Cooperativity of phosphatidylinositol transfer protein and phospholipase D in secretory vesicle formation from the TGN – phosphoinositides as a common denominator?

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Abstract Phosphatidylinositol transfer protein (PITP) and phospholipase D (PLD) stimulate the formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the trans-Golgi network (TGN) in a cell-free system. The stimulatory effects of PITP and PLD are additive. Stimulation by either PITP or PLD is blocked by geneticin, a member of the aminoglycoside antibiotics known to bind to phosphoinositides. Since the PLD we used is insensitive to geneticin, our results suggest that phosphoinositides promote secretory vesicle formation as downstream effectors of both PITP and PLD, possibly via the recruitment of proteins mediating membrane budding and fission.

Key words: Aminoglycoside; Phosphatidylinositol transfer protein; Phospholipase D; Phosphoinositide; Secretory vesicle; Trans-Golgi network

1. Introduction

Inositol phospholipids have emerged as key players in membrane traffic [1–4]. This family of lipids has been shown to exert pivotal roles in both the formation of vesicles from donor membranes [5,6] and their fusion with acceptor membranes [7–9]. Inositol phospholipids have been implicated in membrane trafficking events that constitutively occur in all eukaryotic cells [10,11], as well as in those associated with the regulated secretion mediated by secretory granules [7–9] and synaptic vesicles [12]. Specifically, the machinery operating in these membrane traffic steps has been shown to include several proteins involved in phosphatidylinositol (PI) metabolism, including PI transfer proteins (PITPs) [5,7,9,10,13] as well as various PI kinases [6,8,11] and phosphoinositide phosphatases [12].

Another type of phospholipid-modifying enzyme, phospholipase D (PLD), which catalyzes the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA), has also been implicated in membrane traffic, in constitutive [14,15] and regulated [16,17] pathways, and at the level of vesicle formation [14,15,17] and vesicle fusion [16]. Interestingly, at least two levels of interaction between inositol phospholipids and PLD have been noted that are potentially relevant for the control of membrane traffic [1]. First, mammalian PLD1 requires PI bis phosphate (PIP_2) for full activity [18]. Second, PA, the product of the PLD reaction, activates type I PI 4-phosphate 5-kinase, which generates PIP_2 [19,20]. Given these levels of interaction, it is important to determine whether PLD and proteins involved in phosphatidylinositol metabolism indeed cooperate in specific membrane traffic events, and if so, how.

With regard to vesicle fusion, Cockcroft and colleagues [9], using a cell-free system, have shown that both PITP and ADP-ribosylation factor (ARF), which activates PLD [21], stimulate exocytosis. PIP_2 was proposed as the common denominator, whose synthesis is increased by both PITP [22] and ARF-activated PLD, albeit via distinct mechanisms [9].

With regard to vesicle formation, a possible cooperation of PITP and PLD has not been studied. To investigate this issue, we have exploited the observations that the formation of immature secretory granules (ISGs) from the trans-Golgi network (TGN) is promoted by both PITP [5] and PLD [17]. The latter is thought to be a downstream effector in the stimulation of ISG formation by ADP-ribosylation factor (ARF) [23–25]. Using a cell-free system that reconstitutes the formation of ISGs and constitutive secretory vesicles (CSVs) from the TGN [26], we here investigate whether PITP and PLD cooperate in secretory vesicle formation from the TGN. Furthermore, by using aminoglycoside antibiotics, which bind to PIP_2 [27,28], and an aminoglycoside-insensitive PLD as tools, we examine whether phosphoinositides are a possible common denominator in the stimulation of secretory vesicle formation by both PITP and PLD.

2. Materials and methods

2.1. Materials

Recombinant mouse PITP (α isoform) was prepared from inclusion bodies after expression of the cDNA in *Escherichia coli* [29]. Phospholipase D purified from *Streptomyces chromofuscus* [30] was purchased from Sigma. Analysis of the commercial PLD preparation by SDS-PAGE followed by Coomassie Blue staining confirmed the high degree of purity (>90%) of the PLD (data not shown). 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glyc-

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Abbreviations: ARF, ADP-ribosylation factor; C_6-NBD-PC, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphocholine; C_6-NBD-PA, 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphate; CSVs, constitutive secretory vesicles; hsPG, heparan sulfate proteoglycan; ISGs, immature secretory granules; PI, phosphatidylinositol; PIP2, phosphatidylinositol bis phosphate; PA, phosphatidic acid; PC, phosphatidyl choline; PITP, phosphatidylinositol transfer protein; PLD, phospholipase D; PNS, post-nuclear supernatant; SgII, secretogranin II; TGN, trans-Golgi network

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ero-3-phosphocholine (C_6-NBD-PC) and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphate (C_6-NBD-PA) were obtained from Avanti Polar Lipids. Geneticin was obtained from Gibco BRL.

