The lipid transfer activity of phosphatidylinositol transfer protein is sufficient to account for enhanced phospholipase C activity in turkey erythrocyte ghosts

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Background: The minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) has been implicated in the control of a number of cellular processes. Efficient synthesis of this lipid from phosphatidylinositol has been proposed to require the presence of a phosphatidylinositol/phosphatidylcholine transfer protein (PITP), which transfers phosphatidylinositol and phosphatidylcholine between membranes, but the mechanism by which PITP exerts its effects is currently unknown. The simplest hypothesis is that PITP replenishes agonist-sensitive pools of inositol lipids by transferring phosphatidylinositol from its site of synthesis to sites of consumption. Recent cellular studies, however, led to the proposal that PITP may play a more active role as a co-factor which stimulates the activity of phosphoinositide kinases and phospholipase C (PLC) by presenting protein-bound lipid substrates to these enzymes. We have exploited turkey erythrocyte membranes as a model system in which it has proved possible to distinguish between the above hypotheses of PITP function.

Results: In turkey erythrocyte ghosts, agonist-stimulated PIP_2 hydrolysis is initially rapid, but it declines and reaches a plateau when ~15% of the phosphatidylinositol has been consumed. PITP did not affect the initial rate of PIP_2 hydrolysis, but greatly prolonged the linear phase of PLC activity until at least 70% of phosphatidylinositol was consumed. PITP did not enhance the initial rate of phosphatidylinositol 4-kinase activity but did increase the unstimulated steady-state levels of both phosphatidylinositol 4-phosphate and PIP_2 by a catalytic mechanism, because the amount of polyphosphoinositides synthesized greatly exceeded the molar amount of PITP in the assay. Furthermore, when polyphosphoinositide synthesis was allowed to proceed in the presence of exogenous PITP, after washing ghosts to remove PITP before activation of PLC, enhanced inositol phosphate production was observed, whether or not PITP was present in the subsequent PLC assay.

Conclusions: PITP acts by catalytically transferring phosphatidylinositol down a chemical gradient which is created as a result of the depletion of phosphatidylinositol at its site of use by the concerted actions of the phosphoinositide kinases and PLC. PITP is therefore not a co-factor for the phosphoinositide-metabolizing enzymes present in turkey erythrocyte ghosts.

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Background

The minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) has been implicated in a variety of cellular processes. Hydrolysis of PIP_2 by members of the receptor-activated, phosphoinositide-specific phospholipase C (PLC) family is now recognized as a ubiquitous signalling mechanism that generates two second messengers: inositol 1,4,5-trisphosphate (IP_3), which raises intracellular calcium levels, and diacylglycerol (DAG), which activates some protein kinase C isoforms [1,2]. Growth factor-regulated phosphoinositide 3-kinases use PIP_2 as the principal *in vivo* substrate for the generation of the putative second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [3,4]. PIP_2 has been shown to be necessary for priming of Ca^{2+}-dependent secretion [5] and to be a co-factor for phospholipase D [6,7]. Also, polyphosphoinositides have been shown to bind to, and modulate, the activities of a number of actin-binding proteins *in vitro*, and so may be involved in the regulation of actin polymerization ([8–11] and see [12] for review).

The basal level of PIP_2 is maintained at a steady state by two substrate cycles: firstly, the phosphatidylinositol (PI) ↔ phosphatidylinositol 4-phosphate (PI4P) substrate

cycle is catalyzed by PI 4-kinase and PI4P 4-phosphatase; and then the PI4P $\leftrightarrow$ PIP_2 substrate cycle is catalyzed by PI4P 5-kinase and PIP_2 5-phosphatase [13]. Activation of PLC by agonists has been calculated to be able rapidly to metabolize all cellular PIP_2. PI is synthesized in the endoplasmic reticulum, but these substrate cycles are thought to occur predominantly in the plasma membrane or other sites of utilization of PIP_2 [13]. Therefore, there must be a mechanism for the transport of PI from its site of synthesis to its sites of use by PI kinases in the cell.

