Resynthesis of phosphatidylinositol in permeabilized neutrophils following phospholipase C β activation: transport of the intermediate, phosphatidic acid, from the plasma membrane to the endoplasmic reticulum for phosphatidylinositol resynthesis is not dependent on soluble lipid carriers or vesicular transport

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Receptor-mediated phospholipase C (PLC) hydrolysis of phosphoinositides is accompanied by the resynthesis of phosphatidylinositol (PI). Hydrolysis of phosphoinositides occurs at the plasma membrane, and the resulting diacylglycerol (DG) is converted into phosphatidate (PA). Two enzymes located at the endoplasmic reticulum (ER) function sequentially to convert PA back into PI. We have established an assay whereby the resynthesis of PI could be followed in permeabilized cells. In the presence of [γ -³²P]ATP, DG generated by PLC activation accumulates label when converted into PA. The ³²P-labelled PA is subsequently converted into labelled PI. The formation of labelled PI reports the arrival of labelled PA from the plasma membrane to the ER. Cytosol-depleted, permeabilized human neutrophils are capable of PI resynthesis following stimulation of PLC β (in the presence of phosphatidylinositol-transfer protein), provided that CTP and inositol are also present. We also found

that wortmannin, an inhibitor of endocytosis, or cooling the cells to 15 °C did not stop PI resynthesis. We conclude that PI resynthesis is dependent neither on vesicular transport mechanisms nor on freely diffusible, soluble transport proteins. Phosphatidylcholine-derived PA generated by the ADP-ribosylation-factor-stimulated phospholipase D pathway was found to accumulate label, reflecting the rapid cycling of PA to DG, and back. This labelled PA was not converted into PI. We conclude that PA derived from the PLC pathway is selected for PI resynthesis, and its transfer to the ER could be membrane-protein-mediated at sites of close membrane contact.

Key words: ADP-ribosylation factor (ARF), phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol-transfer protein, phospholipase D.

INTRODUCTION

Receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) is a ubiquitous mechanism of signal transduction that generates the intracellular second messengers, diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃) [1]. The demand for PIP₂ exceeds basal levels, and it can be consumed at a rate of 4–12 times its resting level per min [2]. This high consumption of PIP₂ during cell-surface receptor activation requires that the substrate has to be replenished constantly. PIP₂ also serves as a substrate for phosphoinositide 3-kinase, which is part of a separate signal transduction pathway. Other demands on cellular PIP₂ include a cofactor role for the ADP-ribosylation factor (ARF)/Rho/protein kinase C-activated phospholipase D (PLD), cytoskeletal reorganization and exocytosis [3–5]. In all these cases, PIP₂ is required as an intact lipid, and therefore its levels must be stringently controlled. Levels are maintained at the expense of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PIP) by lipid kinases, which are subject to indirect regulation by cell-surface receptors [6–8]. Known regulators of

PIP 5-kinase Type 1 include phosphatidic acid (PA) and Rho proteins [9,10]. PI, the main inositol-containing lipid, is present in all cellular compartments, with the highest concentrations present in intracellular membranes [11,12]. Nevertheless, the plasma membrane has access to intracellular PI via the action of the soluble phosphatidylinositol-transfer protein (PITP) [13,14]. PI is thus available locally for phosphorylation by the lipid kinases to maintain PIP₂ levels [13,15].

To maintain an adequate supply of PI in the long term, the cell has to resynthesize PI by recycling DG and the inositol moiety of IP₃. DG can be phosphorylated by DG kinase, and the resulting PA is converted back into PI by the sequential action of two enzymes. CDP-DG synthase (CTP:phosphatidate cytidyltransferase, EC 2.7.7.41) catalyses the conversion of PA and CTP to form CDP-DG. The second step is dependent on PI synthase (CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase, EC 2.7.8.11), which catalyses the conversion of CDG-DG and inositol into PI. The hydrolysis of PIP₂ and its subsequent regeneration are known as the PI cycle, and this is summarized in Scheme 1 [16,17].

Abbreviations used: ARF, ADP-ribosylation factor; DG, diacylglycerol; ER, endoplasmic reticulum; FMLP, formylmethionyl-leucylphenylalanine; GTP[S], guanosine 5'-[γ -thio]triphosphate; IP₃, myo-inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PITP, phosphatidylinositol-transfer protein; PLC, phospholipase C; PLD, phospholipase D; POPA, 1-palmitoyl-2-oleoylphosphatidic acid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; Pyr₁₀DG, 1-palmitoyl-2-pyrenedecanoyl diglyceride; Pyr₁₀PA, 1-palmitoyl-2-pyrenedecanoyl phosphatidic acid; Pyr₁₀PC, 1-palmitoyl-2-pyrenedecanoyl phosphatidylcholine; Pyr₁₀PI, 1-palmitoyl-2-pyrenedecanoyl phosphatidylinositol; Tnp-PE, N-trinitrophenyldioleoylphosphatidylethanolamine.

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Scheme 1 Outline of the reactions of the phosphoinositide cycle and their spatial locations

Receptors activate the hydrolysis of PIP_2 at the plasma membrane, and the resulting DG is converted into PA at this location. PA has to traverse the cytosol by some unknown mechanism to reach the ER, where it is converted into PI. PI is subsequently returned to the plasma membrane by the soluble lipid transfer protein, PITP.

