Regulation of PtdIns4*P* 5-kinase C by thrombin-stimulated changes in its phosphorylation state in human platelets

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PtdIns(4,5)P_2 production by the enzyme PtdIns4*P* 5-kinase C (PIPkin C) was examined in thrombin-stimulated human platelets. Thrombin caused a rapid, transient 2–3-fold increase in PIPkin activity and a transient net dephosphorylation of the enzyme. PIPkin C was phosphorylated on serine and threonine residues in unstimulated platelets; no evidence for tyrosine phosphorylation was found. The phosphatase inhibitor okadaic acid promoted PIPkin C hyperphosphorylation and a concomitant marked inhibition of its activity in immunoprecipitates. Activity was restored by treatment with alkaline phosphatase, suggesting the existence of an inhibitory phosphorylation site. In support of this idea, alkaline phosphatase treatment of PIPkin C immunoprecipitated from unstimulated platelets caused a modest (1.6-fold) but significant activation of the enzyme. However, alkaline phosphatase treatment of PIPkin C immunoprecipitated from thrombin-stimulated platelets caused a decrease in activity to approximately the same levels, suggesting that the phosphorylation of PIPkin C also contributes to the observed stimulation. Two-dimensional phosphopeptide mapping of immunoprecipitated PIPkin C revealed that the enzyme is multiply phosphorylated and that, whereas some phosphopeptides are indeed lost on stimulation, consistent with the net dephosphorylation of the enzyme, at least two novel sites become phosphorylated. This suggests that thrombin causes complex changes in the phosphorylation state of PIPkin C, one consequence of which is its activation.

INTRODUCTION

The PtdIns4*P* 5-kinases (PIPkins) are a family of enzymes that synthesize the phospholipid PtdIns(4,5)P_2. This lipid is of central importance in cellular function: in addition to its well-documented role as the precursor of the second messengers Ins(1,4,5)P_3, diacylglycerol [1] and PtdIns(3,4,5)P_3 [2], it is also thought to regulate a number of other cellular processes. These include a requirement for PtdInsP_2 in the mechanism of exocytosis [3,4], in the control of vesicle trafficking [5] and in regulation of the actin cytoskeleton [6]. Additionally, PtdIns(4,5)P_2 might regulate certain protein kinases [7], and, via its ability to bind to PH domains, modify the function of proteins containing this motif [8]. It has also been found to promote dissociation of GDP from the small G-proteins ARF [9] and cdc42Hs [10], leading to the suggestion that it is involved in their activation.

Mammalian cells contain at least two immunologically distinct PIPkin isoforms: the type I [11] and the type II [11] or C [12] PIPkins. Multiple type I PIPkins have been identified by purification [13] and cloning [14]. The type II/C isoform is unable to replace type I in the regulation of exocytosis [4], suggesting that different PIPkin isoforms might regulate functionally distinct pools of PtdIns(4,5)P_2, a phenomenon that is likely to underlie the involvement of PtdIns(4,5)P_2 in many cellular processes.

Signalling mechanisms that impinge on PIPkins to influence PtdIns(4,5)P_2 production are likely to be of great importance in the maintenance and regulation of such functionally distinct lipid pools, but little is known about the mechanisms of regulation of distinct PIPkin isoforms. Phosphatidic acid stimulates type I PIPkins *in vitro* [13] and has been suggested to be a physiological stimulus for PtdIns(4,5)P_2 production by these isoforms. We have previously shown that integrin-mediated signals associated with platelet aggregation promote translocation of PIPkin C to the cytoskeleton, where it regulates a cytoskeletally associated pool of PtdIns(4,5)P_2 [15]. The small G-proteins Rho and Rac have both been reported to increase PtdInsP_2 synthesis when activated [16,17], and direct association of both of these proteins with type I PIPkins has been reported [18,19]. Many other signalling events, including regulation by cAMP-dependent protein kinase [20], protein kinase C [21], tyrosine kinases [22] and integrins [23], have been reported to affect PtdIns(4,5)P_2 synthesis, but in none of these cases has the PIPkin isoform involved been identified unequivocally.

Here we report that PIPkin C is activated during the first few minutes of stimulation of human platelets with thrombin, and that this activation is achieved by complex changes in the phosphorylation state of this isoform.