2.2. Cell-free secretory vesicle formation

Cell-free formation of secretory vesicles from the TGN in a PC12 cell post-nuclear supernatant (PNS, 2 mg/ml protein), separation of post-TGN secretory vesicles from the TGN by differential centrifugation, and quantification of the formation of CSVs and ISGs using [^{35}S]sulphate-labelled heparan sulfate proteoglycan (hsPG) and secretogranin II (SgII) as markers, respectively, were performed, using a previously established system [26,31,32], as described in Fig. 1 of [5], except that vesicle formation is expressed as a percentage of labelled marker in the PNS (rather than P1 fraction [32]) kept at 4°C. The reaction volume was 150 µl. When indicated, recombinant mouse PITPα, *S. chromofuscus* PLD and geneticin were added to the reaction mixture on ice before warming to 37°C, as follows. Recombinant mouse PITPα was added from a stock containing 333 µg/ml recombinant protein and 0.3–0.6 µg/ml BSA which was dialyzed against HBS [31] (0.25 M sucrose, 1 mM EDTA, 1 mM magnesium acetate, 1.6 mM Na_2SO_4, 10 mM HEPES-KOH, pH 7.2) prior to use; controls received the equivalent volume of a stock containing 0.3–0.6 µg/ml BSA only, also dialysed against HBS. PLD was added from a stock in HBS containing either 50 or 100 U/µl according to the manufacturer's specification (one unit of PLD activity corresponds to 1.0 µmole of choline liberated from L-α-phosphatidylcholine (egg yolk) per hour at pH 8.0 at 30°C). Geneticin was added from a neutralized 0.1 M stock in HBS. After setting up the reaction mixtures, aliquots of 50 µl were removed for the determination of PLD activity as described below, and the remaining 100 µl were subjected to cell-free secretory vesicle formation (1 h, 37°C). The data shown are the mean of either four determinations (duplicate cell-free reactions using two independently prepared PNSs) or six determinations (either triplicate cell-free reactions using two independently prepared PNSs, or duplicate cell-free reactions using three independently prepared PNSs). The mean of the duplicate or triplicate control reactions was calculated and set to 100%, and the other values of vesicle formation obtained with a given PNS were expressed relative to this control. The efficiency of vesicle formation in the control condition was usually between 10% and 15%, i.e. 10–15% of the labelled markers were transferred from the TGN to post-TGN secretory vesicles in the cell-free reactions.

2.3. Determination of PLD activity

PLD activity was determined by quantifying the conversion of C_6-NBD-PC to C_6-NBD-PA, using a modification of the method described by Liscovitch and colleagues [18,33]. For each set of PLD assays, an aliquot (464 µl) of C_6-NBD-PC was removed from a 1 mg/ml stock in chloroform, dried under a stream of nitrogen, and dissolved in HBS with the aid of sonication for 5 min at room temperature. Aliquots (≤10 µl) of the C_6-NBD-PC in HBS were added to the 50 µl aliquots of the reaction mixtures of cell-free vesicle formation (see above) to give a final concentration of 0.25–0.3 mM C_6-NBD-PC. After incubation for 15 min at 37°C (or 4°C for the determination of background), reactions were terminated by the addition of 900 µl chloroform/methanol/9 N HCl (1:1:0.006, v/v). Following addition of 450 µl water and phase separation by brief centrifugation, the chloroform phase was collected and dried in a SpeedVac. The residue was dissolved in 20 µl chloroform/methanol (1:1, v/v) and spotted on silica gel 60 TLC plates (Merck). The TLC plates were developed in a solvent mixture containing 2-propanol/ethylacetate/chloroform/methanol/water (50:50:50:21:18, v/v). TLC plates were transilluminated with UV light (wavelength 312 nm) and the fluorescence was recorded by video imaging. The fluorescence intensity of the C_6-NBD-PA-containing bands was quantified using the MacBas 2.3.1 programme. The signal-to-noise ratio (incubation in the absence of exogenous PLD at 37°C vs. 4°C) was at least 5:1. The data shown are the mean of six determinations (triplicate cell-free reactions using two independently prepared PNSs). For any given PNS, the mean of the triplicate samples kept at 4°C was calculated and subtracted from the values obtained after incubation at 37°C. The mean of the resulting values for the triplicate incubation at 37°C in the control condition was calculated, set to 100%, and the other values of C_6-NBD-PA production at 37°C obtained with a given PNS were expressed relative to this control.