PI/phosphatidylcholine transfer protein (PITP) is ubiquitously expressed in eukaryotic cells and can transfer both PI and phosphatidylcholine between membranes [14]. PITP seems to be required for the reconstitution, in permeabilized or broken cell preparations, of cellular processes that require PIP_2 synthesis, including sustained activation of PLCβ and γ isoforms and the ATP-dependent priming of secretion [15–17]. PITP also reconstituted secretory vesicle formation from the trans-Golgi network in a cell-free assay [18], although a requirement for PIP_2 in this process has yet to be determined. PITP was originally proposed to transfer PI from its site of synthesis to its site of use by PLC (see, for example, [19]). Recently, however, an analysis of the kinetics of guanosine 5′-(γ-thio)-triphosphate (GTPγS)-stimulated inositol phosphate production in cytosol-depleted permeabilized HL-60 cells that had been reconstituted with PITP led to the proposal that PITP may control the rate of inositol phosphate production by acting as a 'co-factor' for phosphoinositide-metabolizing enzymes, and thereby control the rate of PIP_2 synthesis [20]. More specifically, this model proposed that it is the limited pool of PI bound to the PITP, rather than the PI resident in the plasma membrane, that is the preferred substrate for the phosphoinositide-metabolizing enzymes.

Turkey erythrocyte ghosts are a well characterized and robust model membrane system and have proved useful for the analysis of the kinetics of receptor and G-protein-regulated PLCβs. The regulation of the PLCβ isoform in turkey erythrocytes is essentially identical to the regulation of PLCβs in mammalian cells, because turkey erythrocytes contain the avian homologues of a P2y purinergic receptor [21], $G\alpha_{11}$ [22] and PLCβ [23]. In addition, as has been shown for mammalian cells [24], a wortmannin-sensitive PI 4-kinase is responsible for maintaining the agonist-sensitive pool of polyphosphoinositides in turkey erythrocytes (R.A.C. and C.P.D., unpublished observations). We have therefore investigated the effect of PITP on phosphoinositide metabolism in turkey erythrocyte ghosts. Our data support a model whereby the loss of polyphosphoinositides by the concerted actions of PLC and the phosphoinositide kinases creates a PI chemical gradient between intracellular membranes which acts as the driving force for the net transfer of PI by the PITP, and do not require a more complex co-factor mechanism for the action of PITP to be invoked.

Results and discussion

PITP prolongs but does not affect the initial rate of GTPγS-stimulated PLC activity in turkey erythrocyte ghosts