MATERIALS AND METHODS

Platelet preparation

Blood (50 ml) was obtained from healthy adults who denied having taken aspirin within the previous 7 days. Platelet-rich plasma was obtained by centrifugation at 150 ***g*** for 20 min. EGTA (1 mM) was added and the platelets were sedimented by centrifugation at 900 ***g*** for 15 min. The pellet was resuspended in platelet buffer [10 mM Hepes (pH 7.4)/10 mM glucose/1 mM $MgSO_4$/145 mM NaCl/5mM KCl] and washed by gel filtration on a Sepharose CL-4B column (Sigma); the protein concentration was adjusted to approx. 1 mg/ml.

Assaying PIPkin C activity in immunoprecipitates

Platelets subjected to different conditions of stimulation were lysed with an equal volume of platelet buffer containing 2% (v/v) Triton X-100, 6 mM EGTA, 10 mM EDTA, 100 mM

Abbreviations used: OA, okadaic acid; PIPkin, PtdIns4*P* 5-kinase.

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NaF, 40 mM β-glycerophosphate, 0.2 mM Na_3VO_4, 2 mM PMSF and 1 μg/ml leupeptin. Lysates were clarified by centrifugation at 10000 ***g*** for 10 min at 4 °C. PIPkin C was immunoprecipitated by using the monoclonal antibody MAC 344 [24] coupled to Protein G–Sepharose beads (Pharmacia) via a mouse anti-(rat κ light chain) antibody (Sigma). Lysates were incubated with the beads for 2 h at 4 °C with mixing. The beads were then washed once with platelet buffer/1 mM EGTA and twice with PIPkin buffer [50 mM Tris/HCl (pH 7.4)/80 mM KCl/10 mM magnesium acetate/2 mM EGTA]. For Western blotting, immunoprecipitates were boiled with SDS sample buffer [2% (w/v) SDS/10% (v/v) glycerol/80 mM Tris/HCl (pH 6.8)]. For PIPkin C assays, PtdIns4*P* (5 μM final concentration) and ATP (5 μM final concentration, containing 10 μCi of [γ^{32}P]ATP per sample) were added and the samples incubated at 30 °C for 10 min. Reactions were quenched with chloroform/methanol (1:1, v/v) and the lipids were extracted and isolated as described [15]. In some cases, washed immunoprecipitates were incubated with or without 200 units/ml alkaline phosphatase (Boehringer Mannheim) in phosphatase buffer for 20 min at room temperature, then washed twice with PIPkin buffer before being assayed for PIPkin activity. Activity was assessed by determining ^{32}P incorporation into PtdIns(4,5)P_2 by phosphorimaging, or as described [15].

Western blotting of PIPkin C immunoprecipitates

^{32}P-labelled platelets (see below) were washed, stimulated with thrombin (1 unit/ml) for the indicated times and lysed as described above. PIPkin C was immunoprecipitated with the monoclonal antibody MAC 334 [24] coupled to Protein G–Sepharose for 2 h at 4 °C. The beads were washed three times with Tris-buffered saline [140 mM NaCl/50 mM Tris (pH 7.4)] and prepared for electrophoresis as described above. SDS/PAGE and Western blotting were performed exactly as described [15].

^{32}P labelling of isolated platelets

Isolated platlelets were labelled with [^{32}P]P_i (Amersham; 2 mCi/ml) for 2 h at room temperature, after which unincorporated label was removed by gel filtration. The washed, labelled platelets were subsequently exposed to thrombin or okadaic acid (OA) for different periods before lysis and immunoprecipitation of PIPkin C with MAC 334 as described above. Incorporation of ^{32}P was assessed by phosphorimaging and corrected for small differences in the PIPkin C content of each lane, assessed by densitometry of autoradiographs of the same blots probed with anti-PIPkin C antibodies and detected by enhanced chemiluminescence.

Two-dimensional phosphopeptide mapping

PIPkin C was immunoprecipitated from ^{32}P-labelled platelet lysates with MAC 334 and subjected to electrophoresis on 10% (w/v) polyacrylamide gels. The PIPkin C band was excised, washed in several changes of 30% (v/v) methanol, then ground up in 50 mM NH_4HCO_3. Trypsin (10 μg; treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one; Fluka) was added and the mixture was incubated overnight at 37 °C. Gel fragments were removed by repeated centrifugation and the sample was freeze–dried. Two-dimensional separation was performed by the method of Boyle et al. [25] with pH 1.9 buffer [formic acid/acetic acid/water (50:156:1794, by vol.)] for the electrophoresis step and n-butanol/pyridine/acetic acid/water (785:607:122:486, by vol.) for the chromatography step. TLC plates (cellulose-coated; Merck) were exposed to autoradiography film for 4 weeks.