In mammalian cells, the biosynthetic machinery for PI synthesis is present at the endoplasmic reticulum (ER) [18]. The first enzyme in the pathway is CDP-DG synthase, which converts PA and CTP to form CDP-DG. The enzyme exists in two forms, one localized at the ER and the other enzyme located on the matrix side of the inner mitochondrial membrane [19,20]. In the mitochondria, the enzyme is involved in cardiolipin and phosphatidylglycerol synthesis. Neither form of mammalian CDP-DG synthase has been purified, although the enzymes from the two sources appear to be differentially sensitive to GTP. Recently, two cDNA sequences for CDP-DG synthase (*cds1* and *cds2*) have been reported [19]. CDP-DG and inositol are then reacted to make PI by PI synthase, the final enzyme in the pathway. Like CDP-DG synthase [20], this enzyme has been found to be localized mainly at the ER [18,21]. Two studies have claimed that, in addition to the ER, an enzyme with different characteristics is also present at the plasma membrane [22,23]; however, this claim has not been substantiated by more recent studies [24]. To date, only a single PI synthase gene has been identified, and this is uniformly expressed in all human tissues examined [19,25]. It encodes a protein of 213 amino acids with a molecular mass of 23400 Da. In the presence of CMP, the inositol on PI can be readily exchanged. This PI:inositol exchange reaction has been reported by many investigators, and was previously thought to be a separate enzyme; however, it is now known to be a feature of PI synthase [19]. CDP-DG synthase and PI synthase are not the critical determinants of cellular PI content in mammalian cells, since the overexpression of either gene alone or in combination does not result in a significant increase in the cellular levels of CDP-DG or PI [19]. In contrast, in flies, overexpression of CDP-DG synthase does influence PIP_2 availability [26].

Whatever governs the synthesis of PI in either resting cells or stimulated cells has not yet been resolved. The enzymic pathway utilized for resynthesis of PI during cell signalling is the same as that for *de novo* synthesis. Several studies have addressed the mechanisms involved in the compensatory PI resynthesis in cells during signalling [14,26,27]. Previously published work has indicated that the rapid synthesis of PI is not directly dependent on the presence of the agonist [28,29]. Two possibilities have been suggested: either the availability of substrate, or the fall in cellular PI, determines the resynthesis of PI. *In vitro* studies indicate that PI synthase can be regulated by its end product, i.e. PI acts as an inhibitor, and its removal would relieve this product inhibition. In a recent study, DG generated from PI hydrolysis

was resynthesized to PI, but not DG generated from an alternative source [30]. From this study, it appears that PI depletion, rather than DG availability, led to activation of PI resynthesis. Finally, it has been suggested that the availability of PA derived from DG phosphorylation occurs within a topologically restricted area [31,32].

It is self-evident that the plasma membrane is the site of agonist-stimulated PLC cleavage of PIP_2 . Replacement of PIP_2 requires five steps: $\text{DG} \rightarrow \text{PA} \rightarrow \text{CDP-DG} \rightarrow \text{PI} \rightarrow \text{PIP} \rightarrow \text{PIP}_2$ (Scheme 1). There is good evidence that the first step and the last two steps catalysed by the lipid kinases occur at the plasma membrane. In the neutrophil, activation by formylmethionyl-leucylphenylalanine (FMLP) causes the activation of PLC, and cells metabolically labelled with [^{32}P]P_i accumulate radiolabelled PA at the plasma membrane [33]. To complete the cycle, PA has to be transported from the plasma membrane to the ER, and the newly-synthesized PI has to be returned to the plasma membrane (see Scheme 1). For the transfer of PI, PITP is ideally suited, and this has been identified as a requirement for PLC-mediated signalling and is thought to supply PI to signalling sites at the plasma membrane for further phosphorylation by the lipid kinases [13]. The issue that remains unresolved is how PA is transferred from the plasma membrane to the ER for PI resynthesis (Scheme 1).

We initially confirmed that PITP could not transfer PA or DG *in vitro*. Secondly, we confirmed that PI synthase is localized at the ER using immunofluorescence. We then used permeabilized human neutrophils to reconstitute the resynthesis of PI subsequent to stimulation of the G-protein-regulated $\text{PLC}\beta$. Resynthesis of PI provides a direct measure of PA translocation from the plasma membrane to the ER. We report here that the resynthesis of PI is dependent on prior activation of the PLC, but not the PLD, pathway. PA generated from the PLD pathway is not utilized for PI resynthesis. PI resynthesis is independent of freely diffusible cytosolic proteins, excluding a role for a soluble carrier protein, or any known form of vesicular mechanism, to mediate the transfer of PA. We speculate that transport of PA might occur by membrane-contact sites, or via large protein complexes that can distinguish between PA produced via the PLC and PLD pathways.

