm$ 0.1
60 s	63 $\pm$ 3 (96 651 d.p.m.)	—	79 $\pm$ 4	1.3 $\pm$ 0.3	47.0 $\pm$ 1.5	50.9 $\pm$ 1.7	0.8 $\pm$ 0.1

thrombin's ability to stimulate the rapid influx of [32 P] P_i by a factor of 5–10-fold [24]. The impact of this effect is diminished after 60 min of labelling, which is consequently more useful for measurements of mass changes than for rates of flux.

These results suggest that the brief labelling conditions and the response to thrombin generated a situation in which the γ -phosphate of ATP had a higher specific radioactivity than the monoester phosphates of the phosphoinositides being analysed, making it more feasible to detect a higher specific radioactivity in the phosphate added to phosphoinositide by an activated kinase. Moreover, the kinetics of PtdIns(3,4,5) P_3 as against PtdIns(3,4) P_2 accumulation provide independent evidence for the initial agonist-stimulated formation of PtdIns(3,4,5) P_3 , and are compatible with the possibility that PtdIns(3,4) P_2 may be a breakdown product of, rather than a precursor for, PtdIns(3,4,5) P_3 . Most of the analyses of the distribution of 32 P among the individual phosphate groups of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 , therefore, utilized a 10 min labelling period prior to stimulation with thrombin. The analysis employed was similar to that of Stephens and colleagues [5]. The inositol phosphates derived from deacylation and deglyceration of PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 revealed homogeneous peaks of 32 P-labelled compounds which co-chromatographed precisely with the 3 H standards. The homogeneity, both of the 32 P-labelled platelet-lipid-derived inositol phosphates, and of the internal 3 H standards, was confirmed by re-chromatography, in the case of Ins(1,3,4,5) P_4 , on a Partisphere w.a.x. column. Such analyses gave a single peak containing both 3 H and 32 P with the mobility expected for Ins(1,3,4,5) P_4 . A flow chart of the methodology employed for analysis of a sample of PtdIns(3,4,5) P_3 is presented in Scheme 1. Recoveries of inositol phosphates through the h.p.l.c., deglyceration and de-salting steps are shown in Table 1. After digestion of Ins(1,3,4,5) P_4 with erythrocyte ghosts (5-PME), the recoveries of desalted [3 H]Ins(1,3,4) P_3 as a percentage of starting [3 H]Ins(1,3,4,5) P_4 were routinely $> 60\%$ (Table 1). The distribution of products, monitored by 3 H label, are also presented in Table 1.

As detailed in Table 1, treatment of Ins(1,3,4) P_3 samples with 4- and 3-PME enzymic activities resulted in between 58 and 98% hydrolysis of the starting material and recovery routinely of 80% of starting 3 H in inositol-containing compounds having the

expected retention times of Ins(1,3,4) P_3 , Ins(1,3) P_2 and Ins1 P , with less than 1% of products as free inositol. 32 P-labelled compounds co-chromatographed precisely with [3 H]Ins(1,3,4) P_3 and [3 H]Ins(1,3) P_2 . By contrast, there was very little 32 P associated with the peak of [3 H]Ins1 P , indicating the very low specific radioactivity of the diester 1-phosphate of the intact lipids, a finding characteristic of such a brief period of labelling. As anticipated, the inclusion of EDTA in these incubations prevented the action of 1-phosphatase on Ins(1,3,4) P_3 , since no 3 H- or 32 P-labelled Ins(3,4) P_2 was observed following chromatography in an h.p.l.c. system that routinely [5] resolves Ins(3,4) P_2 and Ins(1,3) P_2 .

The 32 P/ 3 H ratio was determined for each identified metabolite, and these values, after correcting for decay of 32 P, were used to calculate the relative 32 P contents of the individual phosphate groups, as presented in Table 2. Whether platelets were incubated for 20 or 60 s with thrombin, the 3-position of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 contained the largest amount of 32 P. This was followed by positions 5, 4 and 1 of PtdIns(3,4,5) P_3 , in descending order. In one of the experiments shown, labelling of positions 4 and 5 was not significantly different. The position 3/position 4 specific-radioactivity ratios in PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 were similar (Table 2). In all cases, including studies (not shown) in which labelling was undertaken for 20 min in the presence of 200 μ M [32 P] P_i , the positional distribution of 32 P in PtdIns(3,4) P_2 was $3 > 4 \gg 1$. Under the latter conditions, for two experiments involving triplicate incubations, 53.4 $\pm$ 0.2% of 32 P was in position 3 and 46.6% in position 4 ($< 1\%$ in position 1), at 20 s post-thrombin; at 60 s, 55.5 $\pm$ 0.6% was in position 3, with the remainder in position 4. The 3, 4 and 5 [32 P]phosphorylated positions of PtdIns(3,4,5) P_3 , however, were not significantly different from one another. An extended incubation to 180 s in the presence of both thrombin and 2 mM Ca^{2+} , conditions known to enhance by approx. 2-fold the amount of total PtdIns(3,4) P_2 that accumulates over Ca^{2+} -free controls [26], had no significant effect on the relative labelling of the phosphate positions in this species, implying that Ca^{2+} did not selectively stimulate a 4-kinase acting on PtdIns3 P . The data thus provide clear evidence that PtdIns(3,4,5) P_3 is generated primarily via phosphorylation of the 3-OH position of PtdIns(4,5) P_2 , which arises from the 5-OH phosphorylation of PtdIns4 P , the only

Table 2 Positional distribution of ^{32}P (%)

Results shown are for two separate experiments performed in triplicate for platelets incubated 10 min with [^{32}P]P_i prior to stimulation with thrombin for 20 or 60 s. Data are the means $\pm$ S.D. Results (not ratios) are percentage distributions.

