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Phosphatidylinositol 3-Kinase C2α Is Essential for ATP-dependent Priming of Neurosecretory Granule Exocytosis

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Neurotransmitter release and hormonal secretion are highly regulated processes culminating in the calcium-dependent fusion of secretory vesicles with the plasma membrane. Here, we have identified a role for phosphatidylinositol 3-kinase C2α (PI3K-C2α) and its main catalytic product, PtdIns3P, in regulated exocytosis. In neuroendocrine cells, PI3K-C2α is present on a subpopulation of mature secretory granules. Impairment of PI3K-C2α function specifically inhibits the ATP-dependent priming phase of exocytosis. Overexpression of wild-type PI3K-C2α enhanced secretion, whereas transfection of PC12 cells with a catalytically inactive PI3K-C2α mutant or a 2xFYVE domain sequestering PtdIns3P abolished secretion. Based on these results, we propose that production of PtdIns3P by PI3K-C2α is required for acquisition of fusion competence in neurosecretion.

INTRODUCTION

The analysis of the molecular mechanism controlling neuroexocytosis has been greatly helped by studies on neurosecretory cells (Dunn and Holz, 1983; Sarafian *et al.*, 1987; Martin and Walent, 1989; Monck and Fernandez, 1994; Rettig and Neher, 2002). Early work has shed light on two major steps consisting of the reversible ATP-dependent priming of docked granules followed by their Ca^{2+}-driven fusion with the plasma membrane and release of the granule content (Holz *et al.*, 1989; Hay and Martin, 1992). The spatiotemporal control of intracellular Ca^{2+} concentration together with capacitance measurements has allowed the identification of distinct pools of chromaffin granules involved in these distinct steps (Rettig and Neher, 2002). Further electrophysiological and biochemical approaches, such as the reconstitution of secretion in permeabilized neurosecretory cells (Martin and Walent, 1989), have highlighted the role of key factors involved in these two processes. Cytosolic and membrane proteins have been linked to priming and fusion, including the mammalian orthologues of the *Caenorhabditis elegans* UNC13 and UNC18 gene products, synaptotagmins, and members of the SNARE family and associated proteins (Jahn and Sudhof, 1999; Burgoyne *et al.*, 2001; Brose and Rosenmund, 2002; Rettig and Neher, 2002).

In contrast, less is known about the contribution of lipid dynamics during these processes with the exception of phosphatidic acid and phosphatidylinositol (4,5)-*bis*phosphate [PtdIns(4,5)P_2]. The former is produced by phospholipase D, which is essential for secretion in neurons (Humeau *et al.*, 2001) and in neuroendocrine cells (Vitale *et al.*, 2001). The inhibition of Ca^{2+}-dependent catecholamine release after depletion of phosphatidylinositol highlighted the role of phosphoinositides during the secretory events (Eberhard *et al.*, 1990). Moreover, phosphatidylinositol transfer protein and phosphatidylinositol-4-phosphate 5 kinase (PI4P5K) were shown to be required for the ATP-dependent priming of secretory granules in PC12 cells (Hay and Martin, 1993; Hay *et al.*, 1995). In addition, phosphatidylinositol 4-kinase (PI4K), an integral membrane protein of chromaffin granules and synaptic vesicles, is required for their exocytosis (Wiedemann *et al.*, 1996). Finally, the maintenance of a plasma membrane pool of PtdIns(4,5)P_2 was suggested to be important for the last step of exocytosis (Holz *et al.*, 2000; Cremona and De Camilli, 2001; Osborne *et al.*, 2001).

Phosphatidylinositol 3-kinases (PI3K) have recently been shown to be involved in a variety of cellular functions, including cell migration (Maffucci *et al.*, 2005), axonal guidance (Ming *et al.*, 1999), long-term depression (Daw *et al.*, 2002), and programmed cell death (Brunet *et al.*, 2001). However, a direct involvement of this class of enzymes and their lipid products in neuroexocytosis has been questioned (Chasserot-Golaz *et al.*, 1998; Milosevic *et al.*, 2005), based on little or no observed inhibition of synaptosomal or neurosecretory cell exocytosis by the PI3K inhibitors wortmannin

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Abbreviations used: PI3K, phosphatidylinositol 3-kinase; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; Syt I, synaptotagmin 1; EEA1, early endosomal antigen 1; LDCV, large dense core vesicles; hGH, human growth hormone.

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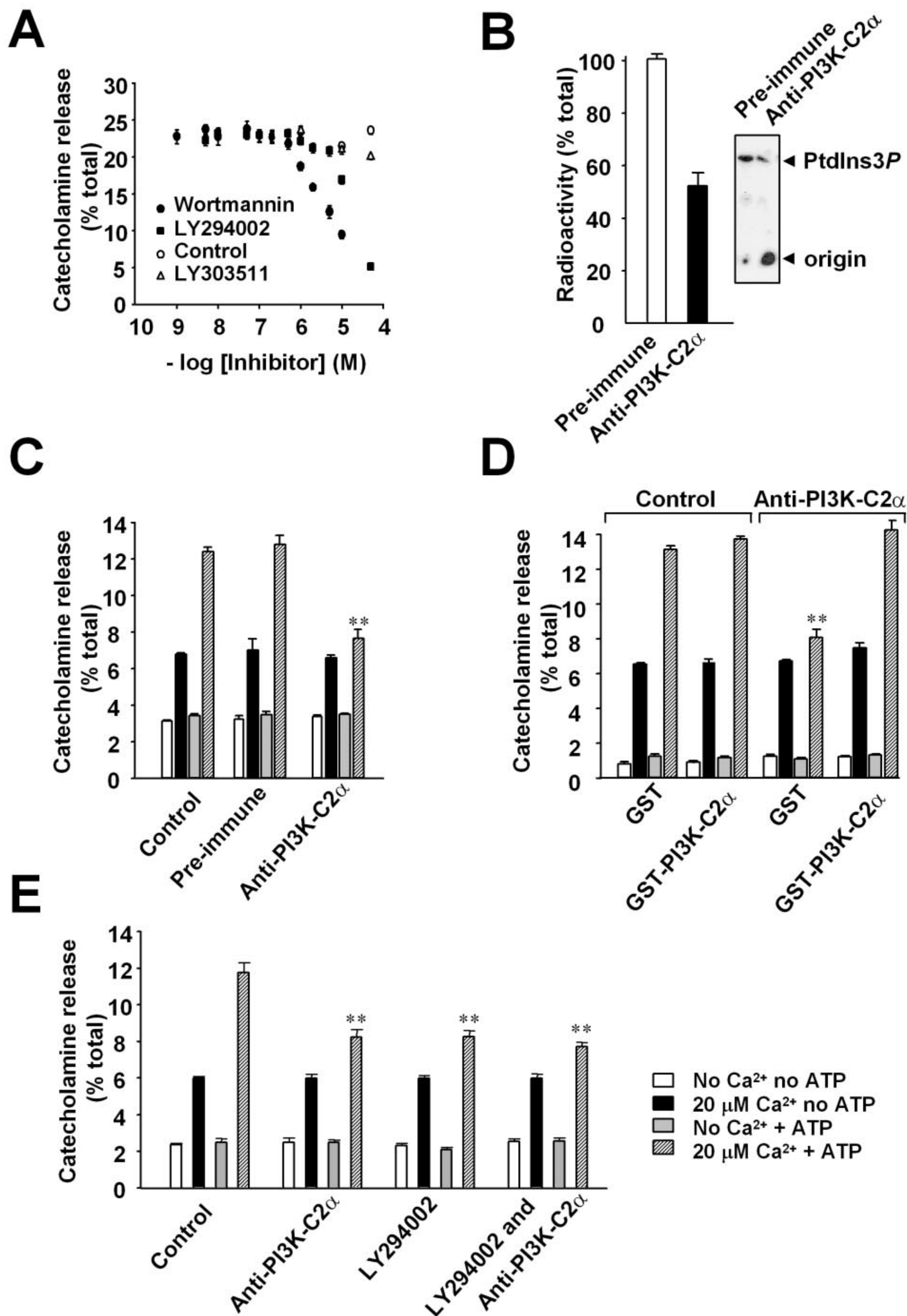


