PIKfyve Negatively Regulates Exocytosis in Neurosecretory Cells*§

Regulated secretion depends upon a highly coordinated series of protein-protein and protein-lipid interactions. Two phosphoinositides, phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3-phosphate, are important for the ATP-dependent priming of the secretory apparatus prior to Ca²⁺-dependent exocytosis. Mechanisms that control phosphoinositide levels are likely to play an important role in priming fine tuning. Here we have investigated the involvement of PIKfyve, a phosphoinositide 5-kinase that can phosphorylate phosphatidylinositol 3-phosphate to produce phosphatidylinositol 3,5-bisphosphate on large dense core vesicle exocytosis from neuroendocrine cells. PIKfyve localizes to a subpopulation of secretory granules in chromaffin and PC12 cells. Nicotine stimulation promoted recruitment of PIKfyve-EGFP onto secretory vesicles in PC12 cells. YM-201636, a selective inhibitor of PIKfyve activity, and PIKfyve knockdown by small interfering RNA potentiated secretory granule exocytosis. Overexpression of PIKfyve or its yeast orthologue Fab1p inhibited regulated secretion in PC12 cells, whereas a catalytically inactive PIKfyve mutant had no effect. These results demonstrate a novel inhibitory role for PIKfyve catalytic activity in regulated secretion and provide further evidence for a fine tuning of exocytosis by 3-phosphorylated phosphoinositides.

Neurotransmitters and hormones are released from their storage vesicles into the extracellular space via calcium-dependent exocytosis. Much work has been directed toward identifying the proteins involved in and regulating the exocytic process. These include members of the SNARE family of proteins, synaptotagmins (1, 2, 4, 5, 13), and a host of regulatory proteins, such as Munc18a (6, 7). In addition to the critical role played by membrane proteins, there is an increasing amount of literature pointing to the importance of lipids, in particular phosphoinositides, in the regulation of membrane trafficking events, including regulated exocytosis (8–13).

Phosphoinositides are generated by the reversible phosphorylation of the inositol head group of phosphatidylinositol (PtdIns)² by an array of kinases and phosphatases at one or more of the 3-, 4-, and 5-OH positions (10). Previous studies have highlighted the importance of a plasma membrane pool of phosphatidylinositol 4,5-bisphosphate for regulated exocytosis of large dense core vesicles (LDCV) (14–17).

We recently demonstrated an involvement of the type II PI3K, PI3K-C2α, and its product PtdIns(3)P in priming, an ATP-dependent step during which LDCV acquire the ability to fuse with the plasma membrane (12). This novel role played by PtdIns(3)P in promoting the priming stage of exocytosis has opened a number of new research avenues regarding the mechanism(s) regulating the concentration of PtdIns(3)P and the potential role played by other 3-phosphorylated phosphoinositides in neurosecretory cells.

Priming is a reversible step and must be tightly controlled to prevent too many vesicles from reaching the primed state. PtdIns(3)P levels could be negatively regulated in either or both of two ways: through dephosphorylation of PtdIns(3)P by members of the myotubularin family of enzymes (18) or by further phosphorylation of PtdIns(3)P on the 5-OH position to generate PtdIns(3,5)P₅. The kinase responsible for this phosphorylation step in mammalian cells is PIKfyve (19, 20). PIKfyve is the mammalian orthologue of the yeast Fab1p (20, 21). In yeast, Fab1p is required for vacuolar membrane integrity and function (20, 21). Similarly, in mammalian cells, suppression of PIKfyve activity, either by overexpressing a kinase-inactive mutant or by siRNA-mediated knockdown results in the accumulation of enlarged internal membrane structures of endocytic origin (22, 23). PIKfyve was shown to be localized on early (24) and late...
endosomes (22). Evidence from an in vitro endosome fusion assay suggested that PIKfyve may act as a negative regulator of endosome fusion (22), whereas siRNA studies were consistent with PIKfyve regulating endosome-TGN trafficking (23). Notably, PIKfyve has also been shown to regulate trafficking of IRAP/GLUT4 vesicles to the plasma membrane in adipocytes (25). This suggests that PIKfyve could play a pleiotropic role in membrane trafficking, including on the exocytic pathway.