		PtdIns(3,4,5) <i>P</i> ₃					PtdIns(3,4) <i>P</i> ₂				
Expt. 1	Position ...	1	3	4	5	Ratio 3:4	1	3	4	Ratio 3:4	
20 s		< 1	39.5 ± 0.4	25.7 ± 0.4	34.8 ± 0.0	1.54	< 1	59.1 ± 0.3	40.9 ± 0.3	1.44	
60 s		< 1	37.4 ± 0.6	28.8 ± 1.4	33.7 ± 0.8	1.30	< 1	57.6 ± 1.6	42.4 ± 1.6	1.36	
Expt. 2											
20 s		< 1	41.9 ± 0.6	28.2 ± 0.6	29.9 ± 0.6	1.48	< 1	57.1 ± 0.0	42.9 ± 0.0	1.33	
60 s		< 1	40.7 ± 0.0	31.0 ± 0.5	28.3 ± 0.5	1.31	< 1	57.8 ± 0.8	42.2 ± 0.8	1.37	

known route for synthesis of PtdIns(4,5) P_2 . The data on PtdIns(3,4) P_2 are consistent with 5-phosphatase acting on PtdIns(3,4,5) P_3 [5] and/or 3-OH kinase acting on PtdIns4P as the source(s) of PtdIns(3,4) P_2 .

The reasons for the discrepancy between the findings reported here and those reported previously [3] for thrombin-stimulated platelets are not immediately evident. The two protocols differed in several respects, the most obvious of which was the 10 as against the 60 min incubation period with [^{32}P]P_i. Had we incubated for 60 min, we would have expected to see no significant differences in labelling among the monoester phosphate positions of PtdIns(3,4,5) P_3 , as was true for the 20 min incubations. We also employed relatively short incubations with thrombin in comparison with the other study, which exposed platelets to thrombin for 10 min where PtdIns(3,4) P_2 was being generated for analysis. Other procedural differences included incubation buffers (Tris/NaCl/glucose; [3]), and termination procedures (addition of HCl, incubation for 30 min on ice, chloroform/methanol extraction of sedimented pellets; [3]). It might also be noted that partially purified inositol polyphosphate 5-PME and 4-PME were utilized in the former studies [3]. Although our enzyme preparations were not purified, the proof of their specificity lies in the nature of their products. In all cases, co-chromatography with 3H standards indicated both that the products generated were homogeneous and that substantial conversion into identified products occurred (Table 1).

Were synthesis of PtdIns(3,4,5) P_3 to proceed by stepwise phosphorylation of PtdIns, PtdIns3P and PtdIns(3,4) P_2 , one would have expected to see transient, or at least early, generation of PtdIns3P and PtdIns(3,4) P_2 . Instead, we saw no significant change in [^{32}P]PtdIns3P [8]; and the present experiment (results not shown) and accumulation of PtdIns(3,4,5) P_3 preceding that of an increasing accumulation of PtdIns(3,4) P_2 (Figures 1a and 1b). Similar findings have been reported for other cells [5,6]. A PtdIns3P 4-kinase has been described in platelets [27], although its activity in comparison with PtdIns4P 3-kinase has not been characterized. If stimulated PtdIns 3-kinase, PtdIns3P 4-kinase, and putative PtdIns(3,4) P_2 5-kinase activities are so tightly coupled that early accumulations of intermediates are prevented, one would expect that such a tight coupling would make differences in positional specific activities undetectable. If, instead, a large, pre-existing and therefore relatively 'cold' (i.e. non-radioactive), pool of PtdIns3P exists, this would obviate initial PI 3-kinase activation. Thus the activation of PtdIns3P 4-kinase utilizing a pre-existing 'cold' pool of PtdIns3P would imply that PI 3-kinase is not stimulated rapidly in platelets exposed to thrombin or in fibroblasts exposed to PDGF, whereas activated PI 3-kinase is found in cytoskeletal fractions from

platelets stimulated for 30–45 s with thrombin [28] and in PDGF–PDGF-receptor immunoprecipitates [1]. In addition, we have recently demonstrated [29] that PI 3-kinase activity that yields PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 from added PtdIns(4,5) P_2 and PtdIns4P respectively, can be stimulated severalfold by guanosine 5'-[γ -thio]triphosphate in soluble platelet fractions, an event dependent upon the small GTP-binding protein rho. Finally, no 5-kinase activity that uses PtdIns(3,4) P_2 as a substrate has been reported to date. The most parsimonious explanation for the data that we present here, which also takes account of the reported agonist-induced stimulation of PI 3-kinase that utilizes PtdIns(4,5) P_2 , is that exposure of platelets to thrombin rapidly activates a PI 3-kinase that first acts upon PtdIns(4,5) P_2 and, subsequently, PtdIns4P, or that PtdIns(3,4) P_2 arises from hydrolysis of newly generated PtdIns(3,4,5) P_3 [5]. In either case, the key regulatory path would appear to be the activation of PI 3-kinase.

This work was supported by National Institutes of Health Grant HL 38622 (to S. E. R.), a Fellowship from the Southeastern Pennsylvania affiliate of the American Heart Association (to R. H.), a Canadian Medical Research Council award (to A. S.), a NATO Collaborative Research Grant #901011 (to S. E. R. and C. P. D.) and an award from the British Medical Research Council (G9017975) (to C. P. D.). We thank Michelle Levinski for assistance with Figures and Tables, and the blood-drawing services of the Blood Bank, Thomas Jefferson University.

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Received 16 December 1993/9 February 1994; accepted 15 February 1994