Figure 1.

and LY294002 (Wiedemann *et al*., 1996; Martin *et al*., 1997; Wiedemann *et al*., 1998). However, at the neuromuscular junction, wortmannin was shown to inhibit both spontaneous and evoked quantal neurotransmitter release (Hong and Chang, 1999). In contrast, high doses of LY294002 were shown to inhibit synaptic vesicle recycling and to increase spontaneous acetylcholine release on the same preparation (Rizzoli and Betz, 2002), raising questions about the susceptibility of certain types of synapses to these inhibitors and on the type of lipid kinases involved.

MATERIALS AND METHODS

Antibodies

We used affinity purified polyclonal antibody against PI3K-C2α (Arcaro *et al*., 2000), anti-hGH (Sigma), anti-Syt I (Matthew *et al*., 1981), anti-P18 (Wendler *et al*., 2001), anti-EEA1 (a kind gift from H.T. McMahon) and anti-clathrin heavy chain (Transduction Laboratories).

Catecholamine secretion from adrenal chromaffin cells

Chromaffin cells were prepared from bovine adrenal glands as previously described (Meunier *et al*., 2002). Intact cells were washed briefly once with buffer A (mM): NaCl, 145; KCl, 5, Na_2HPO_4, 1.2; glucose, 10; HEPES-NaOH, 20 (pH 7.4) and stimulated for 20 min by carbachol (100 μM; Sigma) in the presence of 2 mM $CaCl_2$ or by depolarisation with high K^+ modified buffer A (mM): KCl, increase to 60; NaCl decreased to 90 and 2 mM $CaCl_2$. For permeabilization experiments, chromaffin cells were incubated with 20 μM digitonin (Novabiochem) in KGEP buffer (mM): K-glutamate, 139; glucose, 5; EGTA, 5; and PIPES-NaOH, 20 (pH 6.7) for 10 min with or without addition of antibodies. The supernatant was then discarded and release was immediately triggered for 5 min in four different conditions: KGEP alone, KGEP containing 20 μM free Ca^{2+}, KGEP containing 2 mM free Mg^{2+} and 2 mM ATP and KGEP containing 2 mM free Mg^{2+}, 2 mM ATP and 20 μM free Ca^{2+} in the continuous presence of inhibitors or antibodies. Aliquots of the supernatant were taken at the end of each experiment and cells were lysed with 1% (vol/vol) Triton X-100 (Sigma). Both sets of samples were assayed fluorimetrically for catecholamines, and the amount released expressed as a percentage of the total catecholamine content of the cells (Burgoyne, 1992). Plotted data are representative of experiments carried out in quadruplicate and performed at least four times.

Gel electrophoresis and immunoblotting

Protein samples (20–50 μg) were prepared as previously described (Gasman *et al*., 1998), boiled for 3 min in SDS sample buffer containing β-mercaptoethanol and analyzed on 12% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose (0.2 μm), blocked in phosphate-buffered saline (PBS) containing 3% milk (wt/vol), probed overnight at 4°C with the indicated antibodies and detected with horseradish peroxidase-conjugated secondary antibody (1:2000; Dako) for 1 h followed by enhanced chemoluminescence (SuperSignal; Pierce).

Phosphoinositide kinase activity

Lipid kinase assays were performed in a total volume of 50 μl containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.5 mM EGTA. Phosphatidylinositol (10 mg/ml in 5 mM HEPES-NaOH, pH 7.4) was sonicated and preincubated with each sample at a final dilution of 200 μg/ml for 10 min. Reactions were initiated upon addition of 3.5 mM Ca^{2+} and 42 μM ATP spiked with 0.2 μCi of [γ^{32}P]ATP. Samples were incubated at 30°C for 20 min and then blocked with acidified chloroform:methanol (1:1 vol/vol). The organic layer was isolated, dried and fractionated by TLC. Radiolabeled phosphoinositides were visualized by autoradiography and quantified by densitometry.

Immunocytochemistry

Cells seeded on 18-mm polylysine coated coverslips were fixed with 4% paraformaldehyde for 20 min, washed in PBS and then blocked for 1 h in PBS containing 3% goat serum, 0.05% Triton X-100. In some experiments, cells were treated with wortmannin (Sigma) for 15 min before fixation. Indicated antibodies (all 1:500) were then incubated overnight at 4°C, washed with PBS and revealed using relevant secondary conjugated antibody (Alexa488 and Texas Red; 1:200; Molecular Probes) for 40 min at room temperature in the dark followed by washing and mounting with mowiol (Arco). Samples were imaged by confocal microscopy (LSM 510; Zeiss). To illustrate the degree of colocalization, scatter diagrams were produced using LSM 510 software plotting the pixels intensity in the red and green channels (x and y coordinates respectively). Double stained pixels (intensity >100 A.U.) of the optical sections were highlighted in white.

Constructs

EE tagged PI3K-C2α cDNA in pBluescript vector was used as a template for site directed mutagenesis (QuickChange, Stratagene). Mutant PI3K-C2α contains a single amino acid substitution (R1251P). This residue lies within the DRHNDN sequence that corresponds to the kinase motif present in mammalian PI3Ks. Both wild-type and mutant were inserted in pcDNA3.1 for mammalian expression. GFP-2xFYVE and GFP-2xFYVE^{C215S} from Hrs were prepared accordingly to Pattni *et al*. (2001), whereas GST-2xFYVE was expressed and purified as described previously (Gillooly *et al*., 2000).

hGH release from transfected PC12 cells

PC12 cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum, 10% horse serum. Before transfection, cells were incubated in DMEM antibiotic-free medium for 2 h and in OPTIMEM (InVitrogen) for 30 min. Mammalian expression vectors (1.25 μg/well) and pXGH5 encoding hGH (0.6 μg/well) were cotransfected in PC12 cells with lipofectamine 2000 (InVitrogen) in OPTIMEM according to the manufacturer's instructions. Transfection efficiency was between 30 and 50% and more than 95% of transfected cells coexpressed hGH and the protein of interest. 48 h after transfection PC12 cells were briefly washed in buffer A and incubated either in buffer A (control) or in high K^+ buffer for 20 min. Aliquots of the supernatant were collected and the cells detached using the remaining buffer passing through a 25-gauge needle several times. The amount of secretion from cotransfected cells was determined as % of total hGH/well using a hGH radioimmunoassay kit (Nichols Institute).