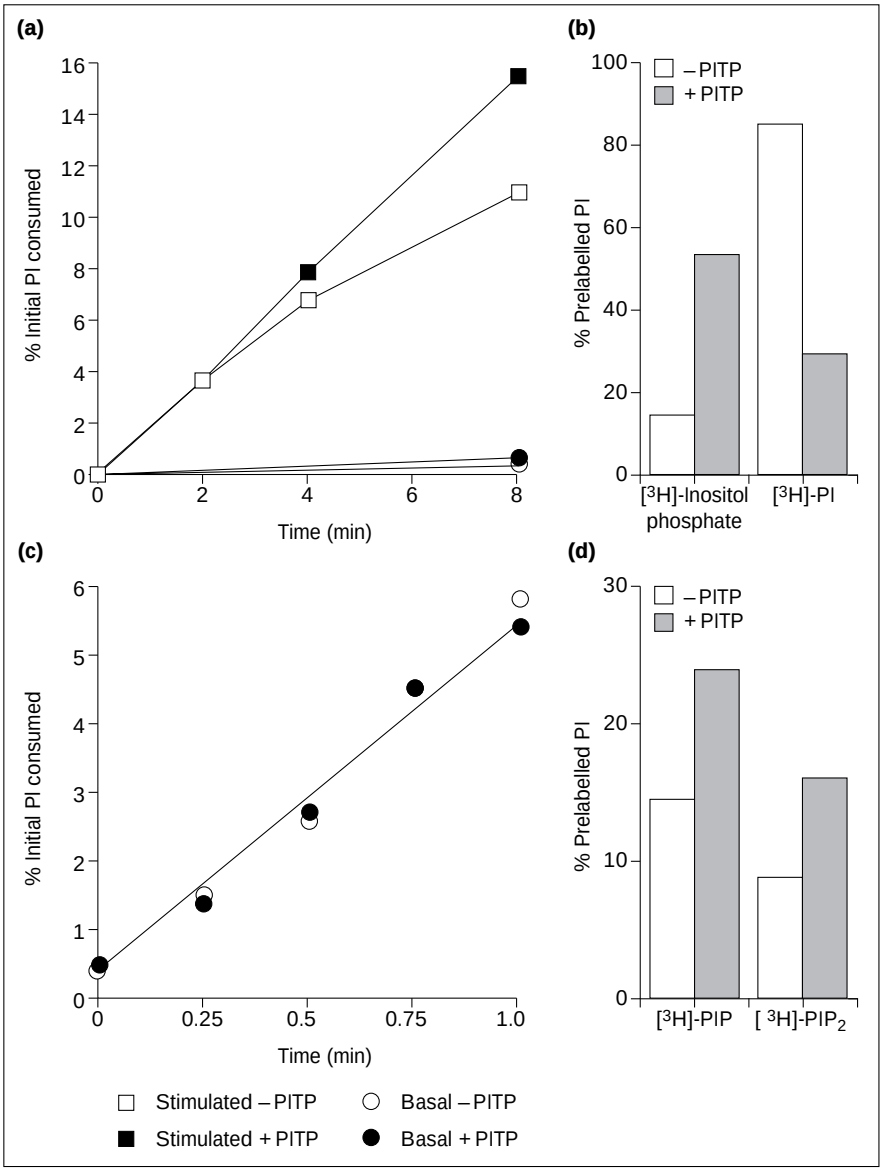
PITP was purified ~260-fold from bovine brain by a modification of the method of Helmkamp *et al.* [25], as described in the Materials and methods. The PITP preparation was devoid of PLC and PI kinase activities; a 35 kDa band, detected with anti-PITP antisera on western blots, comprised at least 30% of the protein as assessed by silver staining (data not shown). Preliminary experiments showed that the effect of PITP on increasing the steady-state level of PI4P was concentration-dependent. All the experiments described below used a maximally effective concentration of PITP, which was equivalent to ~10% of the PITP concentration in turkey erythrocyte cytosol, as determined by PITP activity measurements (data not shown).

We tested the effect of PITP on ATP and GTPγS-stimulated PLC activity in turkey erythrocyte ghosts by monitoring the release of radiolabelled inositol phosphates from prelabelled endogenous inositol phospholipids. As was shown previously for turkey erythrocyte ghosts [26], the rate of inositol phosphate production declined rapidly and ceased when only ~15% of the [^{3}H]-PI had been converted into inositol phosphates (Fig. 1b). However, Figure 1a,b show that, if ghosts were preincubated with PITP to allow penetration of the transfer protein before stimulation with ATP and GTPγS, there was a dramatic increase in the amount of PI that could be converted into inositol phosphates. After 120 minutes (the longest time measured), ~70% of the PI had been used (Fig. 1b), and inositol phosphate accumulation was still proceeding at close to its initial rate (data not shown). However, a significant divergence in the rate of PLC in the presence of PITP was not detected until at least 4 minutes after initiating the reaction (Fig. 1a). Therefore, the presence of the PITP allows the utilization of PI from sites that are not immediately available to the phosphoinositide kinases and PLC, presumably as a result of the lipid transfer activity of the PITP. Also, as PITP does not enhance the initial rate of PLC, it cannot act as a co-factor for this enzyme in turkey erythrocytes.