Phosphoamino acid analysis

^{32}P-PIPkin C immunoprecipitates from washed, unstimulated platelets were subjected to electrophoresis and transferred to PVDF membranes. The PIPkin C band was excised, washed several times with water, heated to 110 °C in the presence of 5.7 M HCl for 1 h, freeze–dried and dissolved in pH 1.9 buffer. Phosphoamino acid standards [phosphoserine, phosphotyrosine and phosphothreonine (Sigma)] were added and the samples were subjected to two-dimensional TLC by the method of Duclos et al. [26] [first dimension, 2-methylpropan-1-oic acid/0.5 M NH_4OH (5:3, v/v); second dimension, propan-2-ol/HCl/water (7:1.5:1.5, by vol.)]. The spots were revealed with ninhydrin and the position of ^{32}P labelling was determined by autoradiography.

RESULTS

The monoclonal antibody MAC 344 can be used to immunoprecipitate PIPkin C activity from cell lysates; PtdInsP_2 production by PIPkin C immunoprecipitated from thrombin-stimulated platelets was therefore compared with that from unstimulated platelets, to determine whether agonists affect the activity of this isoform. This revealed that, after 2 min of stimulation, the PIPkin activity associated with immunoprecipitates was more than doubled (226.1 ± 29.6% of control; $n = 3$). Activation was transient: after 10 min of stimulation the activity had fallen to 150.8 ± 9.3% of control ($n = 3$). The differences in activity between the immunoprecipitates were not due to variations in the amount of PIPkin C present: Western blots of MAC 344 immunoprecipitates generated in parallel with those assayed for activity revealed the presence of identical amounts of PIPkin C in each (results not shown). Stimulation of PIPkin C by thrombin does not involve integrin-mediated signals associated with platelet aggregation, as it occurs irrespective of whether or not the platelets are stirred (results not shown); unstirred platelets do not aggregate and hence integrin-mediated signals to the cell interior are not generated. Moreover the phenomena studied here are distinct in their time course from the integrin-mediated translocation to the cytoskeleton that we have documented previously [15], which is modest after 2 min of stimulation but pronounced after 10 min.

The stimulation of platelets by thrombin causes major changes in the phosphorylation state of many proteins. To determine whether the observed activation of PIPkin C might be due to changes in its phosphorylation, platelets were labelled with [^{32}P]P_i and stimulated with thrombin for different times, after which the degree of phosphorylation of PIPkin C in immunoprecipitates was determined (Figure 1A). This revealed that PIPkin C is phosphorylated in unstimulated platelets and that thrombin causes a transient dephosphorylation of the enzyme to approx. 50% of unstimulated levels. Dephosphorylation is maximal between 30 s and 5 min of stimulation and is again independent of stirring. To determine which amino acid residues were phosphorylated, acid hydrolysis of PIPkin C immunoprecipitated from unstimulated, ^{32}P-labelled platelets was performed and the amino acids were separated by two-dimensional TLC. This revealed that both serine and threonine but not tyrosine residues were phosphorylated (Figure 1B), indicating the presence of at least two phosphorylation sites. The absence of tyrosine phosphorylation was also indicated by the inability of anti-phosphotyrosine antibodies either to immunoprecipitate PIPkin C or to recognize it on Western blots (results not shown).