In this study, we have investigated the expression of PIKfyve in neurosecretory cells and found that it partially localizes to LDCV in an activity-dependent manner. We have used a combination of pharmacological, overexpression, and siRNA approaches to demonstrate that PIKfyve kinase activity plays a critical role in controlling regulated exocytosis in neurosecretory cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—PIKfyve antiserum was raised by inoculating a rabbit with a synthetic peptide corresponding to the last 18 amino acids of mouse PIKfyve (NP_035216). The specificity and selectivity of this antibody for PIKfyve was confirmed by Western blotting extracts of HEK293 cells transiently transfected with pEGFP-C1 or PIKfyve-EGFP. Western blotting and peptide competition and immunocytochemistry on PC12 cells transiently transfected with pEGFP-C1 or PIKfyve-EGFP (Fig. S1). This characterization demonstrated that the anti-PIKfyve antibody is specific for and can detect human (HEK293 cells), rat (PC12), and mouse (overexpressed) PIKfyve by both Western blotting and immunocytochemistry. Other antibodies used were anti-EEA1 (early endosomal antigen 1) (BD Transduction Laboratories), anti-Syt1 (M48) (26), anti-P18 (27), and anti-green fluorescent protein (Invitrogen).

**Gel Electrophoresis and Immunoblotting**—Tissue extracts were prepared by homogenizing tissues at 4 °C in homogenization buffer (20 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 0.32 M sucrose) containing 1 mM dithiothreitol and Complete protease inhibitor mixture (Roche Applied Science) with a Dounce homogenizer. Cellular debris and nuclei were removed by low speed centrifugation, and protein concentrations of the postnuclear supernatants were determined by Bradford reagent (Bio-Rad) using a standard curve of purified IgG. Subcellular fractionation of 3T3-L1 adipocytes (kind gift of R. Parton, Institute for Molecular Bioscience, Queensland, Australia) and PC12 and chromaffin cells was carried out by scraping cells in homogenization buffer as above. Samples were homogenized by repeated passage through a 25-gauge needle, and cellular debris and nuclei were removed by low speed centrifugation. Membrane and cytosolic fractions were separated by centrifugation at 125,000 × g, for 45 min, and protein concentrations were determined as above. 50 μg of protein/fraction was resuspended in SDS sample buffer containing β-mercaptoethanol, heated for 3 min at 95 °C, and analyzed by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (0.2 μm; Bio-Rad), blocked in phosphate-buffered saline (PBS) containing 5% nonfat milk (w/v), probed overnight at 4 °C with the indicated primary antibodies, and detected with horseradish peroxidase-conjugated secondary antibody (1:5000; Pierce) for 1 h followed by enhanced chemiluminescence (SuperSignal; Pierce).

**Reverse Transcription-PCR**—Total RNA was extracted from rat subcutaneous adipose tissue, adrenal glands, and rat pheochromocytoma (PC12) cells (ATCC) using the SV total RNA isolation system (Promega). Poly(A) RNA was reverse transcribed using oligo(dT)18 primers and used as a template for PCR primers designed on the basis of the data base sequence of rat PIKfyve XM_237217: forward (residues 910–931) and reverse (residues 1209–1229). β-Actin primers were chosen as a positive control.