EXPERIMENTAL

Materials

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidic acid (POPA) and *N*-trinitrophenyl-dioleoylphosphatidylethanolamine (Tnp-PE) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). 1-Palmitoyl-2-pyrene-decanoylphosphatidylinositol, -phosphatidylcholine, and -phosphatidic acid (Pyr_{10}PI , Pyr_{10}PC and Pyr_{10}PA respectively) were synthesized as described previously [34]. 1-Palmitoyl-2-pyrene-decanoyldiglyceride (Pyr_{10}DG) was prepared from its corresponding PC via hydrolysis by PLC (*Clostridium prefringens*; Sigma, St. Louis, MO, U.S.A.) followed by purification by HPLC on a silica column using chloroform/methanol/water (30:15:2; v/v/v) as the eluent. All lipids were at least 98% pure, as determined by HPLC and TLC. Recombinant PITP α , $\Delta 5$ -PITP α , PITP β and myr-ARF1 proteins were purified from *Escherichia coli*, as described previously [35,36]. $\text{PLC}\beta 2$ was made in Sf9 cells [37], and all other reagents were obtained as described previously [38]. Calnexin monoclonal antibodies were obtained from Transduction Laboratories, Lexington, KY, U.S.A., and affinity-purified polyclonal antibodies raised against PI synthase were kindly given by Dr. S. Jackowski, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN, U.S.A.

Assay of pyrene-lipid transfer

The assay used has been described previously [39]. Donor vesicle with four different compositions were used: Pyr₁₀PI/POPC/Tnp-PE (0.5:3:0.5 nmol respectively) for PI transfer; Pyr₁₀PC/POPC/POPA/Tnp-PE (0.5:2.5:0.5:0.5 nmol respectively) for PC transfer; Pyr₁₀PA/POPC/POPA/Tnp-PE (0.5:3:0.5 nmol respectively) for PA transfer; and Pyr₁₀DG/POPC/POPA/Tnp-PE (0.5:3:1:0.5 nmol respectively) for DG transfer. The acceptor vesicles consisted of POPC and POPA (50:2 nmol). The medium used was PBS, and the temperature was 25 °C. The measurements were performed with a PTI Quantamaster fluorimeter operated in the digital mode. The initial transfer rates were obtained by a linear fitting procedure of the fluorimeter software.

Preparation of neutrophils

Neutrophils were purified from 50–100 ml of fresh blood obtained from healthy volunteers, and prepared according to established procedures [40]. Anti-coagulated blood (50 ml) was mixed with an equal vol. of a 2% (v/v) solution of dextran in PBS, pH 7.2, to cause the erythrocytes to aggregate. After 20 min at room temperature, the leucocyte-rich upper layer was removed and layered on to 10 ml of Lymphoprep, and centrifuged at 2000 rev./min (800 g) for 20 min to separate neutrophils from other white cells. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were washed twice, and finally resuspended in Pipes buffer [20 mM Pipes/137 mM NaCl/3 mM KCl/1 mM MgCl₂/1 mg/ml glucose/0.1 mg/ml BSA (pH 6.8)], unless stated otherwise.

Immunolocalization of PI synthase in human neutrophils

Human neutrophils were allowed to attach to glass coverslips for 10 min, and then were fixed and stained as described previously [41]. A polyclonal PI synthase antibody (provided by Dr. Jackowski) [19] and a monoclonal calnexin antibody (Transduction Laboratories) were used as primary antibodies; Cy5-labelled anti-rabbit and Cy3-labelled anti-mouse (Amersham, Little Chalfont, Bucks., U.K.) antibodies were used as secondary antibodies. The immunostained cells were observed with a 100× objective (Numerical Aperture, 1.35) using an LSR Digital Imaging System equipped with an Olympus IX70 microscope. Images were captured with a digital 12-bit charge-coupled-device camera, and co-localization of the two markers was analysed with the LSR Ultra-View software.

PI resynthesis in permeabilized neutrophils

Neutrophils (10⁷ cells/ml) in Pipes buffer were permeabilized with 0.4 unit/ml streptolysin O at 37 °C. The Ca²⁺ concentration was maintained at 100 nM using 3 mM EGTA buffer, with 1 mM MgATP also included during the permeabilization. After 10 min, the cells were diluted with ice-cold Pipes buffer and centrifuged at 3000 rev./min (1800 g) for 5 min at 4 °C. The supernatant containing the leaked cytosolic proteins was discarded, and the cells were resuspended in Pipes buffer.

Aliquots (40 µl) of the permeabilized cells were transferred to tubes containing (final concentrations): 1 mM MgCTP, 1 mM inositol, pCa 6, 1 mM MgATP and 1.5 µCi of [γ-³²P]ATP. Other additions were made as follows, unless stated otherwise in the specific Figure legends: FMLP, 1 µM; GTP, 100 µM; guanosine 5'-[γ-thio]triphosphate (GTP[S]), 10 µM; PITPα, 100 µg/ml; myr-ARF, 100 µg/ml. The assay volume was 80 µl, and 10⁶ cells were used per assay. The cells were incubated for 60 min at

37 °C, or as indicated. The reactions were terminated by adding 320 µl of 1 M HCl followed by 960 µl of chloroform:methanol:conc. HCl (200:100:1, v/v/v). After thorough mixing, the lower organic phases were transferred to fresh tubes. The organic phases were washed with 800 µl of methanol:1 M HCl (1:1, v/v), transferred to new tubes, and the chloroform was evaporated under a vacuum. The lipids were dissolved in 50 µl of chloroform, and samples were spotted on to Whatman LK6TLC silica plates. The plates were treated with 1% (v/v) oxalate before use, and subsequently developed in chloroform:methanol:acetic acid:acetone:water (40:13:12:15:8, by vol.), dried at room temperature and imaged using a Fuji Phosphor-Imager. The radioactivity was quantified using appropriate software.