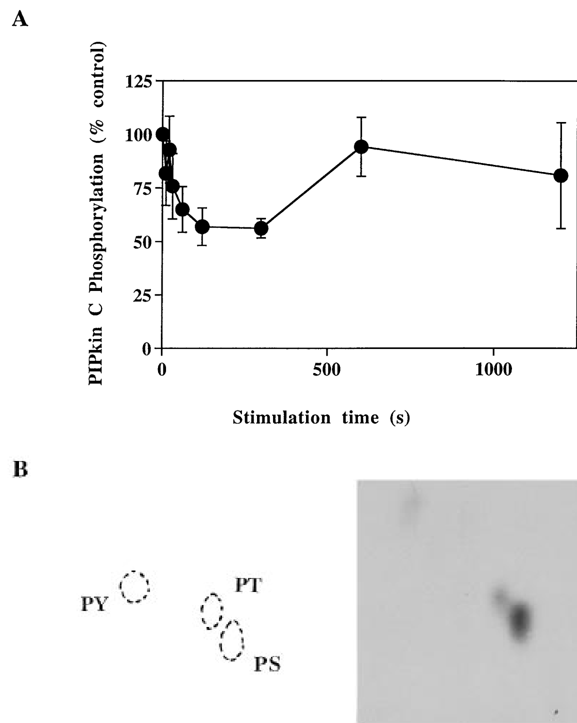

Figure 1 PIPkin C is phosphorylated on serine and threonine residues in unstimulated platelets; thrombin produces transient dephosphorylation of the enzyme

(**A**) Platelets labelled with ^{32}P were washed, warmed to 37 °C in the presence of 2 mM $CaCl_2$, and stimulated with thrombin (1 NIH unit/ml) for the indicated times. Cells were then lysed in the presence of phosphatase inhibitors and the PIPkin C was immunoprecipitated, subjected to electrophoresis and transferred to nitrocellulose. The extent of PIPkin C phosphorylation is shown, expressed as a percentage of that in unstimulated platelets. Results are means ± S.E.M. for three to five independent experiments. (**B**) Autoradiograph of a two-dimensional separation of a PIPkin C hydrolysate obtained from unstimulated ^{32}P-labelled platelets. The positions of the phosphoamino acid standards phosphotyrosine (PY), phosphoserine (PS) and phosphothreonine (PT) are indicated.

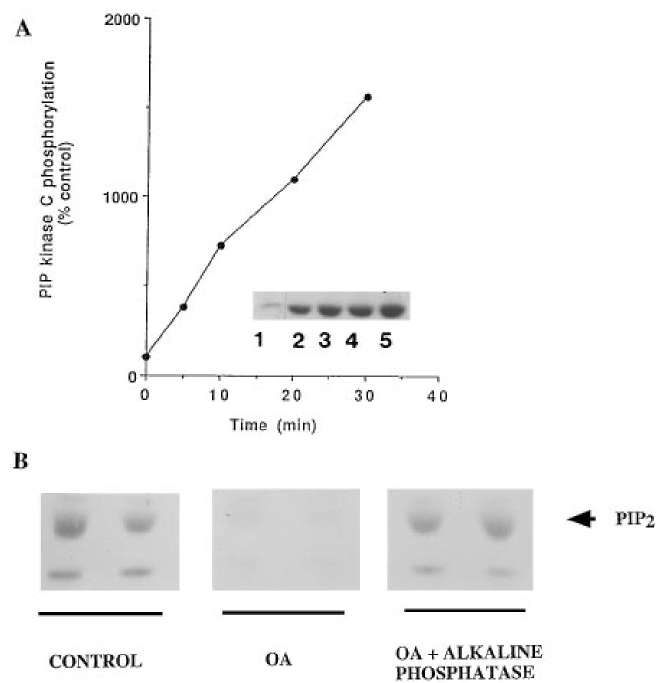

Figure 2 Effects of OA on PIPkin C phosphorylation and activity

(**A**) Washed ^{32}P-labelled platelets were warmed to 37 °C in the presence of 2 mM $CaCl_2$. OA (1 μM) was then added and incubation continued for the indicated times before the amount of ^{32}P incorporated into PIPkin C was determined. Results are expressed as a percentage of the phosphate content of PIPkin C from non-OA-treated, unstimulated platelets. Inset: corresponding autoradiograph of PIPkin C from OA-treated, ^{32}P-labelled platelets. Platelets were incubated with OA for the following times: lane 1, no OA; lane 2, 5 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min. (**B**) PIPkin activity associated with PIPkin C immunoprecipitates from OA-treated and untreated platelets is shown. Platelets were incubated with OA (5 μM) or vehicle [0.1% (v/v) DMSO] for 1 h before lysis and immunoprecipitation of PIPkin C. Washed immunoprecipitates were incubated in the presence or absence of alkaline phosphatase (200 units/ml) for 20 min at room temperature. They were then washed again, and their ability to phosphorylate exogenous PtdIns4*P* was assessed. The position of PtdIns(4,5)P_2 is indicated by PIP_2.