PITP enhances basal levels of polyphosphoinositides in turkey erythrocyte ghosts but does not affect the initial rate of PI 4-kinase

The PI in turkey erythrocyte ghosts can be labelled to high specific radioactivity by the *in vitro* labelling method used in this study [27]. Ghosts that were labelled with [^{3}H]-inositol solely in PI were incubated with ATP in the absence of guanine nucleotides, so that ghost endogenous PI was converted into polyphosphoinositides without hydrolysis to inositol phosphates. The presence of PITP

Figure 1



The effect of PITP on the initial rate of PLC and PI 4-kinase. Well-washed turkey erythrocyte ghosts labelled with [^{3}H]-inositol for **(a,b)** PLC assays or **(c,d)** PI 4-kinase assays were preincubated with PITP for 5 min at 37°C prior to stimulation with 5 mM ATP and (a,b) 100 μM GTPγS or (c,d) 5 mM ATP for the times indicated, and then processed for determination of [^{3}H]-inositol-labelled inositol phosphate and phosphoinositides, as described in Materials and methods; the histograms in (b,d) summarise the data from 120 min incubation. Data are means of duplicate determinations; each assay was repeated three times, with less than 5% difference between the results of each experiment.

in these lipid kinase assays caused the conversion of additional PI into polyphosphoinositides but did not affect the initial rate of conversion of PI to PIP (Fig. 1c,d). PI4P attained a new, higher steady-state level and PIP_2 levels were also increased (Fig. 1d) but did not attain a steady state even after 2 hours of incubation with ATP. Increasing the concentration of PITP from 10% to 50% of that estimated to be present in turkey erythrocyte cytosol had no additional effect. Thus, the effects of PITP are not limited to turkey erythrocyte ghosts in which PLC has been activated. PITP also causes the transfer of PI to the endogenous phosphoinositide kinases under basal conditions, without affecting the activity of the PI 4-kinase; PITP therefore cannot be a co-factor for this enzyme.

The effect of PITP is catalytic

Although the data presented above demonstrate unequivocally that PITP cannot be a co-factor that activates PI 4-kinase or PLC, as envisaged by Cunningham *et al.* [20], they do not exclude the possibility that PI and the polyphosphoinositides produced from it remain bound to the PITP [28] during their metabolism. If this were the case, then because PITP has a single PI-binding site [14], there should be a strict stoichiometry of not greater than one between the molar amount of polyphosphoinositides synthesized in the presence of PITP and the amount of PITP itself in the assay, provided that the newly synthesized lipid is not metabolized by activated PLC. We measured the PI mass in ghosts that had been incubated with

Table 1

The effect of PITP on PI mass in turkey erythrocyte ghosts.

PITP	[^{3}H]-PI (d.p.m.)	PI mass (nmol phosphate)
–	29 030 310	9.8
+	12 729 880	6.4
Percentage change in PI content	44%	65%
Difference		3.4

[3H]-inositol-labelled turkey erythrocyte ghosts were incubated with 5 mM ATP with or without approximately 1 nmol PITP for 2 h. Phospholipids were then extracted and separated on silica-60 HPTLC plates using chloroform:ethanol:water:triethylamine (4:5:1:4 v/v/v/v). The PI band was identified by reference to an authentic standard (Sigma) and extracted from the silica. The PI was then separated from contaminating phospholipids by HPTLC in the same solvent system using borate-impregnated plates. PI was extracted from the silica as before and then refluxed in PCA for 3 h at 230°C. PI mass was then determined by phosphate assay [26].

ATP in the presence or absence of 1 nmol of PITP for 2 hours. As shown in Table 1, the presence of 1 nmol PITP caused the conversion of an additional 3 nmol of PI to polyphosphoinositides, when compared with conversion in the absence of PITP. This establishes that polyphosphoinositides, which are responsible for the enhanced PLC activity upon subsequent stimulation by GTPγS, cannot all remain bound to PITP.

