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Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion

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Permeabilized bovine adrenal chromaffin cells have been used to characterize the MgATP requirement of processes preceding exocytosis. Incubation of primary cultures with the membrane-permeable phenylarsine oxide (PAO) at 20 μM inhibited the phosphorylation of phosphatidylinositol (PtdIns) and completely blocked secretion. This block could be reversed by addition of 2,3-dimercaptopropanol to the permeabilized cells. Simultaneous addition of [γ-^{32}P]ATP and 2,3-dimercaptopropanol permitted a comparison between recovery of secretion and phosphorylation of intracellular components. Recovery of secretion closely correlated with phosphorylation of PtdIns and PtdIns4P. Subcellular fractionation of permeabilized cells after recovery of secretion revealed that the majority of newly phosphorylated PtdIns4P was localized on the chromaffin granules. In accordance with these results, PtdIns 4-kinase activity was found in protein extracts of permeabilized cells as well as associated with purified chromaffin granules, sensitive in both cases to PAO. Additionally, PtdIns 4-kinase activity in these two assays was inhibited by quercetin. In permeabilized cells, quercetin decreased the levels of labeled PtdIns4P and PtdIns(4,5)P_2 and inhibited secretion. Our data suggest that a chromaffin granule-associated PtdIns 4-kinase acts in the priming of exocytosis.

Keywords: chromaffin cells/exocytosis/phosphatidylinositol 4-kinase/polyphosphoinositides

Introduction

The identification of vesicle- and target membrane-specific proteins as molecular tags, that direct membrane interaction and fusion, has greatly advanced our understanding of intracellular membrane traffic and both constitutive and regulated exocytosis (Rothman, 1994). Although the role of these proteins in secretion is indisputable, less well defined is the role of phospholipids in secretion. Recently published studies have indicated an important role for phosphoinositides in this respect. Eberhard *et al.* (1990) have demonstrated that the maintenance of polyphosphoinositides is crucial for regulated exocytosis in chromaffin cells. Furthermore, two cytosolic proteins involved in phosphoinositide metabolism, namely phosphatidylinositol (PtdIns) transfer protein and PtdIns4P 5-kinase are required for stimulated secretion in semi-intact PC12 cells (Hay and Martin, 1993; Hay *et al.*, 1995).

Chromaffin cells of the adrenal medulla and the pheochromocytoma PC12 cell line are used widely to study the molecular mechanisms of stimulated secretion (for a recent review, see Burgoyne and Morgan, 1993). The dependence of Ca^{2+}-stimulated catecholamine release on ATP in these cells was demonstrated in earlier studies (Knight and Baker, 1982; Peppers and Holz, 1986; Schäfer *et al.*, 1987). In recent years, it has been possible to distinguish fast ATP-independent and slow ATP-dependent phases of release (Bittner and Holz, 1992). The fast phase has been attributed to the fusion of a small number of docked granules (Neher and Zucker, 1993; Horrigan and Bookman, 1994), whereas the slow component has been assumed to include docking of granules and priming of the exocytotic machinery (Holz *et al.*, 1989; Bittner and Holz, 1992). A preparation of semi-intact PC12 cells has been used successfully to demonstrate the dependence of the secretory process on distinct classes of cytoplasmic proteins, one class being involved in ATP-dependent priming, and a second indispensable for Ca^{2+}-dependent triggering of exocytotic membrane fusion (Hay and Martin, 1992). The biochemical reactions underlying priming and triggering have remained elusive until very recently. The ATP dependence of secretion has been suggested to reside, at least in part, in maintaining polyphosphoinositide levels in cellular membranes (Eberhard *et al.*, 1990). This earlier notion is supported by the identification of two of the three cytosolic proteins required for priming, as PtdIns transfer protein and PtdIns4P 5-kinase (Hay and Martin, 1993; Hay *et al.*, 1995). Thus, priming is seen as a sequential reaction catalyzed by PtdIns transfer protein, a hypothetical PtdIns 4-kinase and PtdIns4P 5-kinase. The resulting PtdIns(4,5)P_2 would perform a novel role apart from it being a substrate for phospholipase C-mediated generation of diacylglycerol (DAG) and Ins(1,4,5)P_3, neither of which triggers secretion in chromaffin or PC12 cells (Eberhard *et al.*, 1990; Hay *et al.*, 1995).

PtdIns 4-kinase activity has been shown to reside in chromaffin granule membranes, but its function in the secretory process has remained a matter of speculation (Buckley *et al.*, 1971; Phillips, 1973; Husebye and Flatmark, 1988). We now demonstrate that inhibition of this enzyme leads to concomitant loss of PtdIns4P and loss of secretory responsiveness to stimulation. Furthermore, secretory activity is regained simultaneously with phosphorylation of PtdIns on the chromaffin granules. Therefore, PtdIns 4-kinase of chromaffin granules represents a further enzyme of phosphoinositide metabolism that is necessary for the priming of exocytosis.