Statistical analysis

Data analysis was carried out using Student's *t* test. Experiments were performed at least 3 times. Values are expressed as mean ± SEM and data were considered significant at **p <0.01.

RESULTS

Chromaffin cells were preincubated with either 10 nM wortmannin or 1 μM LY294002 for 20 min and thereafter stimulated by depolarisation or by carbachol. Catecholamine secretion was unaffected by either inhibitor (see supplementary material S1). To rule out any long-term effect of PI3K activity on this process, chromaffin cells were treated for 24 h in culture medium with LY294002 (1 μM) without significant consequence on evoked catecholamine release (see supplementary material S1). Together, these experiments seem to exclude any direct involvement of most subtypes of PI3K in neuroexocytosis, as previously established in neurosecretory cells and synaptosomes (Wiedemann *et al*., 1996; Martin *et al*., 1997; Wiedemann *et al*., 1998). However, one PI3K isoform, type II PI3K-C2α, is much less sensitive to these

Figure 1 (facing page). Effect of PI3K inhibitors on catecholamine secretion from chromaffin cells. (A) Chromaffin cells were incubated for 20 min with increasing concentration of inhibitors and stimulated with carbachol (100 μM) in the continuing presence of inhibitors for 20 min. (B) EE-tagged PI3K-C2α was transiently expressed in COS cells and immunoprecipitated with anti-EE monoclonal antibodies followed by protein A-Sepharose. PI3K-C2α was eluted with an excess of EE peptide and aliquots treated at 4°C for 5 h with either preimmune serum or anti-PI3K-C2α antibody. This mixture was then incubated with PtdIns in a standard lipid kinase assay as described in the method section. Phosphorylated products were visualized by autoradiography after separation by TLC. The experiment was done in triplicate. (C) Chromaffin cells were permeabilized with 20 μM digitonin in Ca^{2+}- and ATP-free KGEP buffer alone or containing affinity purified anti-PI3K-C2α (0.38 μg/ml) or preimmune serum (88 μg/ml) for 10 min. (D) GST-PI3K-C2α (5 μg/ml) or GST alone (5 μg/ml) were preincubated for 30 min with or without 0.38 μg/ml anti-PI3K-C2α before addition to permeabilized chromaffin cells for 10 min. (E) Cells were preincubated with LY294002 (50 μM) for 20 min before permeabilization in the continuing presence of inhibitors. (C, D, E) The supernatant was removed and replaced by stimulation KGEP buffers as indicated in the continuing presence of antibodies/inhibitors for 5 min. (A, C, D, E) Aliquots were removed and assayed fluorimetrically for catecholamine content (n = 4 experiments; ** *t* test p <0.01).

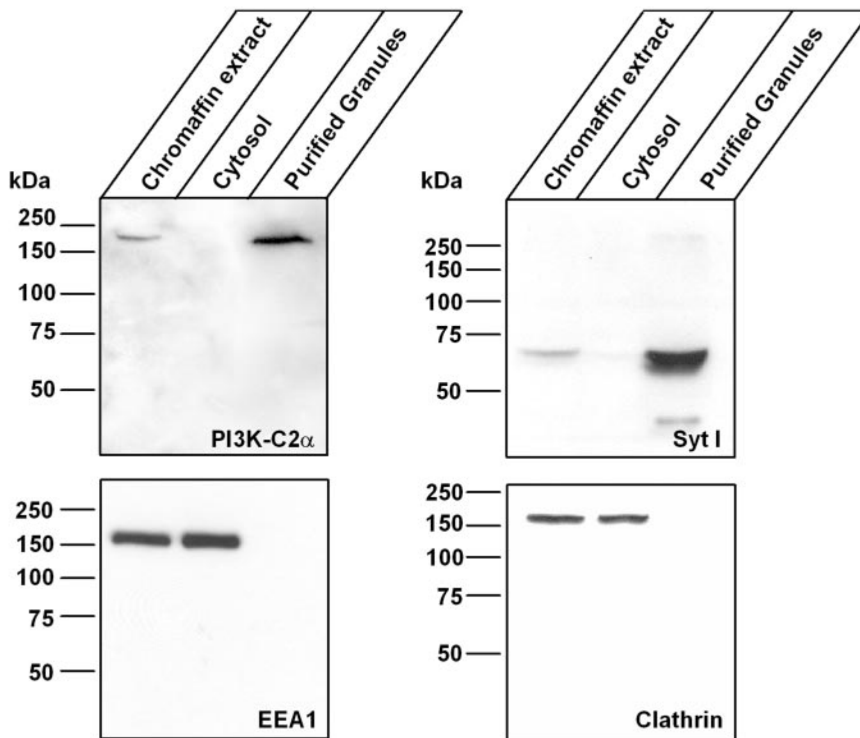


Figure 2. PI3K-C2α associates with secretory granule fraction in chromaffin cells. Equivalent amount of proteins of the indicated fractions (Gasman *et al.*, 1998) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

inhibitors (Domin *et al.*, 1997). Chromaffin cells were pretreated with increasing concentrations of LY294002 or wortmannin before stimulation. Both inhibitors impaired carbachol-induced secretagogue activity in a dose-dependent manner at concentrations that are known to inhibit PI3K-C2α (Figure 1A). However, at these concentrations LY294002 and wortmannin have been reported to inactivate other kinases (Okkenhaug and Vanhaesebroeck, 2001). To exclude this possibility, cells were treated with LY303511, an analogue of LY294002 that does not inhibit PI3K. Carbachol-evoked secretion was not blocked by 50 μM of LY303511, whereas 50 μM LY294002 drastically reduced this response (Figure 1A), suggesting a role for PI3K-C2α during neuroexocytosis.