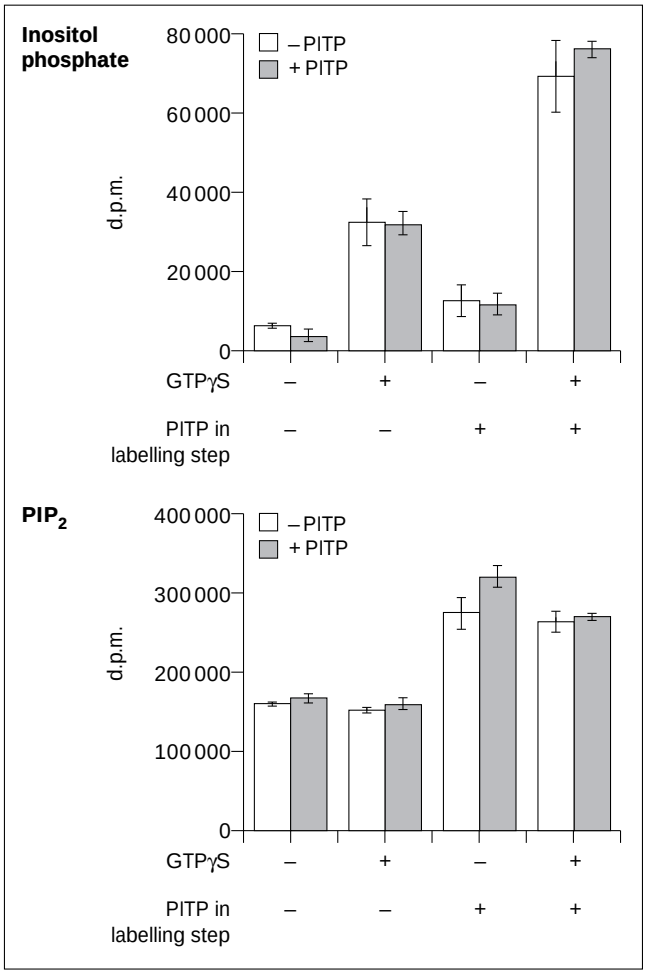
Continued presence of PITP is not necessary for enhanced GTPγS-stimulated inositol phosphate production

Our results suggest that, if PITP is present during the polyphosphoinositide synthesis phase of the assay, the additional PLC substrate levels should lead to enhanced PLC activity if GTPγS is added after removal of PITP by washing the ghosts. Ghosts labelled with [^{3}H]-inositol were incubated for 2 hours with ATP in the presence or absence of PITP, and then the ghosts were collected by centrifugation and washed extensively in lysis buffer. The washed ghosts contained no detectable PITP immunoreactivity and > 99% of the [^{3}H]-inositol-labelled lipids, whereas the first centrifugation supernatants did contain PITP immunoreactivity and had very little labelled lipid (data not shown). Ghosts incubated with PITP during the labelling had double the PIP_2 levels, and subsequent GTPγS-stimulated inositol phosphate production was also initially doubled, as compared with ghosts that were not treated with PITP, irrespective of the inclusion of PITP in the actual PLC assay (Fig. 2). Therefore, PLC appears to act under first-order conditions on lipid that is present in the ghost membranes, and enhanced PLC activity is independent of the presence of PITP.