Results

Inhibition of secretion by PAO is reversible

In primary cultures of bovine adrenal chromaffin cells, phenylarsine oxide (PAO) induces a time- and dose-

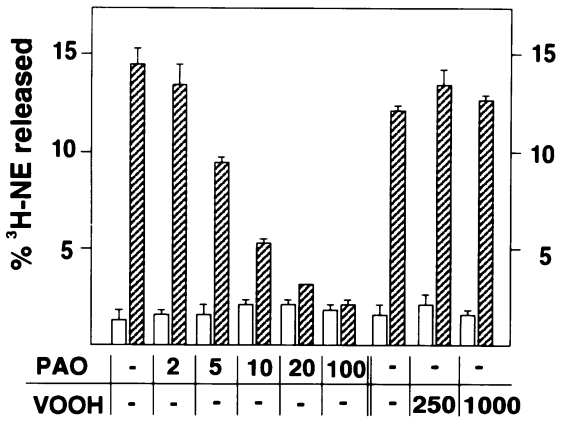

Fig. 1. Inhibition of [^{3}H]NE secretion in digitonin-permeabilized chromaffin cells upon treatment with PAO. Chromaffin cell cultures pre-loaded with [^{3}H]NE were incubated for 15 min in external buffer containing 0.5% DMSO (–), PAO or VOOH at the concentrations indicated (μM). PAO or VOOH solutions were removed, cells were permeabilized with digitonin and washed in internal buffer containing 0.5% DMSO. Cells were incubated in internal buffer (control, open bars) or stimulated with internal buffer containing 10 μM free Ca^{2+} (hatched bars) for 5 min. [^{3}H]NE in the supernatant and in cells solubilized with TX-100 was counted. The results are expressed as the mean ±SD of the percentage of secreted [^{3}H]NE (n = 3). PAO inhibited secretion in a dose-dependent manner and VOOH had no effect on secretion.

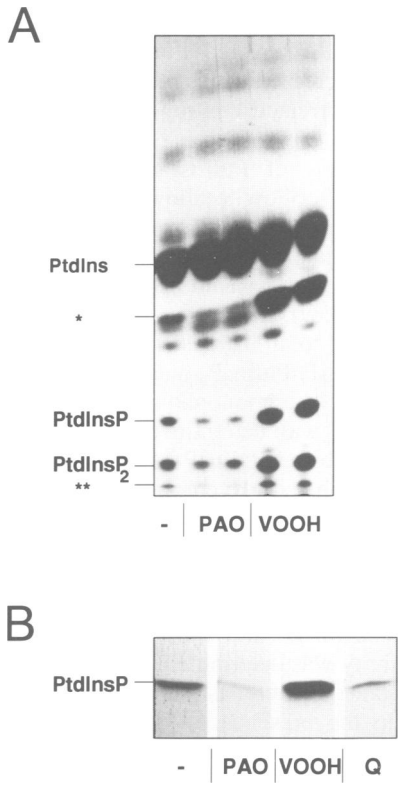

Fig. 2. Influence of PAO, VOOH and quercetin on phosphorylation of phospholipids. (**A**) $^{32}P_i$-labeled chromaffin cells were incubated for 15 min with 0.5% DMSO (–), PAO (20 μM, left lane; 100 μM, right lane) or VOOH (0.1 mM, left lane; 1 mM, right lane). Extracted lipids were separated on TLC plates together with standards [PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol4P; PtdInsP$_2$, phosphatidylinositol(4,5)P$_2$]. On the autoradiogram, phospholipids labeled with (*) and (**) may represent PA and PtdInsP$_3$, respectively. (**B**) Chromaffin cells were incubated for 15 min with 0.5% DMSO (–), 20 μM PAO, 1 mM VOOH or 100 μM quercetin (Q). Proteins were extracted and PtdIns 4-kinase activity determined as described in Materials and methods. On the autoradiogram of the TLC plate PtdIns4P was the only detectable product. PAO and VOOH decreased and increased, respectively, phosphorylation of PtdIns *in vivo* (A) and *in vitro* (B).

dependent inhibition of stimulated catecholamine release (Schäfer *et al.*, 1994). A 15 min pre-incubation with this membrane-permeable arsenical leads to half-maximal inhibition of release at 5 μM and to complete inhibition at 20 μM, measured in digitonin-permeabilized cells (Figure 1). Shorter incubations do not lead to complete inhibition, even at PAO concentrations of 100 μM (Schäfer *et al.*, 1994).

The use of [^{14}C]PAO has enabled us to investigate time- and dose-dependent uptake and subcellular distribution of the arsenical. The intracellular distribution of PAO was determined after incubation of cells with 20 μM [^{14}C]PAO for 3 min, resulting in a partial block of secretion, and for 20 min, inhibiting secretion completely. The majority of [^{14}C]PAO was recovered in the cytosol, which represents the major compartment in terms of protein content. In the crude plasma membrane and mitochondrial fractions, the amount of [^{14}C]PAO did not increase, whereas the amount accumulating in the chromaffin granule fraction doubled from 3 min to 20 min (data not shown).

When PAO was removed from intact or permeabilized cells, the secretory activity was effectively restored. PAO was removed in a dose- and time-dependent manner from blocked cells by 2,3-dimercaptopropanol (British antilewisite, BAL). Treatment of cells for 15 min after permeabilization restored secretion half-maximally at 20 μM BAL, and completely at 100–120 μM BAL in cells previously blocked with 20 μM PAO (Figure 3A).