**Catecholamine Secretion from Adrenal Chromaffin Cells**—Chromaffin cells were prepared from bovine adrenal glands and maintained in 24- or 96-well plates (Nunc) as previously described (31). Intact cells were washed briefly once with buffer A: 145 mM NaCl, 5 mM KCl, 1.2 mM Na2HPO4; 10 mM glucose; 20 mM HEPES-NaOH (pH 7.4), preincubated with the indicated concentrations of inhibitors or inactive analogues for 20 min, and further stimulated for 20 min using high K+ modified buffer A: KCl increased to 60 mM and NaCl decreased to 90 mM in the presence of 2 mM CaCl2. Aliquots of the supernatant were taken at the end of each experiment, and cells were lysed with 1% (v/v) Triton X-100 (Sigma). Both sets of samples were assayed fluorometrically for catecholamines, and the amount released was expressed as a percentage of the total catecholamine content of the cells (12, 28, 29). Plotted data are representative of experiments carried out in quadruplicate and performed at least four times.

**PIKfyve siRNA**—For siRNA targeting, rat PIKfyve cDNA fragments TGACTTGCTCCACTCTCC, encoding the 19-nucleotide siRNA sequence derived from the target transcript and separated from its reverse 19-nucleotide complement by a short spacer, were annealed and cloned in the BglII and HindIII sites in front of the H1 promoter of a modified pXGH5 plasmid encoding for growth hormone named pGHsuper as described previously (30). The human sequence TGATTTGCCTCGATCTCCT was used as control (differences from rat sequence are underlined). Transfection efficiency has been estimated by counting growth hormone-positive cells from 400–500 cells as described previously (30).

**hGH Release from Transfected PC12 Cells**—PC12 cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 7.5% fetal bovine serum, 7.5% horse serum. pXGH5 vector encoding hGH (1.2 μg/well) either alone or together with mammalian expression vectors encoding proteins as indicated in Fig. 5 (1.2 μg/well) were co-transfected in PC12 cells with Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency was between 30 and 50%, and more than 95% of transfected cells co-expressed hGH and the protein of interest. 48 h after transfection, PC12 cells were briefly washed in buffer A and either stimulated in high K+ buffer for 20 min or pretreated in high K+ buffer for 20 min with either inhibitor or inactive analogue prior to stimulation. 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 co-transfected cells was determined as percentage of total hGH/well using an hGH radioimmunossay kit (Nichols Institute) (12). Plotted data are representative of
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experiments carried out in triplicate and performed at least four times.

Immunocytochemistry and Live Cell Imaging—Cells seeded on poly-l-lysine-coated coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, washed in PBS, and then blocked for 1 h in PBS containing 3% normal horse serum, 0.5% bovine serum albumin, 0.05% Triton X-100. For treatments, cells were incubated for 20 min in Buffer A at 37 °C, 5% CO2 with the indicated concentrations of YM-201636 or YM-211387 prior to fixation. A similar experiment was carried out using nicotine stimulation prior to fixation in Buffer A containing 2 mM Ca2+. Coverslips were then incubated overnight at 4 °C with primary antibodies and/or rhodamine-conjugated phalloidin (Molecular Probes), washed with PBS, and revealed using secondary conjugated antibodies (Alexa488 and Alexa546; 1:500; Molecular Probes) for 30 min at room temperature in the dark, followed by extensive washing and mounting with Prolong Gold (Molecular Probes). In other experiments, chromaffin cells (2–3 days of culturing) were permeabilized with digitonin (Sigma) (20 μM) in potassium glutamate buffer (KGEP): 139 mM potassium glutamate, 5 mM glucose, 5 mM EGTA, 20 mM PIPES-NaOH, 2 mM MgCl2, 2 mM ATP in the continuing presence of boiled or unboiled ENTH-GST (6 μg/ml) for 10 min. Cells were washed in KGEP buffer, fixed, and processed for immunocytochemistry as described above and in the figure legends. Samples were imaged with a laser-scanning microscope (LSM 510; Zeiss). To illustrate the degree of colocalization, scatter diagrams were produced using LSM 510 software plotting the pixel intensity in the red and green channels (x and y coordinates, respectively). Double-stained pixel intensity > 100–150 arbitrary units) of the optical sections were highlighted in white. Quantification of the degree of co-localization was performed using the color range tool of LSM 510 software or Laserpix software (Bio-Rad). Briefly, immunopositive puncta were selected first in the green channel as regions of interest. 30–110 regions of interest were selected per cell for at least eight cells. Regions of interest with intensity > 100–150 arbitrary units in both the green and red channels were considered co-localized. The percentage of co-localizing ROI was calculated per image, and the mean and S.E. were calculated. This process was repeated in the red channel. Quantification of the area and size of the early endosomes was carried out by image analysis on EEA1 staining using Laserpix software (Bio-Rad). Briefly, clearly identifiable EEA1-positive structures were outlined, and area and size (length and width) measurement data were acquired using the software.