A model for the action of PITP

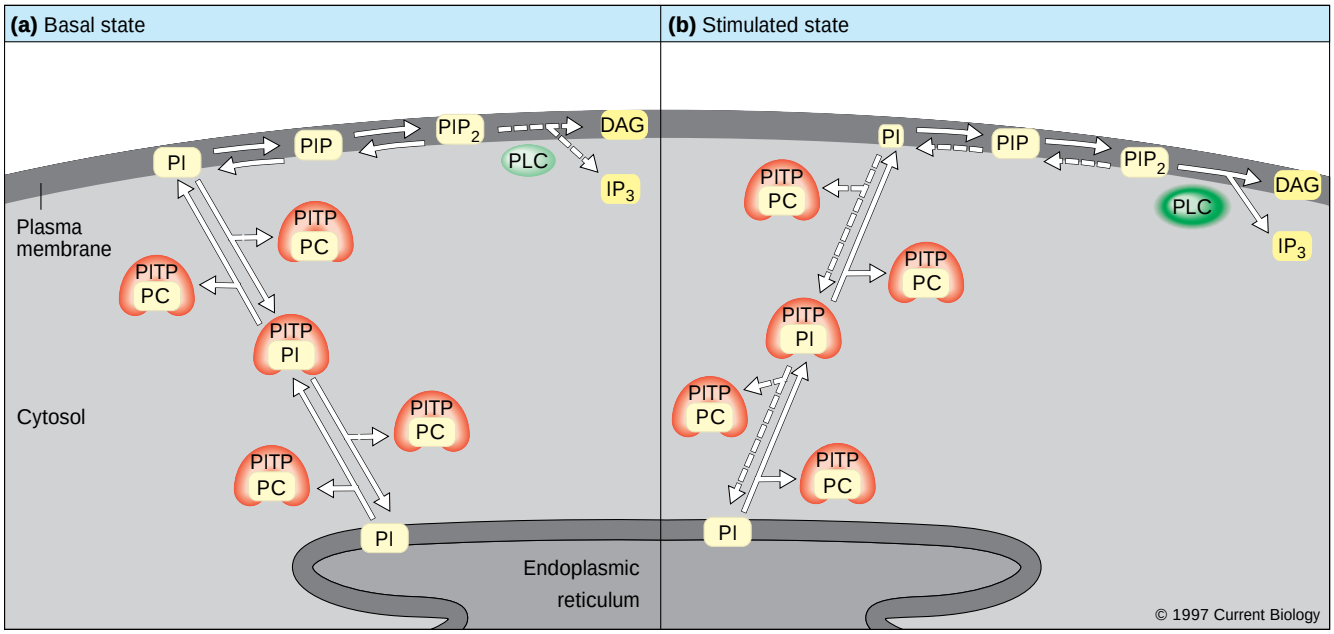
Turkey erythrocyte ghosts have proved to be a valuable model for studies of the mode of action of PITP, because manipulation of the assay conditions allows the endogenous PI kinase and PLC activities to be analyzed separately. PITP does not function as a co-factor for these enzymes in this system. Because the effects of PITP are nonstoichiometric and not immediate, we conclude that PLC and the lipid kinases studied in these experiments use bilayer lipids rather than protein-bound lipids as substrates. Thus, increased polyphosphoinositides are mostly associated with cellular membranes and not with the PITP, which does not stably associate with membranes in turkey erythrocyte ghosts. Consistent with this suggestion is the failure to observe the binding of PITP to plasma membranes by immunocytochemical studies [29].

Figure 2



The continued presence of PITP is not required for the enhancement of inositol phosphate production. Well-washed ghosts labelled with [^{3}H]-inositol in PI were labelled for 2 h with 10 mM ATP in the presence or absence of PITP, as indicated. Ghosts were then washed four times in 20 volumes of lysis buffer and then the PLC was stimulated for 4 min in the presence or absence of PITP before being processed for inositol phosphate and lipid analysis, as described in the Materials and methods section. Points show mean ± standard deviation (n = 3) from a single experiment which has been repeated twice.

Figure 3



Model for the action of PITP. Under basal, unstimulated conditions, the steady-state levels of the polyphosphoinositides are maintained by the two substrate cycles between PI and PIP_2. PITP, by acting to equilibrate the PI:phosphatidylcholine ratio between the cytoplasmic leaflets of intracellular membranes, can be considered as catalyzing an additional 'substrate cycle' in the synthesis of PIP_2. Upon stimulation of PLC, polyphosphoinositides are diverted out of the kinase/phosphatase substrate cycles causing a local depletion of PI in the plasma membrane. The PITP will then catalyze a net transfer of PI to the plasma membrane from other membranes. Erythrocyte ghosts, which have lost their polyphosphoinositide phosphatase activities relative to the kinases, are in effect in a 'pseudo-activated' state, in which any PI which is transferred will be phosphorylated and then trapped as polyphosphoinositides. This means that there will always be a driving force for further supply of PI into the plasma membrane.