PAO has been suggested to be a phosphotyrosine phosphatase blocker (Levenson and Blackshear, 1989; Garcia-Morales *et al.*, 1990). The possibility that the effect of PAO on secretion was mediated through changes in phosphotyrosine phosphorylation was obviated in two ways. First, Western blot analysis with anti-phosphotyrosine antibodies revealed that PAO at up to 20 μM did not alter the content of phosphotyrosine proteins in chromaffin cells. Secondly, and more significantly, the general phosphotyrosine phosphatase blocker vanadyl hydroperoxide (VOOH) (Trudel *et al.*, 1991; Posner *et al.*, 1994), despite causing a large accumulation of tyrosine-phosphorylated proteins over a broad range of molecular mass (data not shown), had no effect on secretory activity (Figure 1).

PtdIns phosphorylation and PtdIns 4-kinase activity are decreased by PAO

In $^{32}P_i$-pre-labeled cells, 15 min incubation with 20 μM or 100 μM PAO caused a striking decrease in the amount of labeled PtdInsP and PtdInsP$_2$ (Figure 2A). Labeled PtdInsP and PtdInsP$_2$ were identified on the basis of their co-migration with unlabeled standards. The effect of PAO versus control solvent (dimethyl sulfoxide, DMSO) was always tested in parallel cultures and quantified by counting the radioactivity present in the respective spots obtained by thin layer chromatography (TLC). For each condition, three experiments were quantified, defining the amount of the labeled phospholipid in the control as 100%

(mean values are reported with SEM ±11%). The amount of labeled PtdInsP recovered after PAO treatment (20 or 100 μM) was decreased to less than half compared with the amount in untreated control cells. Labeled $PtdInsP_2$ was decreased to 20% (Figure 2A). PAO treatment also affected the levels of $PtdInsP_3$, but the radioactivity incorporated was too low to allow reproducible measurement under these experimental conditions. Similar changes caused by PAO treatment were produced in another labeled phospholipid that was (bona fide) identified as phosphatidic acid (PA, see below). In contrast, incubation with 0.1 or 1 mM VOOH led to an increase rather than a decrease in labeled PA, PtdInsP, $PtdInsP_2$ and $PtdInsP_3$.

The nature of the monophosphate form of PtdInsP affected by PAO was determined by HPLC analysis of deacylated ^{32}P-labeled PtdInsP (Auger *et al.*, 1990). The majority of the labeled glycerophosphoinositol was phosphorylated at the D-4 position of the inositol ring, and only 1–4% was labeled at the D-3 position. These results suggest that PAO treatment affects chromaffin cell PtdIns 4-kinase activity. To demonstrate this directly, intact cells were incubated for 15 min with 20 μM PAO and PtdIns 4-kinase activity was determined in protein extracts using lipids of brain extracts as substrate. The assay was performed in the presence of 0.1% NP-40 in order to suppress PtdIns 3-kinase activity (Carpenter and Cantley, 1990). TLC revealed a single product, that co-migrated with unlabeled PtdIns4P on borate TLC plates (Walsh *et al.*, 1991). In extracts of PAO-treated cells, PtdIns 4-kinase activity was reduced by about two thirds (Figure 2B). Quercetin, shown to inhibit PtdIns 4-kinase in A431 cells (Nishioka *et al.*, 1989), inhibited PtdIns 4-kinase activity in extracts of chromaffin cells treated for 15 min with 100 μM quercetin. As a further control, we used 1 mM VOOH to examine whether the results obtained in *in vivo* labeling experiments were consistent with PtdIns 4-kinase activity measured *in vitro*. Indeed VOOH, which had no effect on secretion, increased the kinase activity 2- to 3-fold (Figure 2B).

PtdIns4P is formed during rescue of PAO-treated cells with BAL

The fact that PAO-induced inhibition of secretion is reversed upon addition of BAL to permeabilized cells (see Figure 3A) enabled us to observe the time course of recovery of secretion and of kinase activity. In control cells incubated for 15 min with [γ-^{32}P]ATP after permeabilization, three phosphorylated phospholipids that co-migrate with unlabeled PA, PtdIns4P and $PtdIns(4,5)P_2$ were detected in cellular lipid extracts (Figure 3B, first lane). In extracts of cells pre-incubated with 20 μM PAO followed by incubation with [γ-^{32}P]ATP without BAL, ~25, 10 and 15% of labeled PA, PtdIns4P and $PtdIns(4,5)P_2$, respectively, were present as compared with control (n = 3; SEM ±8%) (Figure 3B, second lane). Concomitant incubation of [γ-^{32}P]ATP with increasing concentrations of BAL (10–100 μM) led to a dose-dependent reappearance of all three phosphorylated phospholipids (Figure 3B). At 100 μM BAL, the amounts of PtdIns4P and of $PtdIns(4,5)P_2$ labeled during the 15 min period were comparable with amounts labeled in untreated control cells. PtdIns4P and $PtdIns(4,5)P_2$ reappeared in parallel with a constant ratio of PtdIns4P to $PtdIns(4,5)P_2$. The