Time Lapse Confocal Microscopy Imaging—Confocal microscope (LSM 510 Meta; Zeiss) laser power was set at <4% for a 488-nm argon laser, and a ×63 water immersion objective (numerical aperture = 0.93) was used. The pinhole was chosen to give rise to confocal z sections less than 2.5 μm. The frequency of acquisition was 2 frames/min over the 10–15 min incubation period. Cells were bathed in Buffer A containing 2 mM Ca2+, and nicotine (100 μM) was applied by injection using a Hamilton syringe.

Analysis of Time Lapse Confocal Images—Labeled organelles that remained in the same optical section throughout the duration of imaging were selected for intensity analysis. The changes in intensity of PIKfyve staining in the cytosol and on identified organelles were followed over time before and after nicotine treatment. The values were expressed as percentage increase of the normalized initial fluorescence intensity of PIKfyve.

PIKfyve Inhibitor—YM-201636 was identified as an inhibitor of PIKfyve by serendipity (31). It was shown to be a potent PIKfyve inhibitor in a counterscreen directed at assessing the specificity of inhibitors of p110α. As for many other kinase inhibitors, this compound appears to be ATP-competitive.

Constructs—PIKfyve cDNA in pBluescript vector (kindly provided by A. Shisheva) was used as a template for site-directed mutagenesis (QuikChange; Stratagene). Mutant PIKfyve contains a single amino acid substitution (K1831M) within the kinase domain. This residue has been demonstrated to be critical for lipid kinase activity (32). Both wild-type and mutant PIKfyve were inserted in pEGFP-C1 for mammalian expression. hGH-EGFP plasmid was kindly provided by I. Robinson (33).

Statistical Analysis—Data analysis was carried out using Student’s t test. Experiments were performed at least three times. Values are expressed as mean ± S.E., and data were considered significant at p < 0.05 (*) and p < 0.01 (**) unless otherwise stated (Student’s t test).

RESULTS

To investigate PIKfyve expression and subcellular localization in neurosecretory cells, a rabbit polyclonal antibody was raised against a peptide corresponding to the last 18 amino acids of mouse PIKfyve. This antibody was expected to cross-react with both rat and human PIKfyve, since the predicted rat PIKfyve orthologue, XM_237127 is identical within this 18-amino acid stretch, and human PIKfyve (32) differs by only 1 amino acid. XM_237217 was identified as rat PIKfyve based on data base searches and sequence alignments; the identity between XM_237217 and mouse PIKfyve (NM_011086) is 93% at the amino acid level, and within the kinase domain, the amino acid identity is 99%, and all of the key residues previously identified as being required for both lipid kinase and protein kinase activity are present (32, 34).

PIKfyve was first identified in adipose tissue and skeletal muscle (35) but has subsequently been reported to have a wider tissue distribution (36). Our result from the antibody characterization suggested that PC12 cells endogenously express PIKfyve (Fig. S1C). To confirm this, PIKfyve mRNA distribution was investigated in rat adrenal glands, rat brain cortex, PC12 cells, and rat adipose tissue as a positive control (35). Using PIKfyve-specific primers, a 310-bp fragment was detected in all samples (Fig. 1A). β-Actin primers were used as a positive control and to standardize the input. No band was detected in samples where the reverse transcriptase was omitted from the reverse transcription reaction, demonstrating an absence of contamination by genomic DNA (data not shown). The identity of the 310-bp amplicon was confirmed as PIKfyve by DNA sequencing (data not shown).