The finding that the transient activation of PIPkin C by thrombin was correlated temporally with overall dephosphorylation of the enzyme suggested a possible causal relationship between the two. To test this, we treated platelets with the protein phosphatase 1/2a inhibitor OA to promote hyperphosphorylation of the enzyme. OA produced a huge increase in the ^{32}P labelling of PIPkin C (Figure 2A); this had not reached maximal levels after 30 min in the presence of 1 μM OA, so a longer incubation (1 h) with a higher concentration of OA (5 μM) was used in subsequent experiments. This procedure markedly decreased PIPkin C activity in immunoprecipitates to 37.0 ± 10.2 % of control ($n = 3$) (Figure 2B), consistent with the existence of an inhibitory phosphorylation site. This idea was supported by the ability of alkaline phosphatase to restore PIPkin activity in immunoprecipitates from OA-treated platelets (to 87.1 ± 5.9 % of control; $n = 3$). Treatment of immunoprecipitates with alkaline phosphatase did not produce detectable proteolysis of the samples (results not shown).

To test whether PIPkin C dephosphorylation leads to its activation *in vitro*, we immunoprecipitated PIPkin C from unstimulated platelets and treated the washed immunoprecipitates with or without alkaline phosphatase. This procedure, which results in the disappearance of PIPkin C radiolabelling when applied to immunoprecipitates from ^{32}P-labelled cells (results not shown), produced a modest but significant activation of the enzyme (to 164.1 ± 21.8 % of control; $n = 10$; $P < 0.05$); it did not, however, produce the 2–3-fold stimulation seen in immunoprecipitates from thrombin-stimulated cells, suggesting that dephosphorylation, although possibly involved in the mechanism, is not solely responsible for the stimulation of activity.

To investigate this idea further, PIPkin C was immunoprecipitated from lysates of platelets that had been incubated for 90 s with or without thrombin after labelling with [^{32}P]P_i. Immunoprecipitates were separated by SDS/PAGE and the ^{32}P-labelled PIPkin C was isolated and digested with trypsin. The resulting peptides were separated in two dimensions by electrophoresis and TLC; labelled peptides were detected by autoradiography. This method revealed the presence of multiple phosphopeptides in PIPkin C even from unstimulated cells (Figure 3). Stimulation with thrombin for 90 s led to the disappearance of certain phosphopeptides (Figure 3, asterisks), in line with the net dephosphorylation of the enzyme shown in Figure 2, and the appearance of three new phosphopeptides (arrows), consistent with the activation of a protein kinase that phosphorylates PIPkin C at distinct residues. Note that the examples shown were obtained from experiments performed on different days; attempts to obtain parallel samples from a single

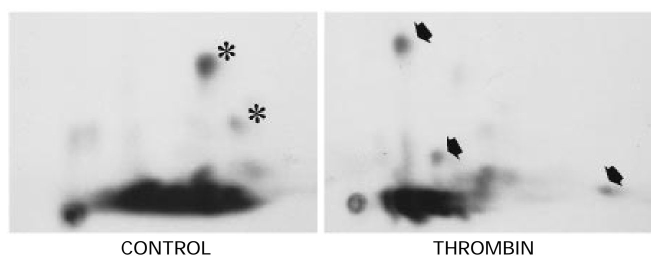

Figure 3 Two-dimensional phosphopeptide maps of tryptic digests of PIPkin C from stimulated and unstimulated platelets

Washed ^{32}P-labelled platelets were incubated at 37 °C in the presence of 2 mM $CaCl_2$ for 5 min. Thrombin (1 unit/ml) or buffer were added for a further 90 s as indicated. Cells were lysed and PIPkin C was immunoprecipitated from the lysates. PIPkin C was digested with trypsin and the peptides were separated in two dimensions; the pattern of phosphopeptides obtained is shown. Differences between the patterns are indicated: asterisks denote phosphopeptides that disappeared on stimulation; arrows indicate novel phosphopeptides. The results are representative of three independent determinations for each condition.

platelet preparation were unsuccessful, because the samples contained too few radioactive counts to generate clear autoradiographs. It is therefore not possible to compare the extent of PIPkin C phosphorylation directly between the two autoradiographs. The poorly resolved material in the autoradiographs consists of one or more highly hydrophilic peptides that migrate poorly in the hydrophobic buffer system used in the second dimension in these experiments.