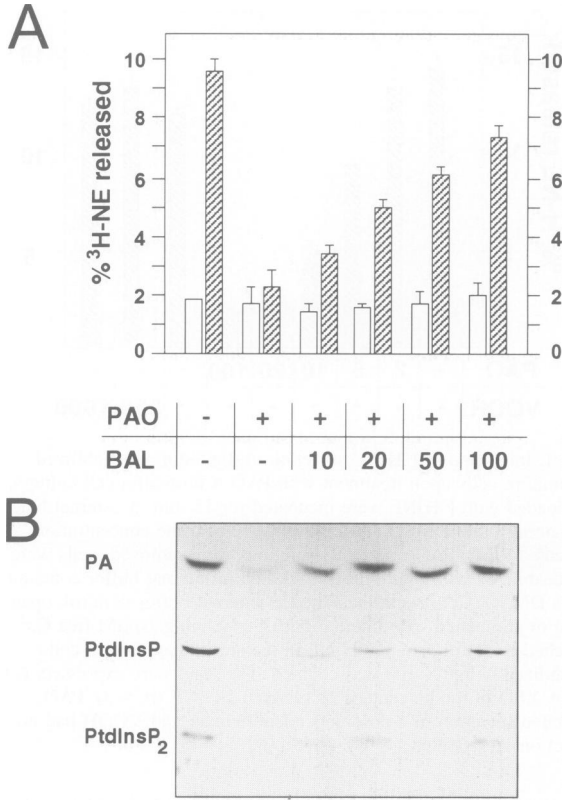

Fig. 3. Recovery of [^{3}H]NE secretion and phosphorylation of phospholipids upon removal of PAO by BAL. Cells were incubated for 15 min with 0.5% DMSO (–) or 20 μM PAO (+). After permeabilization, cells were incubated for 15 min in internal buffer containing 0.5% DMSO (–), or BAL at the concentrations indicated (μM), and $MgSO_4$ instead of MgATP. (**A**) Secretion of [^{3}H]NE was measured as described in Figure 1. Cells were incubated in internal buffer (control, open bars) or stimulated with internal buffer containing 10 μM free Ca^{2+} (hatched bars) for 5 min. The amount of [^{3}H]NE released was lower after washing the permeabilized cells in the absence of MgATP (compare with Figure 1). (**B**) [γ-^{32}P]ATP was added to the internal buffer for 15 min after permeabilization. Without stimulation, lipids were extracted and separated on TLC plates together with standards [PA, phosphatidic acid; PtdInsP, phosphatidylinositol4P; $PtdInsP_2$, phosphatidylinositol(4,5)P_2]. The autoradiogram of a TLC plate is shown. After inhibition by PAO, secretory activity and phosphorylation of PtdIns were restored concomitantly by BAL.

phosphorylation of PtdIns upon removal of PAO by BAL reproducibly correlated with the recovery of secretion determined in parallel release experiments. In contrast, PA was labeled to the same extent as in control cells at low concentrations of BAL (10–20 μM) when secretory activity was still severely inhibited (Figure 3A).

Quercetin blocks PtdIns 4-kinase and secretion in chromaffin cells

To corroborate evidence for the importance of PtdIns 4-kinase activity in the secretory process, the effect of quercetin on PtdIns phosphorylation was compared with its effect on secretion in permeabilized chromaffin cells. The stability of quercetin is optimal at pH 7.2; hence, phosphorylation and release assays were performed at this pH. Moreover, since quercetin and ATP compete for the same site on PtdIns 4-kinase (Cochet and Chambaz, 1986) in both assays, MgATP was replaced with $MgSO_4$ in the

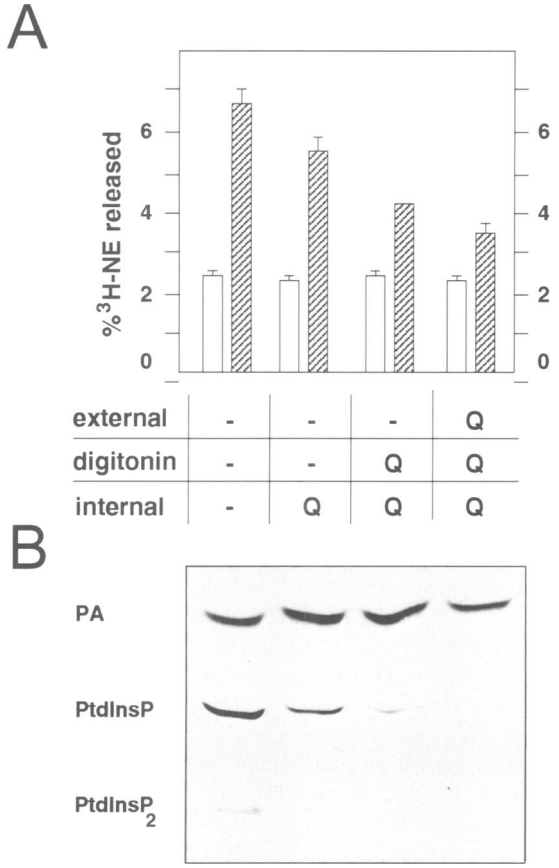

Fig. 4. Time-dependent inhibition of [^{3}H]NE secretion and phosphorylation of PtdIns by 100 μM quercetin. Cells were incubated for 15 min in external buffer, permeabilized for 5 min with digitonin and washed for 15 min in internal buffer with (Q) or without (–) 100 μM quercetin as indicated. The internal buffer contained 1 mM $MgSO_4$, no cold MgATP and was adjusted to pH 7.2. (**A**) Secretion of [^{3}H]NE was measured as described in Figure 1. Cells were incubated in internal buffer (control, open bars) or stimulated with internal buffer containing 10 μM free Ca^{2+} (hatched bars) for 5 min. Under these conditions (no MgATP, pH 7.2) the amount of [^{3}H]NE released was lower than under standard conditions (Figure 1). (**B**) [γ-^{32}P]ATP was added to the internal buffer for 15 min after permeabilization. Lipids were extracted and separated on TLC plates as described in Figure 3. Quercetin concomitantly inhibited secretion and phosphorylation of PtdIns.