Before exocytosis, neurosecretory vesicles are thought to be mobilized from a reserve pool to the plasma membrane, where they can dock and enter the readily releasable pool (Rizzoli and Betz, 2002; Burgoyne and Morgan, 2003; Duncan *et al.*, 2003). To acquire fusion competence, docked vesicles need to be primed, a process requiring ATP (Bittner and Holz, 1992; Osborne *et al.*, 2001; Rettig and Neher, 2002). Permeabilized chromaffin cells are a useful system to investigate these later steps since Ca^{2+} alone triggers a rapid but limited secretion of catecholamine from large dense core vesicles (LDCV) while in the presence of ATP, Ca^{2+}-induced secretion is increased due to recruitment, priming and fusion of additional LDCV (Bittner and Holz, 1992). Addition of an excess of affinity-purified PI3K-C2α antibody to recombinant PI3K-C2α inhibits its kinase activity (Figure 1B) in vitro. We investigated the involvement of PI3K-C2α in neurosecretion by testing the effect of this blocking antibody on LDCV exocytosis in digitonin-permeabilized chromaffin cells. Cells were preincubated for 10 min with anti-PI3K-C2α or preimmune serum and then stimulated for 5 min with each of four indicated intracellular buffers in the continuing presence of antibody. Anti-PI3K-C2α selectively reduced the Ca^{2+} plus ATP-dependent catecholamine secretion to the level obtained with Ca^{2+} alone, whereas the preimmune serum had no effect (Figure 1C). Importantly, the Ca^{2+}-dependent ATP-independent step was unaffected. To assess the specificity of this antibody on priming, anti-PI3K-C2α was preincubated with a molar excess of GST alone or GST fused with the N-terminal portion of PI3K-C2α. Preincubation of anti-PI3K-C2α with the cognate GST-fusion protein rescued priming elicited by ATP in the presence of Ca^{2+} (Figure 1D), whereas anti-PI3K-C2α pretreated with GST alone still inhibited priming. These results demonstrate that by specifically inhibiting PI3K-C2α, this antibody is capable of interfering with the ATP-dependent priming step of catecholamine secretion in chromaffin cells. Although alternative explanations are possible, the strong inhibition seen on priming by treatment with the anti-PI3K-C2α antibodies might be explained by a dominant-negative effect of the PI3K-C2α-antibody complex or envisaging the requirement of a threshold PI3K activity during this process.

Because high concentrations of PI3K inhibitors block carbachol-evoked catecholamine release from intact chromaffin cells (Figure 1A), we examined whether this block was generated at the ATP-dependent level (priming) or at the final Ca^{2+}-dependent step of exocytosis. LY294002 (50 μM) reduced the ATP- and Ca^{2+}-dependent secretion to that of Ca^{2+} alone (Figure 1E), consistent with the effect of anti-PI3K-C2α antibody. Wortmannin (10 μM) had a similar inhibitory effect but also reduced the Ca^{2+}-dependent secretion, suggesting the involvement of other kinases in the latter step of exocytosis (data not shown). In addition, permeabilized chromaffin cells were treated with both anti-PI3K-C2α and LY294002 (50 μM) to test whether an additive effect could be detected. As shown in Figure 1E, anti-PI3K-C2α antibodies and LY294002 alone and in combination give rise to similar inhibition of ATP-dependent priming, indicating that these two reagents act on the same pathway. The blockade of priming by high concentrations of PI3K inhibitors and anti-PI3K-C2α is likely to result from an inhibition

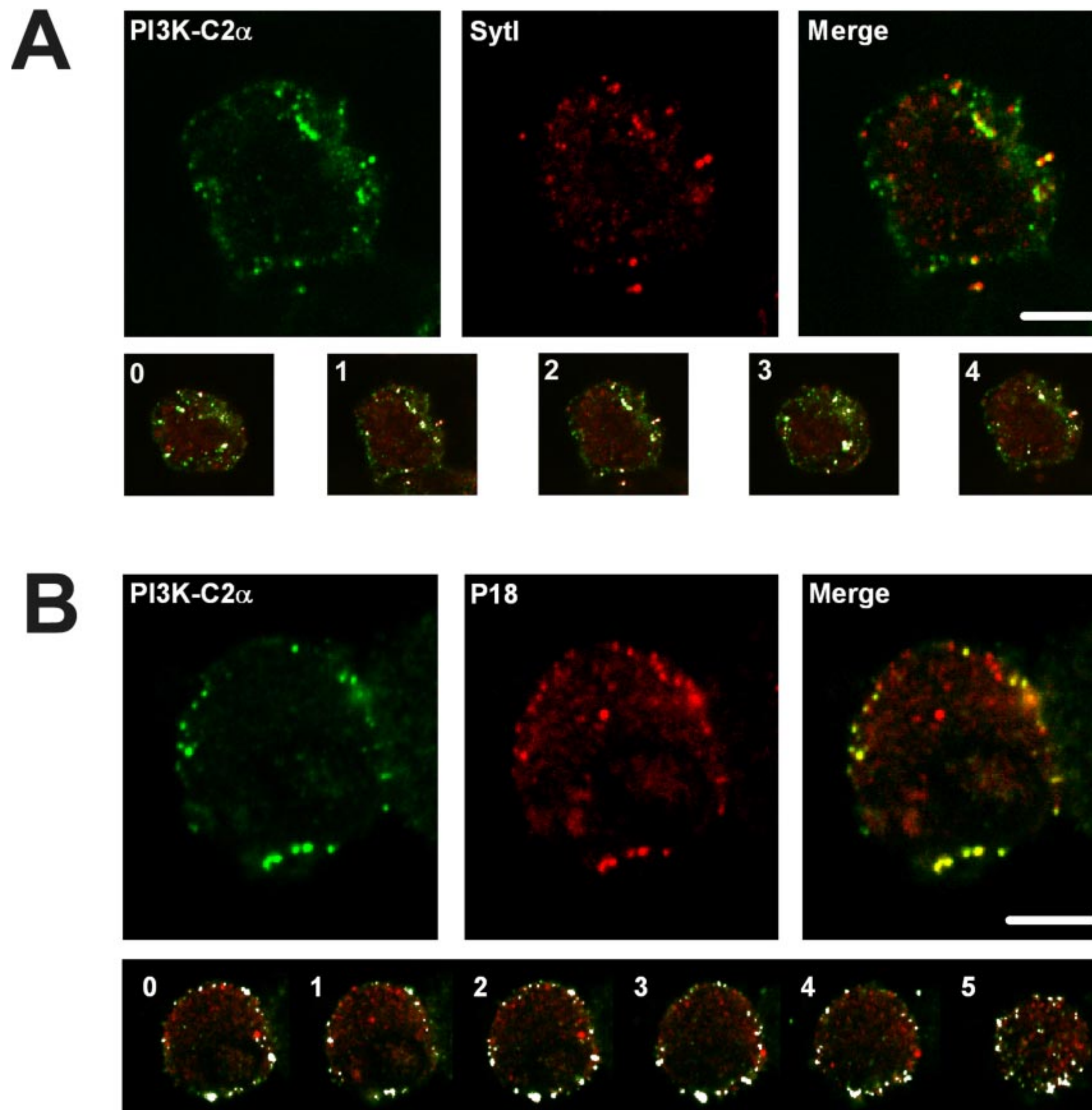


Figure 3. PI3K-C2α localizes to mature secretory granules. Fixed chromaffin cells were immunostained with anti-PI3K-C2α and either anti-Syt I (A) or anti-P18 antibodies (B) and imaged by confocal microscopy. Colocalized pixels (>100 a.u.) were highlighted (in white) in the z-stack series of optical sections shown below. Scale bar: 10 μm.

of PtdIns3P synthesis. We attempted to test this hypothesis by performing the experiment in the presence of [γ^{32}P]-ATP and 1 μM LY294002 to inhibit other PI3K, and then analyzing cellular lipids by HPLC. However, the low level of [^{32}P] incorporation into PtdIns3P under these conditions suggests that the pool of PtdIns3P synthesized by PI3K-C2α in chromaffin cells is too small to be measured with the available analytical techniques.