3. Results and discussion

3.1. The aminoglycoside geneticin blocks the PITP-induced stimulation of secretory vesicle formation from the TGN

Aminoglycoside antibiotics such as neomycin have been shown to bind to phosphoinositides, in particular PIP_2 [27,28]. To investigate whether the stimulation of secretory vesicle formation from the TGN observed upon addition of PITP [5] involved phosphoinositides, we examined the effect of geneticin, an aminoglycoside related to neomycin, in a previously established cell-free system [26,32] that reconstitutes the formation of CSVs and ISGs (collectively referred to as post-TGN secretory vesicles) from the TGN. As shown in Fig. 1, 1 mM geneticin completely blocked the stimulation of CSV (hsPG marker, Fig. 1, top) and ISG (SgII marker, Fig. 1, bottom) formation observed upon addition of 300 nM recombinant mouse PITPα, resulting in values below the control. The aminoglycoside also partially inhibited the basal CSV formation observed in the absence of exogenous PITP and marginally inhibited basal ISG formation (Fig. 1). The extent of this inhibition varied between different PNS preparations (compare Fig. 3A). Significant (≈25%) inhibition of vesicle formation was already observed in the presence of 200 µM geneticin (data not shown).

3.2. Exogenous PLD stimulates secretory vesicle formation from the TGN

PLD is thought to provide an alternative route to PITP to increase phosphoinositide levels [9] because PA, the product of the PLD reaction, stimulates type I PI 4-phosphate 5-kinase [20]. To explore this issue with regard to post-TGN secretory vesicle formation, we first investigated whether PLD stimulates the formation of CSVs and ISGs from the TGN

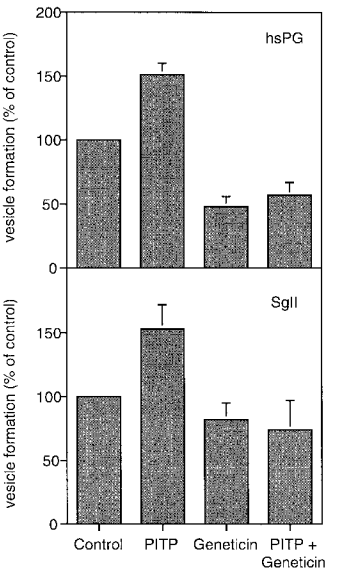

Fig. 1. Geneticin blocks the stimulation of secretory vesicle formation by PITPα. Cell-free secretory vesicle formation was performed in the absence (Control) and presence of 300 nM recombinant mouse PITPα (PITP), 1 mM geneticin (Geneticin) and 300 nM recombinant mouse PITPα plus 1 mM geneticin (PITP+Geneticin). The formation of CSVs (hsPG, top) and ISGs (SgII, bottom) was determined as described in Section 2 and is expressed as a percentage of the control, which was set to 100%. Data are the mean of four determinations; bars indicate the standard deviation.

in our cell-free system, as has been shown by Shields and colleagues [17] for ISGs in another cell-free system. For the purpose of our study, it was important to use a PLD whose activity does not depend on PIP_2 and is unaffected by geneticin. We therefore chose highly purified PLD from *S. chromofuscus* [30], rather than the recombinant human PLD1 previously used for cell-free ISG formation [17], as a tool to study the effect of PA production on post-TGN secretory vesicle formation. Fig. 2A shows that addition of *S. chromofuscus* PLD resulted in a concentration-dependent increase in PA production under the conditions of cell-free secretory vesicle formation. Addition of the bacterial PLD to the cell-free system indeed caused a stimulation of the formation of CSVs and ISGs which approached a plateau at 1 U/μl PLD (Fig. 2B). This stimulation was abolished by pretreatment of the PLD preparation with proteinase K (data not shown), eliminating the possibility that it was due to a non-proteinaceous component in the PLD preparation. Interference with the PLD-catalyzed production of PA by transphosphatidylation, which occurs in the presence of primary, but not secondary, alcohols [17,34], gave inconclusive results because both primary and secondary alcohols (propanol, butanol; 1% final concentration) caused an apparently unspecific inhibition of