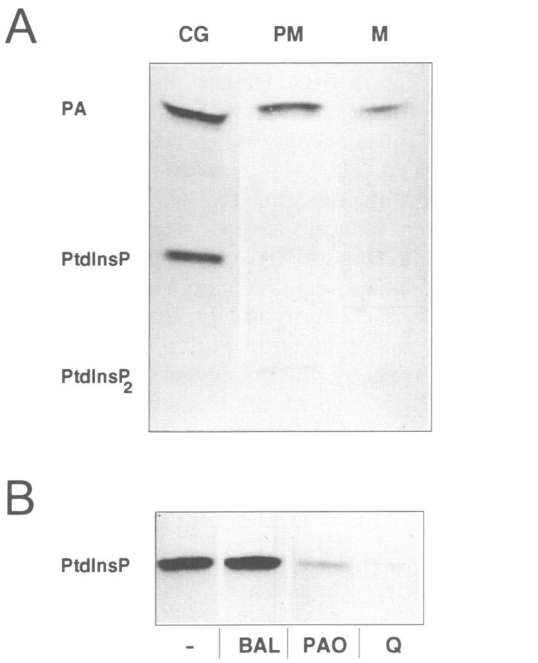

Fig. 5. Subcellular localization of PAO- and quercetin-sensitive PtdIns 4-kinase. (**A**) Cells were incubated for 15 min with 20 μM PAO. After permeabilization, cells were incubated for 15 min in internal buffer containing 100 μM BAL, $MgSO_4$ and [γ-^{32}P]ATP. Cells were removed from the plates and homogenized prior to subcellular fractionation. Lipids of subcellular fractions (CG, chromaffin granules; PM, plasma membrane; M, mitochondria) were extracted as described in Materials and methods and separated on TLC plates as described for Figure 2. PA was formed in all three fractions, whereas phosphorylation of PtdIns during recovery of secretory activity was restricted to the chromaffin granule fraction. (**B**) Purified chromaffin granule membranes were incubated for 15 min with 0.5% DMSO (–), 100 μM BAL, 280 μM PAO or 100 μM quercetin (Q). PtdIns 4-kinase activity was assayed as described by Husebye and Flatmark (1988). PtdIns4P was the only product formed, and chromaffin granule-associated PtdIns 4-kinase was inhibited by both PAO and quercetin.

internal buffer used in the permeabilization step and the subsequent incubation with [γ-^{32}P]ATP. Quercetin blocked secretion and PtdIns phosphorylation with a similar dose and time dependence. The degree of inhibition of secretion increased with the duration of incubation. At 100 μM quercetin, 50% inhibition of secretion was observed upon incubation during permeabilization and the subsequent 15 min wash step, which can be augmented to ~75% inhibition by additional incubation for 15 min prior to permeabilization (Figure 4A). The inhibitory effect of quercetin on PtdIns 4-kinase was reflected by a reduction in the amount of PtdIns phosphorylated during the 15 min period after permeabilization. Again, the duration of incubation with quercetin determined the degree of reduction (Figure 4B). Incubation with 100 μM quercetin together with [γ-^{32}P]ATP for 15 min resulted in a 50% reduction, which was augmented to ~75–90% by additional incubation of the cells with quercetin during permeabilization or during permeabilization and the 15 min preceding permeabilization, respectively (n = 3; SEM ±13%). The amount of PtdIns(4,5)P_2 labeled during the 15 min incubation period with [γ-^{32}P]ATP was too low to be quantified. Quercetin did not block DAG kinase, as the amount of labeled PA was unaffected under these conditions (Figure 4B).

PtdIns present in chromaffin granule membranes is phosphorylated during recovery of secretory activity

Starting from PAO-blocked cells, the subcellular distribution of phospholipids phosphorylated in permeabilized cells during incubation with 100 μM BAL and [γ-^{32}P]ATP was analyzed. Lipid extracts of chromaffin granule, plasma membrane and mitochondrial fractions all contain labeled PA, in a ratio of ~5:4:1 in the respective fractions (Figure 5A). In sharp contrast, PtdIns phosphorylation upon incubation with 100 μM BAL was almost entirely localized on chromaffin granules, with traces of labeled PtdIns4P detectable in plasma membrane and mitochondrial fractions (Figure 5A). The amount of labeled PtdIns(4,5)P_2 was too small to be quantified.