For the experiment shown in Figure 9, the lipids associated with PITP were analysed. In this case, the cells were centrifuged at 3000 rev./min (800 g) for 5 min at the end of the incubation, the supernatant was retained, and then centrifuged further at 100 000 g for 1 h to sediment residual particulate material. The supernatant after this spin was immediately extracted with organic solvents as described above. In separate experiments, the PITP was purified further by gel filtration, yielding identical results.

Wortmannin treatment

Intact cells were incubated at 37 °C for 10 min in the presence of 100 nM wortmannin before use. Under these conditions, wortmannin has been shown to inhibit several vesicular transport steps, including exocytosis [42].

Presentation of data

All experiments presented here are individual experiments that were repeated on at least three separate occasions with similar data. The extent of labelling between experiments varied, and therefore the data could not be pooled. All the experiments were carried out in duplicate (except where time courses were monitored), and the degree of variation between duplicates is shown as error bars in the individual figures.

RESULTS

PITPα binds and transfers PI and PC, but not PA and DG

Previous studies have indicated that PITP does not transfer PA [43]. To confirm this, and also to exclude the possibility that PITP can transfer DG, we measured the transfer of pyrene-labelled PA, DG, PI and PC using a previously developed spectroscopic assay [39]. This assay is based on the time-dependent increase of fluorescence that occurs upon translocation of a pyrene lipid from quenched donor vesicles to unquenched acceptor vesicles. The results are shown in Figure 1. Addition of a small amount (0.4 µg) of PITPα is enough to obtain rapid transfer of Pyr₁₀PI. Pyr₁₀PC is also transferred by PITPα, albeit at a rate lower than with Pyr₁₀PI, as expected from the fact that the affinity of PITPα for PI is about 15 times less than for PI with identical acyl chains [44]. When Pyr₁₀PA-containing donors were employed, there was very little transfer of the fluorescent lipid, even if the amount of PITPα added was 50 times higher than that used with Pyr₁₀PI donors (Figure 1); neither was there any detectable transfer of Pyr₁₀DG with such high concentrations of PITPα. On the basis of the initial slopes and the amount of protein used, we calculated that the relative rates of Pyr₁₀PI, -PC, -PA and -DG were 1, 0.1, 0.0002 and 0 respectively.

We also measured binding of Pyr₁₀PI and Pyr₁₀PA by PITPα. This was achieved by titrating PITPα into the corresponding

Figure 1 Transfer of pyrene-labelled PI, PC, PA and DG by PITP α

Quenched donor vesicles containing the indicated pyrenyl lipid were mixed with an excess of POPC/POPA acceptor vesicles in the cuvette. The increase of fluorescence occurring thereafter is due to (slow) spontaneous translocation of the pyrene lipid molecules from the donor to the acceptor vesicles. Upon addition of PITP α (shown by arrows) the rate is enhanced, provided that the labelled lipid is transported by PITP α . Note that widely different amounts of PITP α were used with different donors.

donor vesicles [39]. In agreement with the transfer data, a robust increase of pyrene fluorescence was observed, which was protein-concentration-dependent with the Pyr₁₀PI donors, whereas no increase at all was observed with the Pyr₁₀PA donors (results not shown). From these experiments we conclude that PITP α is unlikely to mediate significant binding or transfer of PA.

Immunolocalization of PI synthase in human neutrophils

As discussed in the Introduction, the site of PI synthesis is controversial. This is partly due to non-availability of appropriate reagents. Recently, a single PI synthase gene has been identified, and this is uniformly expressed in all human tissues examined [19,25]. Immunolocalization of PI synthase revealed that this enzyme co-localizes with calnexin, an integral membrane protein located in the ER (Figure 2). Analysis of 14 cells demonstrated that PI synthase and calnexin co-localize with a correlation coefficient (r) of 0.977 ± 0.007 .

Establishing the conditions in permeabilized cells for measuring PI resynthesis, i.e. a measure of PA transfer

We initially confirmed the characteristics of PI resynthesis following FMLP stimulation of intact neutrophils [45]. The cells were metabolically radiolabelled with [³²P]P_i to incorporate label into ATP. PA labelling preceded the labelling of PI, and was observed within 30 sec, with a peak after 2 min. The labelling of

Figure 2 Co-localization of PI synthase with the ER-marker calnexin in human neutrophils

(A) Nomarski image; (B) Co-localization is indicated by the yellow colour in the combined image of (C) and (D); (C) immunolocalization of PI synthase (red) and (D) calnexin (green). The correlation coefficient r , which measures the degree of linear association between two values of paired association, is 0.977 ± 0.007 ($n = 14$).