Western blotting using anti-PI3K-C2α antibodies (Arcaro *et al*., 2000) demonstrated that PI3K-C2α is present in chromaffin cell extracts and enriched in the chromaffin granule fraction (Figure 2). In these samples, the anti-PI3K-C2α antibody detected a single band with an apparent molecular weight of 200 kDa, further confirming its specificity. Importantly, no PI3K-C2α was detected in the cytosol, suggesting that in chromaffin cells this enzyme is selectively recruited to intracellular membranes including secretory granules. Synaptotagmin I (Syt I), an integral granule membrane protein, was similarly enriched in the chromaffin granule fraction (Figure 2). Control experiments with anti-early endosomal marker 1 (EEA1) and anti-clathrin antibodies demonstrated that there was no detectable contamination of the granule fraction with clathrin-coated vesicles and early endosomes (Figure 2), two compartments which have been previously shown to recruit PI3K-C2α (Domin *et al*., 1997).

PI3K-C2α was found on a discrete population of vesicles in chromaffin cells (Figure 3A, B). These organelles represented a subpool of secretory granules as shown by colocalization with Syt I (Walch-Solimena *et al*., 1993) (Figure 3A; see 3-D reconstruction in supplementary material S2). Confocal analysis revealed that 40.3 ± 4.0% of the vesicles positive for Syt I were also PI3K-C2α-positive, whereas 36.2 ± 5.1% of the vesicles immunostained for PI3K-C2α were also positive for Syt I. To identify which subset of secretory granules recruits PI3K-C2α, coimmunostaining experiments were performed using a monoclonal antibody (mAb) against the processed form of secretogranin (P18). This fragment is only present in mature secretory granules (Wendler *et al*.,

2001). 73.7 ± 6.4% of the P18-positive granules were also positive for PI3K-C2α, and 59.7 ± 4.4% of the PI3K-C2α positive organelles were coimmunostained by P18 (Figure 3B). These experiments demonstrated that PI3K-C2α is recruited to the membrane of mature secretory granules proximal to the plasma membrane.

To further examine the role of PI3K-C2α in exocytosis, we switched to a pheochromocytoma cell-line (PC12), where plasmids of interest can be cotransfected with a reporter plasmid encoding human growth hormone (hGH). hGH is stored in LDCV (Schweitzer and Kelly, 1985) and is released upon stimulation, serving as a marker for secretion in cotransfected cells (Holz *et al.*, 2000). Plasmids encoding EE-tagged wild-type PI3K-C2α (PI3K-C2α-wt) and PI3K-C2α containing a point mutation in the catalytic domain (PI3K-C2α-R1251P) were transfected in HEK cells where both proteins were expressed at comparable levels (Figure 4). To confirm that PI3K-C2α-R1251P is catalytically inactive, wild-type and mutant kinases were immunoprecipitated with anti-EE tag antibodies (Figure 4A) and tested in a lipid kinase assay using PtdIns as a substrate. A major band corresponding to PtdIns3P was synthesized by immunoprecipitates from PI3K-C2α-wt transfected cells (Figure 4B), as confirmed by HPLC (Domin *et al.*, 1997). In contrast, immunoprecipitates from cells transfected with PI3K-C2α-R1251P or control untransfected cells showed no such activity (Figure 4B).

The sensitivity of hGH secretion to PI3K inhibitors was tested in PC12 cells by incubation with increasing concentrations of wortmannin, LY294002 and its inactive analogue LY303511 for 20 min before stimulation. Both inhibitors block hGH exocytosis at very high concentrations (Figure 5A), reminiscent of their effect on catecholamine secretion from chromaffin cells (Figure 1). Importantly, LY303511 did not alter hGH secretion even at the highest concentration tested (50 μM; Figure 5A).

PI3K-C2α-wt, PI3K-C2α-R1251P or vector alone were then cotransfected with hGH in PC12 cells for 48 h. Immunostaining using anti-hGH monoclonal antibodies demonstrated that overexpression of either PI3K-C2α-wt or PI3K-C2α-R1251P did not affect the level of expression nor the localization of hGH in these cells (Figure 5B). In control samples, depolarisation in the presence of Ca^{2+} triggered the secretion of 11.7 ± 0.3% of the expressed hGH (Figure 5C). Overexpression of PI3K-C2α-wt enhanced hGH secretion to 17.5 ± 0.3%, indicating that PI3K-C2α has a facilitating effect on LDCV exocytosis. In contrast, coexpression of the catalytically-inactive PI3K-C2α-R1251P abolished depolarisation-induced hGH release (5.4 ± 0.4%; compared with 5.6 ± 0.4% of unstimulated cells), indicating that PI3K-C2α activity is required for LDCV secretion (Figure 5C).

To exclude the possibility that overexpression of PI3K-C2α-wt alters the levels of multiple phosphoinositides other than PtdIns3P, we labeled transiently-transfected PC12 cells with [2-^{3}H]inositol, extracted the lipids and analyzed the cellular content of phosphoinositides by HPLC. We observed no changes in the steady state levels of PtdIns4P or PtdIns(4,5)P_2 in cells expressing either wild-type or inactive PI3K-C2α (see supplementary material S3). In contrast, cells expressing PI3K-C2α-R1251P showed a decrease in the steady-state levels of PtdIns3P and PtdIns(3,4,5)P_3. Notably, as the efficiency of transfection in this experiment was low, the decrease in the 3-phosphorylated inositides observed is probably underestimated. In contrast, expression of PI3K-C2α-wt did not produce dramatic changes in PtdIns3P and PtdIns(3,4,5)P_3.