Consistent with the idea that the phosphorylation of PIPkin C at distinct sites in response to thrombin contributes to its activation, alkaline phosphatase causes a decrease in PIPkin C activity immunoprecipitated from thrombin-treated platelets. In experiments where thrombin increased activity to 233.2 ± 29.4 % of control, alkaline phosphatase treatment decreased this to 155.6 ± 15.3 % (mean ± S.E.M., $n = 3$); in this experiment, alkaline phosphatase treatment of immunoprecipitates from unstimulated platelets increased activity to 128.9 ± 12.0 % of control.

DISCUSSION

The results reveal that agonist-mediated signals can activate a defined PIPkin isoform, PIPkin C, in human platelets, and that this activation is achieved via changes in the phosphorylation state of the enzyme. Stimulation with thrombin leads to a doubling in the PIPkin activity associated with anti-PIPkin C immunoprecipitates; this increase can be diminished if the immunoprecipitates are treated with alkaline phosphatase, consistent with activation of the enzyme occurring via its phosphorylation. However, stimulation with thrombin leads to a net dephosphorylation of PIPkin C at the time when it is activated, suggesting the presence of an inhibitory phosphorylation site on the enzyme. This idea is supported by the ability of pretreatment with OA to cause a marked inhibition of PIPkin C activity in immunoprecipitates, and by the ability of alkaline phosphatase both to reverse this inhibition and also to produce a modest increase in enzyme activity compared with untreated samples from unstimulated cells. It should be noted that alkaline phosphatase treatment of PIPkin C immunoprecipitates from OA-treated platelets restored activity to only 87 % of control values: it did not, as expected, increase activity to greater than 100 %. In contrast, alkaline phosphatase treatment of PIPkin C immunoprecipitated from both unstimulated and thrombin-treated platelets resulted in final activities of greater than 100 %. This discrepancy is probably due to the inability of alkaline phosphatase to dephosphorylate the hyperphosphorylated PIPkin C completely.

In agreement with the possible dual effects of thrombin on PIPkin C phosphorylation inferred from the activity studies, thrombin also has complex effects on the two-dimensional phosphopeptide maps of PIPkin C tryptic digests. This again points to the removal of phosphate from some sites and the phosphorylation of PIPkin C on other residues in response to stimulation.

It is clear from the results presented here that PIPkin C is not maximally phosphorylated in unstimulated platelets: the ability of OA to greatly enhance PIPkin C phosphorylation shows that the phosphorylation state of the enzyme is constantly changing, and that it is incompletely phosphorylated in unstimulated platelets. The ability of OA preincubation to markedly reduce PIPkin C activity shows that extensive phosphorylation of the enzyme causes its inhibition. This therefore suggests that, in unstimulated platelets, a fraction of the total PIPkin C is either inactive or of decreased activity, owing to phosphorylation of the inhibitory site. Dephosphorylation of this site permits activation of the enzyme; thrombin-stimulated dephosphorylation would therefore recruit a larger pool of enzyme for the production of PtdIns(4,5)P_2. Our ability to immunoprecipitate more PIPkin C activity from unstimulated platelets than from OA-treated cells suggests that the inhibitory phosphorylation site is incompletely phosphorylated under basal conditions. This presumably reflects a requirement for a basal level of PIPkin C activity in maintaining PtdIns(4,5)P_2 levels in the face of constant conversion to PtdIns4P by 5-phosphatases. It should be noted that platelets contain unusually large amounts of PIPkin C compared with any other cell type that we have tested [27]. It will be extremely interesting to determine agonist-stimulated effects on both the phosphorylation state and the level of activity of PIPkin C in cell types that contain more moderate amounts of the enzyme.

The existence of multiple phosphorylation sites on PIPkin C is intriguing. Although the presence of both inhibitory and stimulatory phosphorylation sites is demonstrated by the current results, it is possible that other changes in phosphorylation state might also regulate the subcellular distribution of the enzyme [15]. It is clear that the regulation of PIPkin C by changes in its phosphorylation state is likely to be a very complex issue, and this complexity potentially underlies the apparent involvement of PtdIns(4,5)P_2, and of the PIPkins, in the regulation of many diverse cellular processes.

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