Figure 3 PI resynthesis is dependent on inositol and CTP

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins, and stimulated with GTP[S] in the presence of PITP α to activate the endogenous PLC β . Incorporation of label derived from [γ - 32 P]ATP into PA (**A**) and PI (**B**) was monitored over a period of time in the presence and absence of inositol and CTP (1 mM). At the end of the incubation, the lipids were extracted and analysed by TLC. This experiment is representative of one of three performed.

PI was slower and reached a plateau at 30 min (results not shown).

We then established conditions for reconstitution of the resynthesis of PI in permeabilized neutrophils following PLC activation. G-protein-regulated phospholipases C β 2/ β 3 present in these cells were activated with either GTP[S] or FMLP [46]. PITP had to be replaced in the permeabilized cells to obtain a robust stimulation of PLC activity [46]. We added 32 P-labelled ATP to monitor the incorporation of label into PA and PI. In our initial studies, we observed that the addition of the stimulus increased the labelling of PA, but not of PI. This indicated that the DG derived from PIP $_2$ hydrolysis could be metabolized by DG kinase to PA, but could not be converted into PI (Figure 3A). We reasoned that the enzymes CDP-DG synthase and PI synthase, which require CTP and inositol respectively, were limited by the availability of these soluble factors in the permeabilized cells. Addition of CTP and inositol was sufficient for PI resynthesis to occur. PI resynthesis occurred at the expense of PA, as observed by a decrease in the amount of labelled PA (Figure 3B). A precursor-product relationship is evident, with maximal incorporation of label into PA preceding the incorporation of label into PI, as observed previously for intact cells [45]. Figure 4 establishes that addition of both CTP and inositol is required for conversion of PA into PI in basal and under stimulated conditions. In the presence of CTP only, the in-

Figure 4 Addition of CTP or inositol alone is insufficient to support PI resynthesis

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins, and stimulated with GTP[S] in the presence of PITP α to activate the endogenous PLC β . PI resynthesis was monitored in the presence and absence of inositol (1 mM) alone, CTP (1 mM) alone or in the presence of both CTP and inositol (1 mM each). At the end of the incubation, the lipids were extracted and analysed by TLC. This experiment is representative of one of three.

intermediate, CDP-DG, was found to accumulate (results not shown).

We next examined whether PI resynthesis was dependent on prior activation of the PLC pathway by using neomycin and a deletion mutant of PITP [47,48]. Neomycin blocks the hydrolysis of PIP $_2$ due to sequestration of the substrate [47], and we found that it also blocked the incorporation of label into PI (Figure 5). This indicates that the increase in PI resynthesis was dependent on PLC activation, rather than GTP[S] activating any of the subsequent enzymes in the PI biosynthetic pathway. Deletion of five amino acids from the C-terminus of PITP produced a mutant protein (Δ 5-PITP α), which no longer supports IP $_3$ production despite retaining some transfer activity [48]. Δ 5-PITP α was unable to support PI resynthesis either (Figure 6), thus reinforcing the observation that activation of PLC was the driving force for PI resynthesis.

GTP[S] is a global activator of all GTP-binding proteins, and has many effects including the inhibition of vesicular transport. We therefore tested whether FMLP was also capable of supporting PI resynthesis in permeabilized cells. FMLP binds to its receptor at the plasma membrane, and transmits its signal by the receptor interacting with the heterotrimeric G-proteins of the G $_i$ family. Thus FMLP can only activate a subset of GTP-binding proteins, unlike GTP[S]. As for GTP[S], FMLP was able to stimulate PI resynthesis, but the response was not as robust as that seen for GTP[S] (Figure 7A).

Mechanism of PA transport from the plasma membrane to the ER

From the results described so far, cytosol-depleted cells are capable of converting PA into PI. PA is a product of DG phosphorylation, which is generated at the plasma membrane by receptor-regulated PLC β hydrolysis. The two biosynthetic enzymes responsible for converting PA into PI reside at the ER, CDP-DG synthase and PI synthase. Thus the PA produced at the plasma membrane has to be transported to the ER. There are

Figure 5 Optimal resynthesis of PI is dependent on GTP[S] and P1TP α : neomycin blocks the resynthesis of PI

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins and incubated with either GTP[S] (10 μ M) or P1TP α (100 μ g/ml), or with both for 1 h. The assay included inositol (1 mM) and CTP (1 mM), MgATP (1 mM), and 1.5 μ Ci of [γ - 32 P]ATP. At the end of the incubation, the lipids were extracted and analysed by TLC. The incorporation of label into PI was used as a measure of PI resynthesis. This experiment was repeated on four separate occasions with similar results.

Figure 6 Resynthesis of PI is not supported by a deletion mutant of P1TP α

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins and incubated with GTP[S] (10 μ M) and P1TP or Δ 5-P1TP α (D5) (100 μ g/ml), as indicated. The assay included inositol (1 mM) and CTP (1 mM), MgATP (1 mM) and 1.5 μ Ci of [γ - 32 P]ATP. At the end of the incubation the lipids were extracted and analysed by TLC. The incorporation of label into PI was used as a measure of PI resynthesis. This experiment was repeated on three separate occasions with similar results.

potentially three mechanisms that a cell can utilize in the transport of membrane lipids: (1) a vesicular method of transport (this would not be specific for a particular lipid, but would depend on bulk flow); (2) by soluble, specific lipid-transport proteins; or (3) by membrane proteins acting at membrane-contact sites. To examine which of the three mechanisms were responsible for PA transfer, we used a number of experimental approaches.