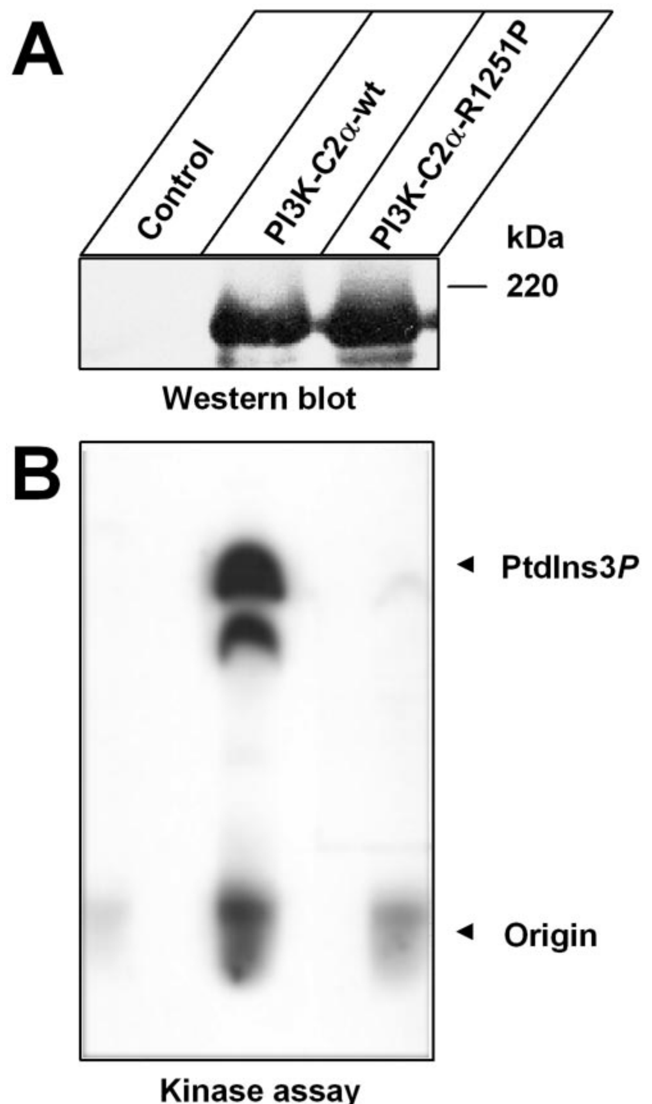
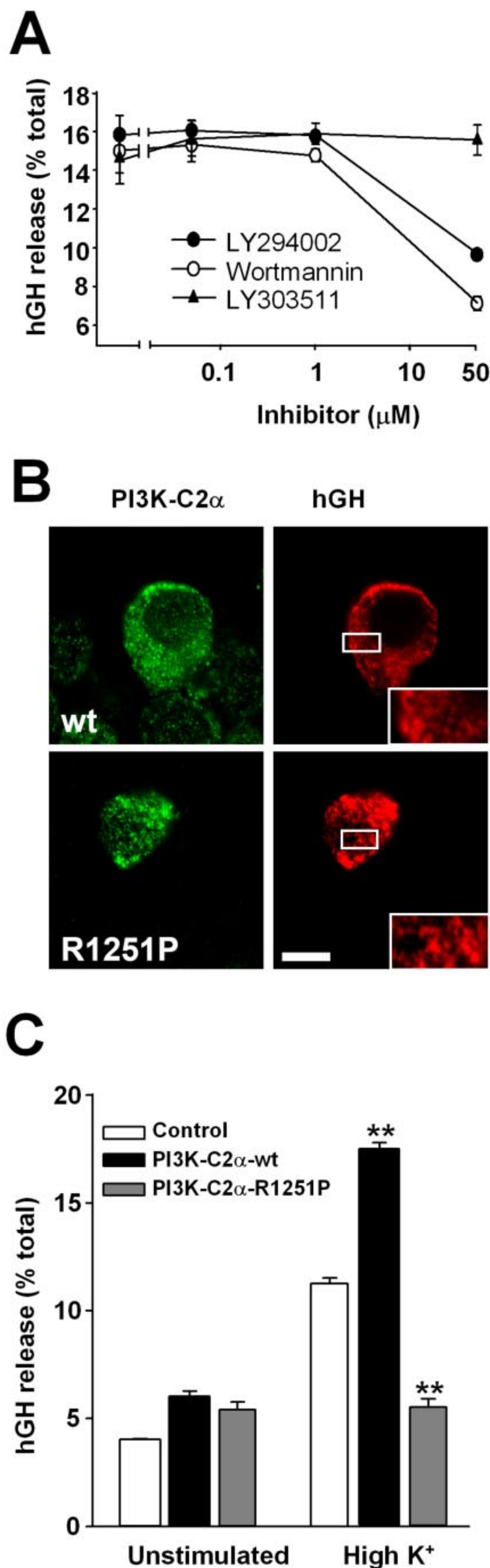


Figure 4. Expression and kinase activity of PI3K-C2α-wt and mutant PI3K-C2α-R1251P. HEK 293 cells were transfected with control vector, PI3K-C2α-wt or mutant PI3K-C2α-R1251P. After 72 h, recombinant enzymes were isolated from cell lysates using an anti-EE mAb and protein A-sepharose beads and blotted with anti-EE antibody (A) or used in a lipid kinase assay using PtdIns as substrate (B).

The effect of PtdIns3P sequestration on neuroexocytosis was then assessed by cotransfection of PC12 cells with hGH and GFP-2xFYVE. To exclude that overexpression of GFP-2xFYVE or its PtdIns3P binding-impaired mutant (GFP-2xFYVE^{C215S}) (Pattni *et al.*, 2001) was altering hGH processing, its distribution in secretory granules was assessed by immunocytochemistry. As shown in Figure 6A, hGH immunoreactivity appeared indistinguishable in single- (hGH only) and double-transfected (hGH and GFP-2xFYVE or GFP-2xFYVE^{C215S}) PC12 cells. However, when these cells were stimulated by depolarisation, GFP-2xFYVE overexpression blocked hGH release (Figure 6B). In contrast, GFP-2xFYVE^{C215S} mutant had no effect on secretion (Figure 6B). Finally we tested whether 2xFYVE domain-induced sequestration of PtdIns3P was capable of selectively blocking ATP-dependent priming in chromaffin cells. Digitonin-permeabi-



lized chromaffin cells were incubated with either bacterially-expressed GST-2xFYVE or GST alone (17 μM) for 15 min before Ca^{2+}-dependent stimulation of exocytosis. Our data indicate that 2xFYVE domain does indeed inhibit Ca^{2+}- and ATP-dependent exocytosis to the level observed for Ca^{2+} alone stimulation (Figure 6C). Altogether, these findings demonstrate that the maintenance of a PtdIns3P pool on secretory granules is necessary for neurosecretion.

DISCUSSION

To our knowledge, this study reveals for the first time a major role for PI3K-C2α in the ATP-dependent priming of neurosecretory vesicles - a crucial step during which vesicles acquire the competence to fuse with the plasma membrane upon Ca^{2+} entry.

Differential sensitivity to PI3K inhibitors suggest a role for PI3K-C2α in neuroexocytosis

Wortmannin and LY294002 are two cell permeable inhibitors possessing good selectivity and potency for PI3K types I, II and III (Okkenhaug and Vanhaesebroeck, 2001). One advantage of LY294002 over wortmannin is its longer half-life (Okkenhaug and Vanhaesebroeck, 2001), which allowed not only acute but also long-term inhibition of PI3K activities, a feature used in our experiment to address possible long-term effects of 3-phosphorylated lipids on secretion. In our hands, neither acute nor chronic exposure to PI3K inhibitors modified catecholamine secretion from chromaffin cells. Nevertheless neurosecretion from chromaffin cells was sensitive to both PI3K inhibitors at high concentrations. A recent study has highlighted a role for PI3K during synaptic vesicle exocytosis via a direct interaction of p85 with synapsin (Cousin *et al.*, 2003). Interestingly, the concentration of PI3K inhibitors used in this study far exceeded that usually needed to inhibit PI3K type I.