The distribution of PIKfyve expression in different rat tissues was also investigated at the protein level by Western blotting (Fig. 1B). Equal amounts of postnuclear supernatants of the
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The ability of PIKfyve to regulate LDCV exocytosis was investigated using a novel, specific inhibitor of PIKfyve, YM-201636 (31). This enabled us to investigate both the acute and chronic effects of PIKfyve inhibition on exocytosis. Chromaffin cells were preincubated for 20 min (Fig. 2, B–D) or overnight (data not shown) with 800 nM YM-201636 or YM-211387, an unrelated PI3K type I inhibitor, prior to stimulating secretion by KCl-evoked depolarization. In acute treatments, YM-201636 treatment significantly potentiated catecholamine secretion by 35% (Fig. 2, B–D), whereas YM-211387 had no effect (Fig. 2, C and D). A similar potentiation was observed upon overnight incubation with YM-201636 (data not shown). Increasing amounts of YM-201636 or YM-211387 were used to

FIGURE 1. PIKfyve is expressed in neurosecretory tissues and cell lines. A, reverse transcription-PCR analysis of RNA extracted from rat adipose, adrenal, and PC12 cells using PIKfyve-specific primers. β-Actin primers were used as a positive control. B, equal amounts of protein (50 μg) from the rat tissues shown were separated by 8% SDS-PAGE and analyzed by Western blotting using anti-PIKfyve antibodies. C, equal amounts of protein (50 μg) from membrane (M) and cytosol (C) fractions from 3T3-L1 adipocytes, PC12, and bovine adrenal chromaffin cells and chromaffin granule membranes (CG) were separated by 8% SDS-PAGE and Western blotted with anti-PIKfyve antibodies.

PIKfyve is distributed between cytosolic and membranous compartments in 3T3-L1 adipocytes (34, 37). Membrane and cytosolic fractions were prepared from 3T3-L1 adipocytes, PC12 cells, and bovine adrenal chromaffin cells, and PIKfyve distribution was determined by SDS-PAGE and Western blotting using anti-PIKfyve antibodies (Fig. 1C). As previously reported, we found more PIKfyve in 3T3-L1 adipocyte cytosol than associated with cellular membranes (37). In both PC12 and adrenal chromaffin cells, PIKfyve was present in membrane and cytosol fractions. We also ran an equal amount of purified chromaffin granule membranes prepared as previously described (12, 38, 39). Importantly, PIKfyve immunoreactivity was also detected in the granule fraction, suggesting that PIKfyve may be recruited to large dense core vesicles and could play a role in secretory granule function.

To investigate a role for PIKfyve in exocytosis, we initially focused on bovine adrenal chromaffin cells, a primary cell that is a widely used model for studying neurosecretion. The localization of PIKfyve on chromaffin granules observed by Western blotting (Fig. 1C) was confirmed by immunocytochemistry using anti-PIKfyve and anti-P18 antibodies (Fig. 2A). P18 is a proteolytic fragment of secretogranin II and a marker of mature secretory granules (27). PIKfyve immunoreactivity shows both a diffuse and punctate localization, partially co-localizing with P18 positive secretory granules (Fig. 2A).