Several cytosolic proteins have been identified as being required for vesicle formation and subsequent fusion, and these include ARF, P1TP, *N*-ethylmaleimide-sensitive fusion protein (NSF)

and soluble NSF-attachment protein (or SNAP) [13,49]. The experiments described here have been carried out in cells that have been depleted of freely diffusible proteins, such as ARF and P1TP. Previous studies have demonstrated that streptolysin O makes lesions that permit the near-complete release of lactate dehydrogenase [50], P1TP [51,52], ARF [51] and phosphoinositide 3-kinase γ [52] within 5–10 min. P1TP is added back, since it is an essential component of the PLC pathway [13]. The cells had been permeabilized for 10 min, conditions in which PLC β is still retained [46]. To deplete the cells of their cytosolic components

(A) 10 min permeabilization **(B) 40 min permeabilization**

Figure 7 Extended permeabilization with streptolysin O for 40 min did not diminish the ability of neutrophils to resynthesize PI after stimulation of PLC activity with GTP[S] or FMLP

Human neutrophils were permeabilized for either 10 min or 40 min with streptolysin O, and washed free of the leaked cytosolic proteins. PLC activity was stimulated with GTP[S] (10 μ M) or FMLP (1 μ M) plus GTP (100 μ M). For the 10-min-permeabilized cells, the assays were supplemented with P1TP α (100 μ g/ml), and for the 40-min-permeabilized cells, recombinant PLC β 2 was also included to compensate for its loss in the extended permeabilized cells. The assay included inositol (1 mM) and CTP (1 mM), MgATP (1 mM) and 1.5 μ Ci of [γ - 32 P]ATP. At the end of the incubation the lipids were extracted and analysed by TLC. The incorporation of label into PI was used as a measure of PI resynthesis. This experiment was repeated on three separate occasions with similar results.

more extensively, we permeabilized the cells for a longer period. Under these conditions, loosely associated proteins, such as PLC β , are now lost to the external medium [46]. To test whether PI resynthesis could still occur in these cells, we had to add back not only P1TP α , but also PLC β , to obtain a reasonable level of PIP $_2$ hydrolysis. The experiment in Figure 7(B) illustrates that even after permeabilization for 40 min, the cells are still capable of PI resynthesis following stimulation with FMLP or GTP[S]. This result strongly indicates that freely diffusible cytosolic proteins are not required for the transport of PA to the ER for PI resynthesis.

To exclude further the possibility of a role for vesicular transport, we used both wortmannin and a 15 $^{\circ}$ C temperature

Figure 8 PI resynthesis is not inhibited by wortmannin (A), and does occur at 15 $^{\circ}$ C (B)

(A) Intact neutrophils were incubated with 100 nM wortmannin, permeabilized for 10 min, and washed. PI resynthesis was monitored in the presence of CTP (1 mM), inositol (1 mM), P1TP α (100 μ g/ml) and 1.5 μ Ci of [γ - 32 P]ATP. The cells were stimulated with GTP[S] (10 μ M) or FMLP (1 μ M) plus GTP (100 μ M). (B) Permeabilized neutrophils were stimulated with 10 μ M GTP[S] in the presence of CTP (1 mM), inositol (1 mM), P1TP α (100 μ g/ml) and 1.5 μ Ci of [γ - 32 P]ATP at 37 $^{\circ}$ C, or at 15 $^{\circ}$ C for 1 h. The lipid images are shown at the top of the panel.

block. Although wortmannin has been reported to inhibit PI 4-kinase activity, this requires higher concentrations than those used for inhibition of phosphoinositide 3-kinase activity. Wortmannin at 100 nM only partially reduced PI resynthesis (Figure 8A). This concentration of wortmannin was sufficient to inhibit many membrane-trafficking events, including endocytosis [53–57]. Since many intracellular vesicular transport systems show a sharp temperature-dependence [58–60], we examined the temperature-dependence of PA transport. Reduction in temperature was again unable to inhibit PI resynthesis (Figure 8B). A decrease was observed, but this was accounted for by a corresponding decrease in PLC activation (results not shown). Finally, GTP[S] is reported to be inhibitory to most forms of membrane traffic and yet, in the majority of experiments described here, we have used GTP[S] to stimulate PLC activity.

Figure 9 P1TP α becomes loaded with radiolabelled PI, but not PA under stimulated conditions

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins, and incubated with GTP[S] (10 μ M) and P1TP α (100 μ g/ml), added as indicated. The assay included inositol (1 mM) and CTP (1 mM), MgATP (1 mM), and 1.5 μ Ci of [γ - 32 P]ATP. At the end of the incubation, the cells were sedimented by centrifugation, and the supernatant containing the P1TP was retained. The supernatant was centrifuged at 100 000 g for 1 h before the lipids were extracted, and subsequently analysed by TLC. The lipid image (upper panel) and quantification of the data (lower panel) are shown. This experiment was repeated on three separate occasions with similar results.