These results have shifted our attention to PI3K-C2α, which is more than ten times less sensitive to both inhibitors (Domin *et al.*, 1997). PI3K-C2α is expressed in chromaffin cells, where it was found on a subpopulation of chromaffin granules. The colocalization of PI3K-C2α with the processed form of secretogranin (Wendler *et al.*, 2001) on vesicles abutting the plasma membrane provides the first demonstration that PI3K-C2α is present on mature secretory granules. However, PI3K-C2α is selectively recruited on a subpool of secretory granules as PI3-kinase C2α colocalization with this LDCV marker is partial. Although we cannot formally exclude that our anti-PI3K-C2α antibody is capable of recognizing only a fraction of PI3K-C2α-positive structures, the use of a polyclonal antibody detecting multiple PI3K-C2α epitopes makes the above-mentioned possibility unlikely. Furthermore, the percentage of the granules stained was remarkably constant in different sets of experiments where

Figure 5. PI3K-C2α enhances hGH secretion from PC12 cells whereas its catalytically-inactive mutant is inhibitory. (A) PC12 cells transfected with a human growth hormone (hGH)-expressing vector (pXGH5) were treated with indicated concentrations of wortmannin, LY294002 or its inactive analogue LY303511 for 20 min prior stimulation with high K^+ buffer A containing Ca^{2+} for 20 min in the continuous presence of the inhibitors. (B, C) PC12 cells were cotransfected with pXGH5 and with wild-type PI3K-C2α or PI3K-C2α-R1251P. (B) Cells were fixed and coimmunostained for PI3K-C2α and hGH. Scale bar: 10 μm. (C) Transfected PC12 cells were stimulated in high K^+ buffer A containing Ca^{2+} for 20 min. (A, C) Aliquots were removed and assayed for hGH secretion, ** $p < 0.01$).

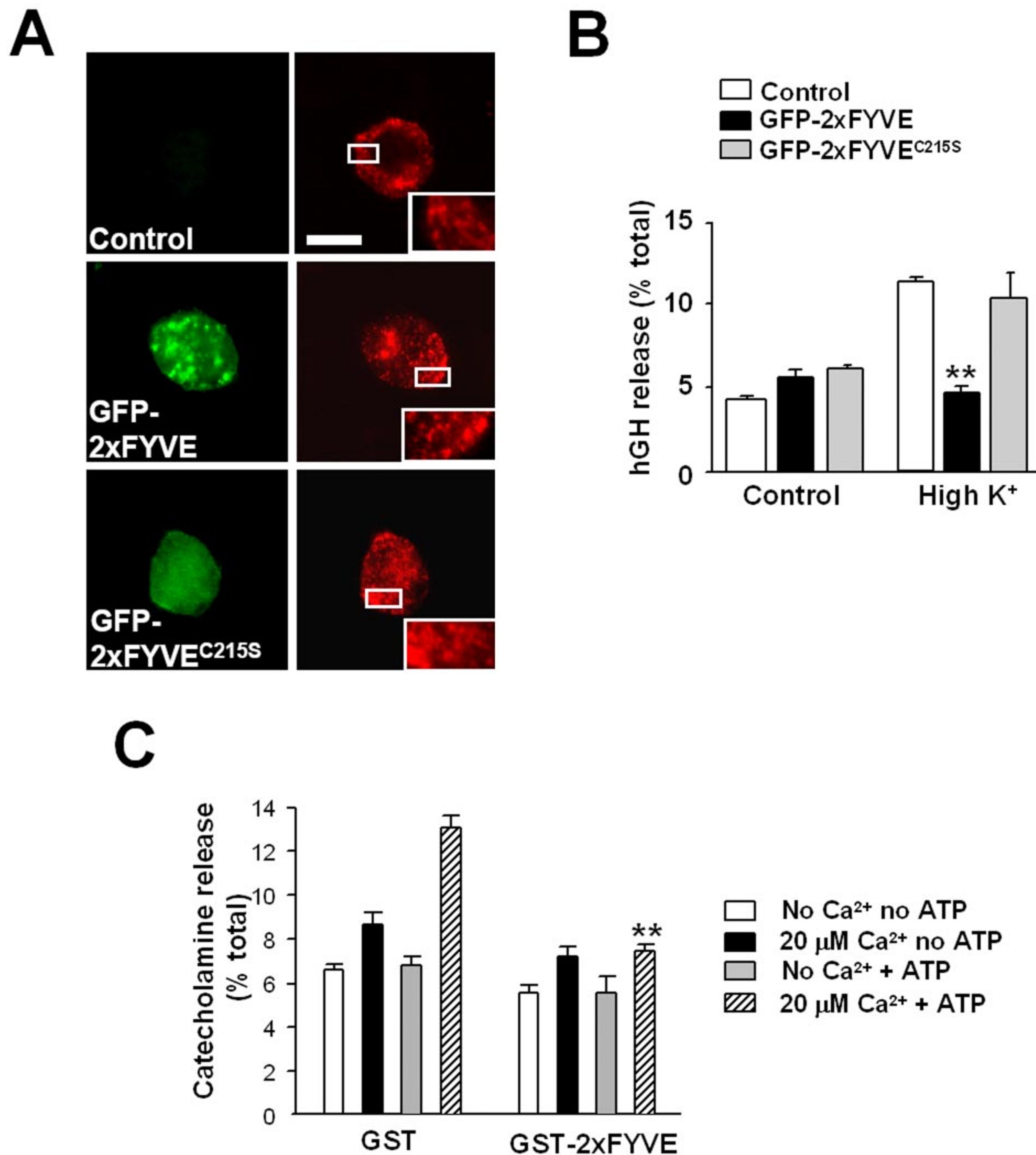


Figure 6. PtdIns3P is required for neurosecretion in PC12 cells. (A, B) PC12 cells cotransfected with pXGH5 encoding hGH and GFP-2xFYVE or GFP-2xFYVE^{C215S} for 48 h and then immunostained for hGH (A). Insets show high-magnification details of the hGH-containing structures (white rectangle). Scale bar: 10 μm. (B) Cotransfected PC12 cells were briefly washed and stimulated in high K^+ buffer A containing Ca^{2+} for 20 min. Aliquots were removed and assayed for hGH secretion, which was expressed as a percentage of the total hGH content. (C) Chromaffin cells were permeabilized with 20 μM digitonin in Ca^{2+}- and ATP-free KGEP buffer in the presence or absence of bacterially expressed GST or GST-2xFYVE (17 μM) and incubated for 15 min. The supernatant was then removed and replaced by stimulation KGEP buffers as indicated, in the continuing presence of recombinant proteins for 5 min. Aliquots were removed and assayed fluorimetrically for catecholamine content (n = 4 experiments, ** $p < 0.01$).

antibody concentration and protocols of fixation and permeabilization varied widely.