FIGURE 2. PIKfyve inhibits calcium-dependent secretion in bovine adrenal chromaffin cells. A, top, paraformaldehyde-fixed chromaffin cells were co-immunostained with anti-PIKfyve (green) and anti-P18 (red). Bottom, control experiment using anti-P18 (green) and cortical actin network with Texas Red-conjugated phalloidin (red). Images are 0.5-μm confocal z sections; scale bar, 10 μm. B, chromaffin cells were treated with 800 nM YM-201636 compound for 20 min prior to stimulating exocytosis by KCl-evoked depolarization (n = 4). C, chromaffin cells were treated with 800 nM YM-201636 or YM-211387, an unrelated class Ia PI3K inhibitor, prior to stimulating exocytosis by KCl-evoked depolarization (n = 4). D, chromaffin cells were preincubated with increasing concentrations of YM-201636, YM-211387, or vehicle alone prior to KCl-evoked depolarization (n = 4). E, chromaffin cells were treated with 800 nM YM-201636 or YM-211387 for 20 min prior to digitonin-permeabilization and stimulation using Ca2+- and ATP-containing buffer in the continuing presence of inhibitors (n = 16).
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PIKfyve localizes to punctate structures in PC12 cells. Anti-PIKfyve antibody (green) was used for indirect immunofluorescence on paraformaldehyde-fixed PC12 cells. Co-localizations were performed with anti-P18 antibodies (red). Partial co-localization (yellow) in the merge image can be observed, which is particularly visible in the enlarged panels (arrows). Co-localized pixels (>150 arbitrary units (A.U.)) on a scatter plot were highlighted (in white) in the optical sections shown below. Images are 0.5-μm confocal z-sections; scale bar, 5 μm.

FIGURE 4. PIKfyve inhibitors stimulate hGH exocytosis from PC12 cells. A, PC12 cells transiently transfected with hGH were incubated for 20 min with 800 nM YM-201636 or YM-211387 prior to stimulating with High K+-evoked depolarization (20 min). *, p < 0.05 (Student’s t test, one-tailed; n = 3). B, PC12 cells were treated with vehicle or 1 μM YM-201636 or YM-211387 for 20 min prior to fixation. Cells were immunolabeled using anti-P18 antibodies. Images are 0.5-μm confocal z-sections; scale bar, 10 μm.

construct a concentration-response curve, which demonstrated that 1 μM YM-201636 caused maximal potentiation of release (Fig. 2D). However, no potentiation was observed when the concentration was raised to 10 μM. This could be due to a compound effect of the YM-201636. Since release experiments on intact cells do not differentiate between an effect on the release machinery or effects on voltage-gated calcium channels, chromaffin cells were preincubated with 800 nM YM-201636 or YM-211387 as before and permeabilized with digitonin, and exocytosis was stimulated using Ca2+- and ATP-containing buffer to bypass plasma membrane calcium channels (Fig. 2E). A similar and significant potentiation of exocytosis was observed with YM-201636 but not YM-211387, as was seen with intact cells.

To investigate the effects of overexpressing PIKfyve constructs on secretion, we turned to the neuroendocrine PC12 cell line. The effect of candidate protein overexpression on secretion can be assayed by co-transfection with a plasmid encoding hGH. The hGH is packaged into secretory granules, and exocytosed hGH can be detected using a specific radioimmunoassay (40, 41). First, co-localization studies were carried out by confocal microscopy to confirm that endogenous PIKfyve associates with secretory granules in this cell line as in chromaffin cells (Fig. 3). PC12 cells were immunostained with anti-PIKfyve antibodies together with antibodies against P18 (Fig. 3). PIKfyve immunostaining was found to be both diffuse throughout the cytosol and punctate as expected from the subcellular fractionation (Fig. 1C). A clear degree of co-localization was detected between punctate PIKfyve and P18 (Fig. 3). However, PIKfyve punctate staining did not completely co-localize with P18, suggesting that PIKfyve is present on multiple organelles in PC12 cells. Similarly, a fraction of P18 immunostaining did not co-localize with PIKfyve, suggesting that PIKfyve is only recruited on a subpopulation of mature secretory granules under resting conditions. The immunostaining data, together with the subcellular fractionation (Fig. 1C), demonstrate that PIKfyve associates with a pool of mature LDCV.