From the available data, we can exclude a mechanism involving vesicular transport as well as involvement of cytosolic lipid transfer proteins, since PI resynthesis is not dependent on cytosolic proteins. P1TP has not been found to bind PA and transport this lipid *in vitro* (Figure 1, and [43]). Nonetheless, we examined the possibility that PA might be transported through the cytoplasm by P1TP in the cell-based assay used here. The cells were permeabilized and washed free of the cytosolic proteins. GTP[S] and P1TP α were added to the cells to stimulate the hydrolysis of PIP $_2$ in the presence of γ -labelled [32 P]ATP. After 10 min, the supernatant containing P1TP was obtained and the lipids were analysed (Figure 9). In the absence of added P1TP, very little radiolabelled PI or PA was found in the supernatant of

either control or stimulated cells. Some labelled PIP and PIP $_2$ were noted, but this was probably bound to PIP $_2$ -binding proteins such as profilin, gelsolin and actinin. (In other experiments, P1TP was re-isolated from the supernatant by gel filtration, and labelled PIP and PIP $_2$ were decreased.) Upon addition of P1TP to the cells, radiolabelled PI was associated with P1TP, and this was substantially increased when the cells had been stimulated with GTP[S]. However, very little radiolabelled PA was found associated with P1TP under any conditions, despite a considerable increase in PA labelling observed in the cellular pellet upon stimulation. From these data, we can formally exclude P1TP as a lipid transporter of PA; moreover, they confirm that, in cells, P1TP is capable of transferring newly synthesized PI from the ER to target membranes. We have previously suggested that P1TP might provide substrate to the lipid kinases either sequentially or by channelling PI to a molecular complex containing the lipid kinases [13]. The absence of binding of PIP or PIP $_2$ to P1TP does not eliminate these possibilities, because the P1TP bearing the highly phosphorylated inositol lipids might no longer be freely soluble and would not have been extracted in the procedure used here.

Is the PA derived from PLD activity available for PI synthesis?

In neutrophils, PLC and PLD activation both occur at the plasma membrane [33,61]. Whereas PLC hydrolyses PIP $_2$, PLD hydrolyses PC to produce PA and choline. In the experiment in Figure 3, it can be noted that, in the presence of CTP and inositol, only some of the PA was converted into PI, and we reasoned that the remainder of the PA was derived from an alternative source. In neutrophils, GTP[S], by regulating the activity of ARF and Rho proteins, can stimulate PLD activity, resulting in the production of PA [42,62]. Although the PA formed initially from PC would be unlabelled, PA could accumulate label by initial dephosphorylation followed by rephosphorylation, owing to the sequential action of a plasma membrane phosphatidate phosphohydrolase and DG kinase respectively. We formally tested for this possibility by adding ARF1 to the permeabilized cells to stimulate PLD activity (Figure 10A). GTP[S] led to an increase in PA labelling, and ARF increased it further. From these data, we can conclude that the PA generated by the PLD pathway is subjected to degradation by phosphatidate phosphohydrolase, and the resulting DG can acquire radiolabel by subsequent phosphorylation. Thus the PA derived from PLD is cycled between DG and PA. To confirm further that the radiolabelled PA seen with ARF was derived originally from ARF-stimulated PLD activity, we used ethanol. Ethanol effectively blocked the increase in label observed in PA, indicating that, when PLD activation produces phosphatidylethanol rather than PA as the initial product, PA is no longer available for dephosphorylation (phosphatidylethanol is more stable than PA) (results not shown).

It is clear that the PA derived from the PLD pathway does acquire label, and therefore we could examine whether it was utilized for PI resynthesis by tracking the formation of labelled PI. It is observed that the increase in PA labelling due to ARF and GTP[S] is not reflected in a substantial increase in PI labelling (Figure 10B). In contrast with ARF1 and GTP[S], in the combined presence of P1TP α and GTP[S], there is not a substantial build-up of PA, although there is a substantial increase in PI labelling. Thus the fate of PA from the PLD and PLC pathways is different. Whereas PA from the PLC pathway is utilized for PI resynthesis, the PA produced from the PLD pathway is not efficiently converted into PI. The fate of this PA was not investigated further in this study, but studies in intact

Figure 10 PA derived from the PLD pathway is not converted into PI, but is cycled between DG and PA

Human neutrophils were permeabilized for 10 min with streptolysin O, washed free of the leaked cytosolic proteins and incubated with GTP[S] (10 μ M), myristoylated ARF1 and P1TP α (100 μ g/ml) added as indicated. The assay included inositol (1 mM) and CTP (1 mM), MgATP (1 mM) and 1.5 μ Ci of [γ - 32 P]ATP. At the end of the incubation, the lipids were extracted and analysed by TLC. The lipid images are shown at the bottom of the individual panels. This experiment was repeated on three separate occasions with similar results.

cells have indicated that the PA is re-utilized for PC synthesis [63].