The effects of PITP in turkey erythrocytes can be explained by the lipid supply model as originally proposed by Michell [19]. The levels of polyphosphoinositides in cells are maintained at steady state by two substrate cycles ([13]; Fig. 3). In turkey erythrocyte ghosts, these substrate cycles are disrupted because the ghosts are depleted of their polyphosphoinositide phosphatases relative to the opposing kinases (data not shown), and therefore any polyphosphoinositides that are formed by incubation of ghosts with ATP will be effectively trapped. This would be expected to deplete the plasma membrane of PI in the vicinity of the PI 4-kinase and so create a gradient of PI between this site and other membranes that do not contain a PI 4-kinase. As the kinetic parameters of PITP are such that it should act to maintain the typically observed ratio of PI:phosphatidylcholine in cell membranes [30], anything that caused a localized depletion of PI could obviously be the driving force for the net transfer of PI and could provide the basis for the reconstituting abilities of PITP. In an intact cell, this model predicts that steady-state levels of polyphosphoinositides would be maintained until the activation of PLC, which would divert PIP_2 out of the substrate cycles. PIP_2 would be maintained at the expense of PI in the vicinity of the phosphoinositide kinases, creating a gradient of PI which the presence of a PITP would tend to correct. If this model is correct, it would explain the need for the substrate cycles, as without the phosphatases, even a small amount of phosphoinositide kinase activity would eventually lead to all the PI in a cell being converted into polyphosphoinositides.

The reason for the differences between the mechanism of PITP action in turkey erythrocyte ghosts and the mechanism that was proposed for PITP in HL-60 cells permeabilized with streptolysin O is not readily apparent, but might result from differences in the protocols used. In the experiments described here, ghosts were preincubated with PITP; in the experiments of Cunningham *et al.* [20], however, the PITP was added at the same time as the GTPγS and the subsequent enhancement of inositol phosphate production occurred after a short lag period, which would deplete PI in the vicinity of the phosphoinositide kinases/PLC, therefore creating a driving force for the transfer of PI by the transfer protein. Moreover, the earliest time points described in the experiments of Cunningham *et al.* [20] began approximately 2 minutes after stimulation, whereas the initial rates of PI 4-kinase activity were readily measurable within the first minute after ATP addition to turkey erythrocyte ghosts.

The rates measured by Cunningham *et al.* [20] may therefore not be reliable estimates of the true initial rates of phosphoinositide kinases or PLC. Given the extensive homologies between the components that regulate PLCβs in turkey erythrocytes and in mammalian systems, it seems unlikely that our failure to observe a co-factor-like activity is due to some cell-type-specific mechanism. Also, as both mammalian PITPs and the structurally unrelated *Saccharomyces cerevisiae* PITP, Sec14p, can all reconstitute equally well in permeabilized HL-60 cells when matched for PI transfer activity [28], it seems more likely that it is the PI transfer activity, rather than any specific co-factor function, which is the critical component in this action of PITP.

Our recent results [31] suggest that PLCβ and inositol lipid kinases form a coupled enzyme system, the structural relationships of which are maintained by association with the actin cytoskeleton. We propose that PITP fuels this system by supplying PI down a chemical concentration gradient between membranes. Although PITP does not directly stimulate the relevant lipid kinases or PLC, our results do not rule out the possibility that PITP might be targeted to sites of signalling by reversible association with a suitable docking protein. The PI 4-kinase itself would be a good candidate for such a docking protein as PI could then be channelled to its sites of phosphorylation. This idea may explain the finding that PITP is associated in a signalling complex with both the epidermal growth factor receptor and a PI 4-kinase activity [16]. Nevertheless, it now seems likely that the lipid kinases themselves use membrane-bound, rather than PITP-bound, substrates.

Conclusions

We have investigated the effect of PITP on phosphoinositide metabolism in turkey erythrocyte ghosts. In this system, PITP functions solely as a lipid transfer protein without any discernible co-factor activity. In our model, the transfer protein catalyzes the continuous transfer of PI between biological membranes. Under unstimulated conditions in intact cells, polyphosphoinositide levels are maintained at steady-state and there is no net transfer of PI between membranes. Any biochemical change that disrupts the steady-state levels, such as activation of a PLC, would cause a depletion of polyphosphoinositides, which in turn would create a PI gradient, thereby allowing net transfer of PI into the depleted membrane. In permeabilized cells, the substrate cycles may be disrupted by the loss of polyphosphoinositide phosphatases, which would exaggerate any early effects of PITP on subsequent inositol phosphate production.