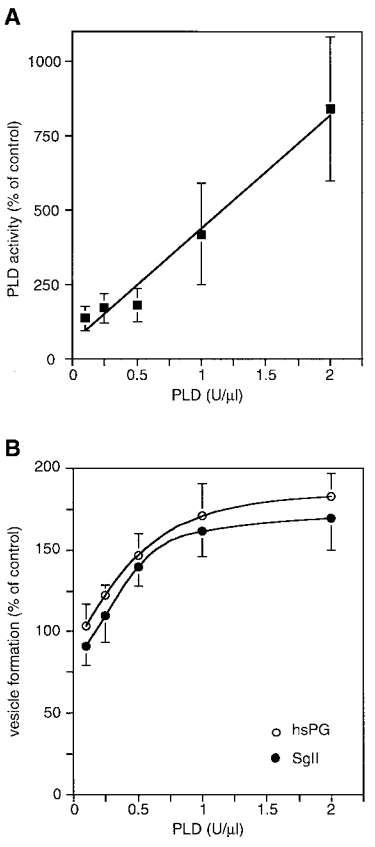

Fig. 2. Exogenous PLD stimulates secretory vesicle formation. PNS was incubated under the conditions of cell-free secretory vesicle formation in the presence of the indicated concentrations of PLD from *S. chromofuscus*. A: Aliquots of the PNS were analyzed for PLD activity as described in Section 2. B: Aliquots of the PNS were analyzed for the formation of CSVs (hsPG, open circles) and ISGs (SgII, filled circles) as described in Section 2. A, B: PLD activity and secretory vesicle formation are expressed as a percentage of the values obtained in the absence of added PLD, which were set to 100%. Data are the mean of six (1 U/μl, nine) determinations; bars indicate the standard deviation.

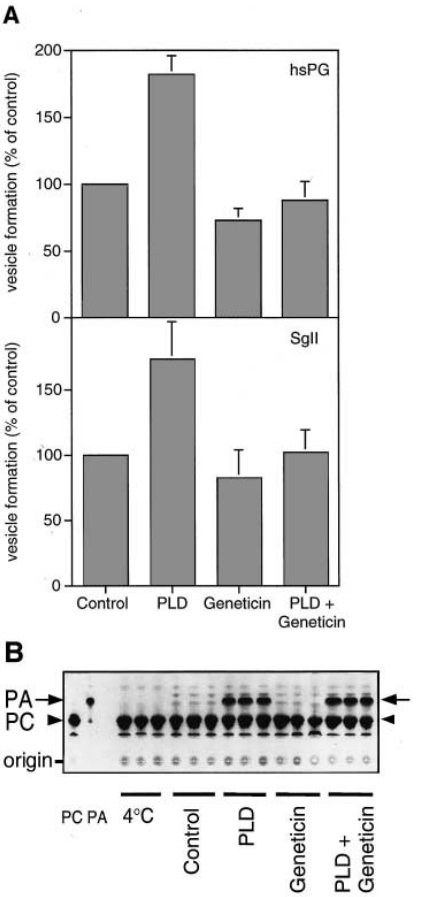

Fig. 3. Geneticin blocks the stimulation of secretory vesicle formation by exogenous PLD without affecting its activity. PNS was incubated under the conditions of cell-free secretory vesicle formation in the absence (Control) and presence of 1 U/μl *S. chromofuscus* PLD (PLD), 1 mM geneticin (Geneticin) and 1 U/μl *S. chromofuscus* PLD plus 1 mM geneticin (PLD+Geneticin). A: Aliquots of the PNS were analyzed for the formation of CSVs (hsPG, top) and ISGs (SgII, bottom) as described in Section 2. Vesicle formation is expressed as a percentage of the control, which was set to 100%. Data are the mean of six determinations; bars indicate the standard deviation. B: Aliquots of the PNS were analyzed for PLD activity as described in Section 2. The thin-layer chromatogram of triplicate reactions is shown. PC, C_6-NBD-PC standard (arrowheads); PA, C_6-NBD-PA standard (arrows); 4°C, incubation as in the control condition except that 4°C rather than 37°C was used.

secretory vesicle formation in our cell-free system (data not shown).

3.3. Geneticin blocks the stimulation of secretory vesicle formation by exogenous PLD without inhibiting its activity

If the stimulation of post-TGN secretory vesicle formation by exogenous PLD involved a PA-mediated increase in phosphoinositide levels, one would expect this stimulation to be sensitive to aminoglycosides. Indeed, as shown in Fig. 3A, addition of 1 mM geneticin to the cell-free system completely blocked the stimulation of both CSV and ISG formation observed in the presence of 1 U/μl *S. chromofuscus* PLD. Importantly, geneticin did not inhibit the PLD-catalyzed increase in PA levels (Fig. 3B). We conclude that the PLD-induced increase in PA levels in turn leads to an increase in an effector of secretory vesicle formation that is sensitive to aminoglycosides, such as PIP_2.