DISCUSSION

We have used permeabilized neutrophils to identify the mechanism of PA transfer. PLC activity was stimulated by activation of the G-protein with GTP[S] or FMLP in the presence of P1TP. From our previous studies, we have established that removal of cytosolic proteins by permeabilization for 10 min results in the loss of G-protein-regulated PLC β activity, and this can be regained by addition of exogenous P1TP [46]. The addition of γ -labelled ATP ensured that the newly formed PA produced by phosphorylation of DG was labelled, and therefore could be tracked through the biosynthetic pathway. We initially used immunolocalization to confirm that PI synthase is localized at the ER, and therefore transfer of PA to the ER is a prerequisite for PI resynthesis. The resynthesis of PI at the ER served as a

measure of PA transfer. Despite the removal of the freely diffusible cytosolic proteins, the resynthesis of PI occurred unimpaired in the permeabilized cells, provided that CTP and inositol were present. Depletion of the cytosol for 10 min only removes the freely diffusible proteins, and therefore depletion was extended to 40 min. Under these conditions, membrane-associated proteins now exit from the cells and can be removed, including PLC β . By re-addition of PLC β and P1TP to these extensively permeabilized cells, we could still restore PI resynthesis. Thus transfer of PA to the ER is independent of cytosolic proteins.

Eukaryotic cells utilize a variety of vesicular transport mechanisms to import molecules into the cell interior, including uptake by caveolae, non-clathrin-coated vesicles, clathrin-coated pits and vesicles, macro-pinosomes and phagosomes. These different vesicle types are used for different functions and are thought to have unique protein compositions relevant to their specialized roles. Although it is generally accepted that the protein compositions of the various vesicle types are different from one another, the distribution and internalization of plasma membrane lipid components are unclear. Some studies have suggested that certain lipids might be organized in microdomains, either within the plane of the plasma membrane or enriched in certain membrane specializations, such as caveolae. Because we can eliminate a requirement for cytosolic proteins, all forms of vesicular transport can be eliminated. Additionally, we have used GTP[S] to drive the activation of PLC. GTP[S] is a non-hydrolysable analogue of GTP, and is known to inhibit vesicular transport processes by blocking the action of GTPases. These data collectively exclude both vesicular forms of transport and PA transport by soluble lipid transfer proteins.

In addition to vesicle transport systems, lipids can be transported by lipid transport proteins. An example of one ubiquitous protein is P1TP, as discussed above. The possibility that PA could be transported by P1TP was eliminated by examining whether any labelled PA was associated with P1TP. Data *in vitro* have shown that P1TP does not bind PA, and neither does it transfer it (Figure 1). Nonetheless, in the cellular context P1TP might be able to carry out such a role. We were unable to find any evidence for labelled PA associated with P1TP, thus excluding this possibility.

Having eliminated the two most likely mechanisms that could account for transport of PA to the ER, we are left with the possibility that PA can be retrieved selectively from sites of PLC signalling by a tightly associated membrane protein that is present at the ER. Regions of close contact between the ER and the plasma membrane have been noted, and these membrane-contact sites could be responsible for the exchange of lipid by a protein-mediated mechanism. In phototransduction in the retina of *Drosophila*, hydrolysis of PIP $_2$ by a G $_q$ -regulated PLC β enzyme is part of the signalling cascade. In this case, P1TP is anchored at the rhabdomeres, which are closely apposite to the plasma membrane [13]. Thus a precedent for lipid exchange by an integral membrane protein is present in this highly specialized phototransduction cascade. Membrane exchange at contact sites is a concept that has gained credence from studies where the synthesis of phosphatidylethanolamine from PS has been examined. In this case, PS that is synthesized at the ER is imported to the mitochondria for decarboxylation to form phosphatidylethanolamine. PS transfer to mitochondria occurs independently of cytosolic proteins, is an ATP-dependent process and requires a mitochondrial membrane protein [64].

PA is also a product of the PLD pathway, which is activated in many cells by the appropriate agonists acting at the cell surface. PLD activity is regulated by the small GTPases, ARF

and Rho proteins. In the permeabilized cells used here, ARF proteins have been removed during permeabilization, but Rho proteins remain associated with the cells; therefore GTP[S] does activate PLD activity to a limited degree. The further addition of ARF greatly increases the activation of PLD, owing to the synergy between ARF and Rho proteins [42]. The PA that is initially formed is not labelled, since it is derived directly from PC. In the presence of γ -labelled ATP, PA accumulates label (Figure 10A). This labelled PA did not enter the PI synthesis pathway. The basis for the distinction between PA formed by the PLC and PLD pathways lies in differences in the fatty acid composition. The PA derived from the PLC pathway is predominantly enriched in stearic and arachidonic acids, whereas the PA derived from the PLD pathway contains a mixture of palmitic, stearic, linoleic and oleic acids [65,66].

In summary, we have established a permeabilized cell system to study the resynthesis of PI from PA at the ER following PLC activation at the plasma membrane. We have defined the cellular requirements for this process, and find no role for 'freely diffusible' cytosolic proteins. This excludes soluble lipid transfer proteins and all forms of vesicular transport. We cannot exclude the possibility that large cytosolic protein complexes are involved, because these would be retained in the permeabilized cells. We suggest that a potential mechanism for PA transfer from the plasma membrane to the ER is by membrane-contact sites, where a membrane-bound lipid transfer protein could mediate lipid transfer.

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