In subcellular fractions of chromaffin cells, PtdIns 4-kinase activity can be detected by using the endogenous PtdIns as a substrate (Husebye and Flatmark, 1988). PAO- and quercetin-sensitive activity was found associated with

the granules. Chromaffin granule membranes labeled with [γ-^{32}P]ATP selectively contained labeled PtdIns4P in lipid extracts (Figure 5B). Higher phosphorylated forms of phosphoinositides or PA were not detectable under the conditions of our assay. When chromaffin granules were pre-incubated for 15 min with high concentrations of either PAO or quercetin, the amount of phosphorylated PtdIns was reduced to about one fifth or one tenth, respectively (Figure 5B). In control incubations, BAL alone did not affect the chromaffin granule-associated PtdIns 4-kinase activity *in vitro*. In further control experiments, phosphorylation of PtdIns on chromaffin granules was inhibited by the monoclonal anti-PtdIns 4-kinase antibody 4C5G, which inhibits selectively type II PtdIns 4-kinase activity (Endemann *et al.*, 1991). Incubation of chromaffin granule membranes with this antibody at 2–50 μg/ml caused an inhibition of 70–90% of the granule-associated PtdIns 4-kinase (data not shown).

Discussion

The ATP dependence of the secretory process in chromaffin cells has long been recognized (Knight and Baker, 1982; Peppers and Holz, 1986; Schäfer *et al.*, 1987), but only very recently has a crucial enzymatic step been characterized. In semi-intact PC12 cells, stimulated secretion has been shown to depend on a compulsory priming process (Hay and Martin, 1992). This priming has been demonstrated to include the phosphorylation of PtdIns4P by a soluble PtdIns4P 5-kinase (Hay *et al.*, 1995), a finding that emphasizes the importance of polyphosphoinositides in the exocytotic fusion process (Eberhard *et al.*, 1990; Hay and Martin, 1993). Here we present evidence that PtdIns4P, generated in the chromaffin granule membrane by a membrane-associated PtdIns 4-kinase, plays a crucial role in priming of exocytosis.

We have found previously that PAO inhibits secretion of [^{3}H]norepinephrine ([^{3}H]NE) in PC12 and chromaffin cells and that this inhibition is reversible upon treatment with small dithiols such as BAL (Schäfer *et al.*, 1994). This combination provides an excellent tool to interrupt cellular processes temporarily. The only cellular targets of PAO characterized so far are two membrane-bound phosphatases essential for signal transduction in the glucose transport system in 3T3-L1 adipocytes (Liao *et al.*, 1991), a process that also involves the exocytotic fusion of glucose transporter (GLUT4)-containing vesicles. PAO previously has been demonstrated to act as a phosphotyrosine phosphatase blocker in fibroblasts (Levenson and Blackshear, 1989) and in lymphocytes (Garcia Morales *et al.*, 1990). In chromaffin cells, we show that PAO does not block phosphotyrosine phosphatases, as judged by immunoblotting with anti-phosphotyrosine antibodies, and indeed VOOH, a general phosphotyrosine phosphatase inhibitor, had no effect on secretion. This suggests that changes in phosphotyrosine phosphorylation do not contribute to modulation of secretion in this system.

Pre-incubation of chromaffin cells with the lowest dose of PAO leading to complete inhibition of secretion significantly lowered the amount of labeled PA, PtdIns4P and PtdIns(4,5)P_2 in intact and permeabilized cells (Figures 2A and 3B). Permeabilized cells provide an excellent model to characterize phosphorylation reactions occurring during recovery from the PAO-induced block of the secretory process. All three phospholipid species were phosphorylated upon release of the block by BAL, with only PtdIns4P and PtdIns(4,5)P_2 appearing in a time- and dose-dependent manner similar to recovery of secretion. Importantly, we are able to exclude a rate-limiting role for PA in the recovery of secretion, because the reappearance of labeled PA follows different kinetics compared with the recovery of secretion. Furthermore, quercetin, which has been shown to inhibit PtdIns 4-kinase *in vitro* (Cochet and Chambaz, 1986; Nishioka *et al.*, 1989), did not affect phosphorylation of DAG to PA, but inhibited secretion concurrently with phosphorylation of PtdIns. Our results on changes in PtdIns4P and PtdIns(4,5)P_2 are therefore consistent with the earlier postulate describing a dependence of secretion on the presence of phosphoinositides (Eberhard *et al.*, 1990).

PtdIns 4-kinase activity has been detected previously on chromaffin granules, and therefore its product, PtdIns4P, tentatively has been implicated in exocytosis (Buckley *et al.*, 1971; Phillips, 1973; Husebye and Flatmark, 1988). When starting from PAO-blocked cells, [^{32}P]PtdIns4P formed during recovery of secretion induced by BAL was found almost exclusively in the chromaffin granule fraction (Figure 5A). The relatively minor amount of PtdIns4P formed in the plasma membrane and mitochondrial fractions may be due to contamination of this fraction by disrupted chromaffin granules. However, at this point, we cannot rule out a role for PtdIns4P in the plasma membrane.

Our results from lipid analysis of subcellular fractions of BAL-recovered cells are consistent with our results from *in vitro* kinase assays with chromaffin granule membranes. Purified chromaffin granule membranes contained PtdIns 4-kinase activity that was sensitive to PAO and quercetin and was inhibited by the monoclonal antibody 4C5G specific for the PtdIns 4-kinase type II. Interestingly, the dose-dependent inhibition of the granule-associated kinase by this antibody is similar to inhibition of detergent-solubilized type II PtdIns 4-kinase from bovine brain (Endemann *et al.*, 1991). This result is an indication of a type II PtdIns 4-kinase on the chromaffin granule membrane. PAO blocked the formation of PtdIns4P, albeit at higher concentrations than those used on intact cells. Experiments with [^{14}C]PAO revealed a slow accumulation of PAO in chromaffin granules over time, that, together with the requirement for a high concentration needed to block PtdIns 4-kinase, may account for the slow onset of inhibition. The other parameter governing the kinetics of inhibition is the rate of PtdIns4P turnover. PtdIns4P may be phosphorylated to PtdIns(4,5)P_2 or hydrolyzed by a phosphatase or a phospholipase. Our experiments with [γ-^{32}P]ATP suggest a continuous turnover of phosphoinositides at the expense of ATP, even in permeabilized cells.