Having established that PIKfyve immunoreactivity partially co-localizes with P18 in chromaffin cells (Fig. 2A) and in PC12 cells (Fig. 3), we checked whether PIKfyve inhibition could also affect exocytosis from PC12 cells. PC12 cells expressing hGH were treated with 800 nM YM-201636 or YM-211387 and stimulated by depolarization (Fig. 4A). YM-201636 significantly potentiated depolarization-evoked hGH secretion by a similar amount (42 ± 9%) to that observed in chromaffin cells (35 ± 3%), whereas YM-211387 had no effect on secretion (Fig. 4A). Neither YM-201636 nor YM-211387 (1 μM) affected the overall distribution of LDCV by confocal microscopy (Figs. 4B and S2).

We next examined the effect of reducing endogenous levels of PIKfyve on hGH release from PC12 cells using RNA interference. The level of knockdown was found to be around 80% after 72 h of transfection with plasmids expressing PIKfyve-targeted siRNAs versus control and mutated siRNAs (Fig. 5A). Impor-
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FIGURE 5. PIKfyve negatively regulates exocytosis of hGH from PC12 cells. A, PC12 cells were transfected with either the pGHsuper vector (No siRNA), the pGHsuper-PIKfyve siRNA vector (PIKfyve siRNA), or the pGHsuper-PIKfyve mutated siRNA vector (mutated siRNA). 72 h after transfection, proteins were extracted, and 20–μg samples were analyzed by Western blot using anti-PIKfyve and anti-β-actin antibodies. A, semiquantitative analysis of the PIKfyve levels detected in PC12 cells transfected with the various vectors. Quantification was performed using scanning densitometry analysis of the Western blots and is presented as mean values ± S.E. of three independent experiments. Data have been normalized for transfection efficiency (60–65%). B, 72 h after transfection, the cells were incubated for 10 min in the absence (Resting; open bars) or presence of 59 mM K (Stimulated; closed bars). hGH release is expressed as the percentage of total hGH present in the cells before the 10-min stimulation period. C, PIKfyve-EGFP expression inhibits hGH exocytosis from PC12 cells. PC12 cells transiently transfected with hGH and either control plasmid (EGFP-C1), PIKfyve-EGFP, PIKfyve(K1831M)-EGFP, or Fab1p-EGFP were incubated for 20 min in the absence or presence of 59 mM K buffer A to stimulate exocytosis. hGH release is expressed as the percentage of total hGH present in the cells before the 10-min stimulation period. Data are given as the mean values ± S.E. obtained in three experiments performed on three different cell cultures (n = 3). *, p < 0.05; **, p < 0.005 (Student’s t test, one-tailed; n = 3).

PIKfyve has been implicated in regulation of endomembrane integrity, and in particular, the overexpression of the PIKfyve(K1831E) mutant causes enlarged endosomal structures positive for PIKfyve and EEA1 (22) and at later time points vacuolation (42). PC12 cells transfected with PIKfyve(K1831M)-EGFP did contain enlarged PIKfyve(K1831M)-EGFP-positive structures, the extent of which correlated with the levels of overexpression (Fig. 6A and Table 1). These structures were also positive for EEA1 (Fig. 6A), as described in other cell types (22). The appearance of such enlarged EEA1-positive structures can also be induced by short (20 min) incubations with 10 μM YM-201636 but not YM-211387 in untransfected PC12 cells (Fig. S3) and, to a similar extent, in PC12 cells overexpressing PIKfyve-EGFP (Fig. 6 and Table 1). Importantly, lower concentrations of YM-201636 that maximally potentiate calcium-dependent catecholamine and hGH release (Figs. 2D and 4A) did not result in the formation of enlarged EEA1-positive structures (Fig. S3). This strongly suggests that PIKfyve regulation of exocytosis and endosomal size is independent. In contrast to other cell types, vacuolation visible by phase contrast was not observed in PC12 cells overexpressing PIKfyve-EGFP (Fig. 6 and Table 1). Furthermore, PIKfyve has been previously shown to functionally substitute for each other (20). Importantly, overexpression of Fab1p-EGFP inhibited depolarization-induced secretion to a similar extent as PIKfyve-EGFP (35 ± 9%) (Fig. 5C). Total hGH levels were not affected by overexpressing either the wild-type or catalytically inactive kinases (data not shown).