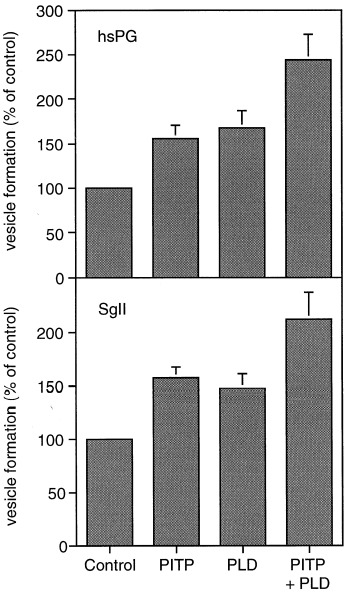

Fig. 4. Additive stimulatory effects of PITPα and PLD on secretory vesicle formation. Cell-free secretory vesicle formation was performed in the absence (Control) and presence of 300 nM recombinant mouse PITPα (PITP), 1 U/μl *S. chromofuscus* PLD (PLD) and 300 nM recombinant mouse PITPα plus 1 U/μl *S. chromofuscus* PLD (PITP+PLD). The formation of CSVs (hsPG, top) and ISGs (SgII, bottom) was determined as described in Section 2 and is expressed as a percentage of the control, which was set to 100%. Data are the mean of six determinations; bars indicate the standard deviation. Vesicle formation in the presence of both PITPα and PLD is significantly different from that in the presence of either PITPα or PLD alone (Student's *t*-test, $P \leq 0.005$).

3.4. PITP and PLD cooperate in promoting secretory vesicle formation from the TGN

The blockade by geneticin of both the PITP-induced and the PLD-induced stimulation of secretory vesicle formation from the TGN is consistent with a central role of PIP_2 in these membrane traffic events. If, in analogy to the stimulation of exocytosis [9], the stimulation of secretory vesicle formation by PITP and that by PLD reflect two distinct mechanisms of increasing the level of PIP_2, PITP and PLD should exert additive stimulatory effects on vesicle formation. Indeed, the combined addition of 300 nM recombinant mouse PITPα and 1 U *S. chromofuscus* PLD per μl to the cell-free system resulted in a significantly greater stimulation of post-TGN secretory vesicle formation than that observed with either protein alone (Fig. 4).

3.5. Conclusions

We provide evidence for cooperativity between PITP and PLD in secretory vesicle formation from the TGN. With regard to PLD, our data confirm the conclusion by Shields and colleagues [17] that PLD stimulates ISG formation and extend this notion to the other class of secretory vesicles formed in the TGN, the CSVs. Our observation that the aminoglycoside geneticin blocks both the stimulation of secretory vesicle formation by PITP and that by PLD (but not the PLD activity itself) suggests that phosphoinositides, known targets of aminoglycosides, are downstream effectors in both pathways of stimulation. The inhibition of PITP-stimulated and PLD-stimulated secretory vesicle formation by geneticin is unlikely to reflect interference with the coatomer machinery [35,36] because (i) secretory vesicle formation from the TGN does not involve coatomer [24] and (ii) geneticin neither precipitates coatomer (in contrast to neomycin) nor inhibits its binding to Golgi membranes at the concentrations used here [37]. It is also highly improbable that the inhibition of PITP-stimulated and PLD-stimulated secretory vesicle formation by geneticin is due to binding of ATP, which has been reported for the related aminoglycoside neomycin [38]. ATP is required not only for PITP-stimulated and PLD-stimulated, but also basal post-TGN secretory vesicle formation [26], which still occurred in the presence of geneticin.

The observation that at least half of the basal post-TGN secretory vesicle formation was not inhibited by geneticin does not necessarily imply a lack of involvement of phosphoinositides in basal vesicle formation. In our cell-free system, about half of the post-TGN secretory vesicle formation seen under control conditions is refractory to inhibition by brefeldin A, which completely blocks the formation of these vesicles in intact cells [39], and such refractoriness may also apply to inhibition by geneticin.

In conclusion, phosphoinositides are pivotal components of the machinery mediating post-TGN secretory vesicle formation. Given that phosphoinositides promote the membrane binding of numerous cytoplasmic proteins including components of the cytoskeleton [3,4], an attractive, though not exclusive, function of phosphoinositides may lie in the recruitment to the TGN membrane of proteins mediating vesicle budding and fission.

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