The localized formation of PtdIns4P on chromaffin granules may be responsible for the fusion competence of chromaffin granules, or it may serve as a membrane-anchored receptor for PtdIns4P binding proteins. Interestingly, inhibition by PAO of glucose uptake in adipocytes, which depends on the fusion of GLUT4-containing vesicles also comprising a PtdIns 4-kinase (Del Vecchio and Pilch, 1991; Liao *et al.*, 1991), suggests that PtdIns4P

may be required on secretory vesicles in other secretory systems.

PtdIns4P may serve as a substrate for a 3-kinase, which seems to be a soluble enzyme. Speculations on a role for 3-phosphorylated phosphoinositides in vesicle traffic have been boosted by the identification of Vps34p as a yeast PtdIns 3-kinase essential for protein sorting (Stack and Emr, 1994). The identification of insulin-stimulated PtdIns 3-kinase activity on intracellular membranes in adipocytes (Kelly *et al.*, 1992) certainly favors the involvement of a PtdIns 3-kinase in intracellular membrane traffic.

PtdIns 4-kinase may also function in concert with the soluble PtdIns transfer protein and PtdIns4P 5-kinase (Hay and Martin, 1993; Hay *et al.*, 1995), constituting a pathway involved in priming of exocytosis. One working hypothesis is that PtdIns transfer proteins have to present PtdIns and its phosphorylated forms to the respective kinases, or to phospholipase C (Kauffmann-Zeh *et al.*, 1995). Indeed, several studies suggest a function of PtdIns(4,5)P_2 as a structural element in the secretory process (Liscovitch *et al.*, 1994). PtdIns(4,5)P_2 has been found to be a co-factor of phospholipase D, and has been proposed to act in neuronal signal transduction and membrane transport (Liscovitch *et al.*, 1994). Phospholipase D is not present in chromaffin cells (Purkiss *et al.*, 1991), but the recent demonstration of specific binding of PtdIns(4,5)P_2 to pleckstrin homology domains (Harlan *et al.*, 1994) may be taken as an indication of a more general role for PtdIns(4,5)P_2 as an activator or anchor of other enzymes putatively involved in the secretory process. In addition, PtdIns(4,5)P_2 binds a number of cytoskeletal proteins, and has therefore been postulated to represent a membrane anchor of the cytoskeleton. This is interesting in view of the fact that alterations in the interaction between cytoskeleton and the chromaffin granule membranes seem to be necessary for the docking of granules at the plasma membrane (for review, see Burgoyne and Morgan, 1993; Hughes and Michell, 1993). Clarification of the role of PtdIns(4,5)P_2 requires definition of the subcellular localization of PtdIns transfer protein and PtdIns4P 5-kinase activities. Our data do not exclude a localization of those activities on chromaffin granules, leading to the production of PtdIns(4,5)P_2. The observed lack of labeled PtdIns(4,5)P_2 in the chromaffin granule fraction (Figure 5A) could be caused either by fast degradation of PtdIns(4,5)P_2 by a phosphatase or a phospholipase or by slow formation of PtdIns(4,5)P_2, which would leave the main pool of PtdIns(4,5)P_2 unlabeled under our assay conditions.

Here we demonstrate the involvement of the chromaffin granule membrane-associated PtdIns 4-kinase in the secretory process. Whether PtdIns4P indeed performs a function in exocytosis by itself, or acts as a precursor of polyphosphoinositides, is a subject for further investigation. Certainly, PtdIns 4-kinase activity on chromaffin granules, identified here as one of the MgATP-dependent and rate-limiting priming steps, is obligatory for stimulated secretion in this system.

Materials and methods

Materials

PAO, BAL, sodium orthovanadate and bovine brain extract (type I) were obtained from Sigma. PAO was dissolved in DMSO and titrated according to Zahler and Cleland (1968). VOOH was prepared from orthovanadate and H_2O_2 according to Trudel *et al.* (1991) and used within 1 h. Digitonin was obtained from Calbiochem. [7,8-^{3}H]Norepinephrine (37 Ci/mmol), $^{32}P_i$ (200 mCi/mmol), [γ-^{32}P]ATP (10 Ci/mmol and 3000 Ci/mmol) and custom-made [U-^{14}C]PAO (52 mCi/mmol) were from Amersham. PtdIns, PtdIns4P, PtdIns(4,5)P_2, PA and quercetin were obtained from Fluka. Silica gel 60 TLC plates were from Merck. Cell culture media were from GIBCO, and Primaria cell culture dishes were from Falcon.

Cell cultures

Bovine adrenal medullary chromaffin cells were isolated as described (Burgoyne *et al.*, 1988). For release measurements, they were seeded at a density of ~3×10^5/cm^2 in 6-well or 24-multiwell Primaria plates and cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse and 5% fetal calf serum. They were used 3–5 days after isolation. For ^{32}P-labeling, the cells were seeded at the same density into 6 cm or 10 cm Primaria dishes. They were used 2 days after isolation.