Materials and methods

Purification of PITP

PITP was purified from bovine brains by a method similar to that described by Helmkamp *et al.* [25]. The protein was purified 260-fold from the 100 000 × *g* supernatant of a bovine brain homogenate by ammonium sulphate fractionation followed by dialysis and sequential chromatography on fast flow Q–sepharose, Bio-Gel HPT–hydroxyapatite and Sephadex G-100 gel filtration.

Assay of PITP activity

PITP activity was assayed in 200 μl 10 mM HEPES (pH 7.0), 10 mM $MgCl_2$ using 1 mg ml^{-1} [^{3}H]-inositol-labelled ghosts as donor membranes and 1 mg ml^{-1} phosphatidylcholine:PI vesicles (49:1 w/w) as acceptor vesicles. Reactions were initiated by addition of 50 μl of column fractions to 200 μl of the ghost/vesicle mix, incubated for 30 min at 37°C and terminated by centrifugation for 30 sec at 13 000 r.p.m. in a microfuge followed by quenching 125 μl supernatant in 750 μl $CHCl_3$:MeOH:conc. HCl (40:80:1 v/v/v). A phase split was achieved by addition of 250 μl $CHCl_3$ and 350 μl 0.1 M HCl followed by vortexing and centrifugation as above. An aliquot of the lower phase was mixed with Opti-Phase and radioactivity determined by scintillation counting.

Labelling of turkey erythrocyte ghosts and assays of phosphoinositide kinase and PLC

Well-washed turkey erythrocyte ghosts were prepared as described [27]. Labelling of the phosphoinositides in well-washed ghosts with [^{3}H]-inositol and subsequent PLC assays were performed as described [27] with 5 mM ATP and 100 μM GTPγS as agonist. Phosphoinositide kinase assays were essentially identical to the PLC assays except that the ghosts were labelled with [^{3}H]-inositol only in PI and incubated with ATP in the absence of GTPγS. Labelled ghosts were pre-incubated with PITP for 5 min at 37°C before initiation of the reactions. Reactions were terminated with equal volumes of 1 M trichloroacetic acid (TCA) and samples processed for inositol phosphates and lipids as described [32].

Measurement of PI mass

Ghosts (3 mg protein) labelled with [^{3}H]-inositol were incubated with or without 1 nmol PITP for 2 h at 37°C in 3 ml intracellular buffer containing an ATP-regenerating system as described above. Reactions were terminated by addition of an equal volume of 1 M TCA. The precipitate was collected by centrifugation and extracted with 1.5 ml $CHCl_3$:MeOH: conc. HCl (40:80:1 v/v/v). A phase split was achieved by addition of 0.5 ml $CHCl_3$ and 0.9 ml 0.1 M HCl followed by vortex mixing and centrifugation. The lower phase was removed, dried *in vacuo,* resuspended in $CHCl_3$:MeOH (2:1 v/v) and loaded onto a silica-60 HPTLC plate (Merck) in a 2 cm streak. Lipids were resolved in $CHCl_3$:EtOH:H_2O:triethylamine (4:5:1:4 v/v/v/v) and visualized by iodine staining. The band corresponding to PI, as determined by co-migration with an authentic lipid standard (Sigma), was cut out, the lipid was eluted from the silica, dried down and re-chromatographed on borate-impregnated silica-60 HPTLC plate. PI was resolved in the same solvent system and re-extracted. The purified PI was then refluxed in perchloric acid at 240°C for 3 h and PI mass was determined by phosphate assay [26]. Recovery of PI was assessed by the recovery of [^{3}H]-label in the phosphate assay compared to the amount of [^{3}H]-PI present in an aliquot of the original sample. Recovery ranged between 30% and 50%.

Materials

All chemicals were from Fisons, BDH or Boehringer and were AnalaR grade or higher. Bovine brains were from Advanced Protein Products Ltd. Fast flow Q–sepharose was from Pharmacia, Bio-Gel HTP from Bio-Rad and Sephadex G-100 from Sigma. [^{3}H]-Inositol was from NEN. Anti-PITP antiserum was a gift from S. Cockcroft.

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