In fibroblasts, PI3K-C2α has been shown to be enriched on the TGN and clathrin-coated vesicles (Arcaro *et al.*, 2000). On the TGN, PtdIns3P has been shown to be required for the formation of constitutive transport vesicles (Jones and Howell, 1997). In chromaffin cells, PI3K-C2α may therefore control the biogenesis of secretory vesicles at the level of the TGN as well as later stages. In these cells, PI3K-C2α partially colocalizes with clathrin-coated vesicles (data not shown). Because clathrin can regulate PI3K-C2α enzymatic activity (Gaidarov *et al.*, 2001), PI3K-C2α could also be involved in

the clathrin-dependent budding that is required for granule maturation (Tooze *et al.*, 2001). Therefore, PI3K-C2α may play pleiotropic roles in regulating the function of secretory and recycling vesicles in neurosecretory cells. This pleiotropy is apparently shared by PI4K IIα, which was recently shown to regulate vesicle budding from the Golgi in nonsecretory cells (Wang *et al.*, 2003), as well as being required for synaptic vesicle exocytosis (Guo *et al.*, 2003).

Involvement of PI3K-C2α enzymatic activity in neurosecretion

Two distinct strategies were used to investigate the role of PI3K-C2α and its enzymatic product(s) in secretion. First, we exploited an antibody-based approach in permeabilized chromaffin cells to determine whether sequestration of PI3K-C2α interferes with catecholamine release. This method allowed us to show that PI3K-C2α antibodies specifically impair the ATP-dependent step of secretion, but not the Ca^{2+}-dependent fusion of already primed LDCV with the plasma membrane. Moreover, this inhibitory effect suggests that the PtdIns3P generated by PI3K-C2α on mature secretory granules might be directly responsible for the acquisition of their fusion-competence. The ATP-dependent step preceding Ca^{2+}-dependent fusion comprises both recruitment of LDCV to the plasma membrane and priming (Dunn and Holz, 1983; Sarafian *et al.*, 1987). PI3K-C2α activity might be important for either one or both processes. For example, it could be implicated in ARF6-dependent vesicular movements via cortical F-actin, which involves the Arp2/3 complex, tyrosine kinase activity, PLD and 3-phosphoinositides (Schafer *et al.*, 2000; Vitale *et al.*, 2001).

Our second strategy was to transiently transfect PC12 cells with PI3K-C2α or its catalytically-inactive mutant in conjunction with hGH. Because hGH is stored in secretory vesicles (Schweitzer and Kelly, 1985), this method has been successfully used to elucidate the role played in neurosecretion by many proteins of interest (Holz *et al.*, 2000). The enhancement of evoked secretion following overexpression of wild-type PI3K-C2α suggests that PI3K-C2α activity controls the progression of LDCV toward a fusion-competent state. Importantly, transient expression of a catalytically-inactive PI3K-C2α mutant completely abolished hGH secretion, demonstrating that PI3K-C2α is essential to the release mechanism. Although the molecular mechanism responsible for this phenomenon remains elusive, it is possible that the overexpression of the catalytically-inactive mutant may lead to the sequestration of PI3K-C2α regulators resulting in an inhibition of docking and/or priming.

PI3K-C2α catalyzes the production of PtdIns3P in vivo (Domin *et al.*, 1997 and this study). In addition to its well-established role in endosomal traffic (Gillooly *et al.*, 2001), PtdIns3P, produced by the class II PI3K-C2β, can also act as a signaling molecule, with levels increasing upon lysophosphatidic acid stimulation during cell migration (Maffucci *et al.*, 2005). Furthermore, this agonist-stimulated pool of PtdIns3P is on the plasma membrane. Thus, multiple intracellular pools of PtdIns3P exist in different cell-types. Our results add to this expanding list by identifying a novel pool of PtdIns3P in chromaffin and PC12 cells that is required for exocytosis of LDCV.

The ATP-dependent priming process remains quite enigmatic since little is known about the dynamics of the molecular events involved. Several proteins have been shown to participate in the priming of LDCV via controlling SNARE complex formation. A main player in this process is Munc18, which holds syntaxin in a closed conformation thereby preventing the assembly of the SNARE bundle. The dissociation of Munc18-syntaxin complex is believed to have an important role in the early events of priming (Misura *et al.*, 2000). In contrast, Munc13–1 overexpression accelerates the rate of LDCV priming by promoting the open conformation of syntaxin (Ashery *et al.*, 2000). Acetylcholine-SNAP and NSF were also shown to increase the priming rate (Xu *et al.*, 1999). Several kinases such as PKA, protein kinase C (PKC), Ca^{2+}-regulated myosin light chain kinase have been shown to modulate neurotransmitter release (Kumakura *et al.*, 1994; Capogna *et al.*, 1995; Mochida, 1995). Finally, PtdIns(4,5)P_2 formation by the conjoint ATP-dependent activities of PI4K and PI4P5K has been shown to be required for LDCV priming (Martin *et al.*, 1997) In contrast to its role in signal transduction, where it acts as a substrate for the generation of diacylglycerol and $InsP_3$, PtdIns(4,5)P_2 itself is likely to serve as a membrane anchor or modulator for several proteins of the secretory apparatus, such as synaptotagmins and CAPS (Walent *et al.*, 1992; Schiavo *et al.*, 1996). PtdIns(4,5)P_2 has also been shown to be required for synaptic vesicle recycling (Cremona and De Camilli, 2001; Bai *et al.*, 2004). Interestingly, PtdIns(4,5)P_2 was found predominantly associated with the plasma membrane in chromaffin cells (Holz *et al.*, 2000) and is absent from vesicular structures, indicating that a precise mechanism regulating its synthetic machinery exists in neurosecretory cells. To our knowledge, our findings provide the first evidence that phosphoinositides other than PtdIns(4,5)P_2 may play a role in Ca^{2+}-regulated secretion and suggest that PtdIns phosphorylation by PI3K-C2α leading to PtdIns3P production is an essential step during priming of secretory vesicles. How does PtdIns3P control priming? To answer this question, a crucial step in future investigations will be the identification of PtdIns3P effectors, which bind to mature secretory granules upon PI3K-C2α recruitment.

In conclusion, we have demonstrated a critical function for PI3K-C2α and its enzymatic product, PtdIns3P, during the ATP-dependent priming step preceding Ca^{2+}-dependent exocytosis. The generation of PtdIns3P by PI3K-C2α on the vesicular membrane adds to the repertoire of phosphoinositide synthetic processes, which are crucial for regulated secretion. In this light, different phosphoinositides may carry out distinct functions in a single trafficking step, revealing a more complex level of regulation for the acquisition of fusion-competence.

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