Release assay

Cultures were pre-labeled with 1 μCi/ml of [^{3}H]NE overnight. The standard protocol involved three washes of the cultures with external buffer (120 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES, pH 7.2), 5 min each. PAO, VOOH or quercetin were added during these washes from 200-fold concentrated stock solutions. Cells were permeabilized with 20 μg/ml of digitonin in internal buffer (139 mM sodium glutamate, 5 mM MgATP, 5 mM EGTA, 20 mM PIPES, pH 6.6) for 5 min. Cells were washed for 3 min with internal buffer, and then stimulated for 5 min with internal buffer containing 4.43 mM $CaCl_2$, resulting in 10 μM free Ca^{2+}. Controls were exposed to Ca^{2+}-free internal buffer to assess basal release. BAL or quercetin was added during permeabilization and/or the wash step as indicated. Both supernatants and cells solubilized in 1% TX-100 were counted in a scintillation counter. The results are expressed as the percentage of total [^{3}H]NE released into the supernatant.

$^{32}P_i$ Labeling of phospholipids

Cells on 10 cm dishes were incubated with 100 μCi of $^{32}P_i$ for 2 h. They were then washed three times for 5 min each in external buffer supplemented with PAO or VOOH. Cells were scraped from dishes, pelleted and resuspended in methanol:HCl (100:1). Lipids were extracted and analyzed as described below.

[γ-^{32}P]ATP labeling of phospholipids

Cells on 6 cm dishes were treated as described for the release assay. The wash step after permeabilization was extended to 15 min. Buffer did not contain cold MgATP, but instead contained 1 mM $MgSO_4$ and 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) per dish. Reactions were stopped by removing the buffer and putting the dishes on ice. Cells were scraped from the dishes in ice-cold internal buffer and pelleted. Pellets were resuspended in 400 μl of 1 M HCl. Lipids were extracted and analyzed as described below.

In vitro PtdIns 4-kinase assay

Cells on 10 cm dishes were incubated for 15 min in external buffer containing DMSO, PAO, quercetin or VOOH. These agents were present at the same concentrations throughout the assay. Cells were extracted on the dishes with 1 ml of ice-cold extraction buffer according to Susa *et al.* (1992). The protein extract was diluted 1:20 in kinase assay buffer containing 0.1% NP-40. As a substrate in the kinase assay, 1.7 μg/μl of phospholipids (brain extract) were used. In our assay, the ATP solution contained 10 μCi of [γ-^{32}P]ATP (10 Ci/mmol) per assay, and 60 μM cold ATP. The assays were performed by mixing 10 μl of phospholipids with 10 μl of diluted protein extract. After 2 min, 30 μl of the ATP solution were added and the reaction was stopped after 5 min by addition of 400 μl of 1 M HCl. Lipids were extracted and analyzed as described below.

PtdIns 4-kinase assay with chromaffin granules

Chromaffin granules were prepared as described below. For each assay, 50 μg of chromaffin granule protein were used. Shock-frozen chromaffin granules were thawed on ice and hypotonically shocked. Then, chromaffin granule ghosts were pelleted and resuspended in assay buffer, pH 7.0, containing 5 mM $MgCl_2$ as described by Husebye and Flatmark (1988). Samples were diluted 1:5 in assay buffer supplemented with drugs or antibodies and incubated at room temperature for 15 min. In this assay, the ATP solution contained 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) per

assay and 5 mM cold MgATP. To 300 µl of chromaffin granule suspension, 30 µl of ATP solution were added for 2 min, and the reaction was stopped by addition of 800 µl of chloroform:methanol:1 M HCl (20:40:1). An additional 200 µl of chloroform was added and lipids were extracted and analyzed as described below.

Lipid extraction and TLC analysis

For extraction of phospholipids, 800 µl of chloroform:methanol (1:1) were added to the acidified samples. The chloroform phase was extracted twice with 320 µl of methanol:1 M HCl (1:1) and then evaporated. TLC plates were pre-run in oxalate solution (1% potassium oxalate, 2 mM EDTA, 50% ethanol) and baked at 100°C for 1 h. Evaporated lipids were resuspended, spotted and developed in chloroform:methanol:4 M NH_4OH (9:7:2). Plates were exposed on film overnight at –70°C. For quantitative measurement, radioactive spots were scraped from the plates and counted in a scintillation counter, or the autoradiograms were analyzed by the use of a phosphorimager program. Radioactive PtdInsP obtained in the PtdIns kinase assays was analyzed on borate plates according to Walsh *et al.* (1991).

Subcellular fractionation of chromaffin cells

Chromaffin granules, mitochondria and plasma membrane fractions were obtained from bovine adrenal medullary tissue as described (Hodel *et al.*, 1994). Corresponding subcellular fractions of permeabilized cells were prepared by a modification. Cells were scraped from 15 cm dishes in ice-cold buffer (0.3 M sucrose, 5 mM EDTA, 10 mM morpholinopropane-sulfonic acid, pH 7.2) and pelleted. Subcellular fractionation then followed the protocol described, but chromaffin granules and plasma membrane fractions were not purified over Percoll or sucrose gradients.

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