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PIKfyve Negatively Regulates Exocytosis

localization with a LDCV marker, Syt1, is significantly increased upon stimulation of exocytosis by nicotine treatment, 20.0 ± 5.2% in unstimulated cells (n = 9 cells) versus 45.5 ± 8.5% in stimulated cells (n = 10 cells), suggesting that upon stimulation of exocytosis, PIKfyve is indeed recruited from the cytosol to a subpopulation of LDCV. Since PIKfyve plays a role in exocytosis, it is likely that its recruitment takes place on mature secretory granules. PIKfyve-EGFP was found to partially co-localize with P18 immunostaining, a marker of mature secretory granules. Importantly, the level of co-localization was significantly enhanced in nicotine-stimulated PC12 cells (Fig. S4).

Our results demonstrate that PIKfyve is located on secretory granules. Since PIKfyve uses PtdIns(3)P as substrate to generate PtdIns(3,5)P$_2$, we investigated the presence of PtdIns(3,5)P$_2$ on secretory granules using the ENTH domain from yeast Ent3p, a specific PtdIns(3,5)P$_2$ effector (43). In permeabilized chromaffin cells, ENTH staining was punctate. A good degree of co-localization was found with Syt1, strongly suggesting that PtdIns(3,5)P$_2$ could indeed be present on a subpopulation of secretory granules (Fig. 8).

Together, the data using inhibitors and overexpression indicates that PIKfyve kinase activity is involved in negatively regulating calcium-dependent exocytosis of secretory granules in neuroendocrine cells.

**DISCUSSION**

In this study, we have used overexpression studies, RNA interference, and a novel inhibitor to demonstrate a role for the phosphoinositide kinase, PIKfyve, in LDCV exocytosis from neurosecretory cells.

We have first demonstrated that PIKfyve mRNA and protein are present in neuroendocrine tissues, including rat brain and adrenal glands. In PC12 and chromaffin cells, PIKfyve is both cytosolic and associated with punctate structures. From our co-localization studies, it appears that there are multiple vesicular pools of PIKfyve in neurosecretory cells, including a pool co-localizing with P18 and Syt1 on LDCV. In PC12 cells, PIKfyve is dynamically regulated. Upon nicotine stimulation, there is a transient recruitment of PIKfyve from the cytosol to punctate structures, including LDCV. A previous study demonstrated an insulin-dependent relocation of PIKfyve from a cytosolic to low density membrane fraction biochemically (37). Thus, recruitment of PIKfyve to cellular membranes in response to extracellular stimuli may be an important regulator of membrane trafficking events.

The combination of pharmacological inhibitor, RNA interference knockdown, and overexpression evidence points to PIKfyve playing a negative role in regulated secretion. Indeed, overexpression of PIKfyve and its yeast orthologue, Fab1p, inhibits hGH secretion in PC12 cells, whereas acute treatment

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**TABLE 1**

Measurement of various parameters of PC12 cells early endosomes (EE) labeled with EEA1

Results of the image analysis shown are representative of the average ± S.E. of the indicated parameters.

| PC12 cells                      | Early endosomal parameters |
|---------------------------------|----------------------------|
|                                 | Area          | Length       | Width        |
|                                 | $\mu$m$^2$    | $\mu$m       | $\mu$m       |
| Untransfected (6 cells, 263 EE) | 0.169 ± 0.007 | 0.462 ± 0.017 | 0.325 ± 0.011 |
| Transfected PIKfyve(K1831M) (3 cells, 71 EE) | 0.434 ± 0.061 | 0.789 ± 0.069 | 0.622 ± 0.048 |
| Transfected PIKfyve-WT (3 cells, 83 EE) | 0.115 ± 0.002 | 0.370 ± 0.004 | 0.244 ± 0.004 |
| Untransfected YM-201636 (10 μm) (5 cells, 153 EE) | 0.513 ± 0.0194 | 0.871 ± 0.007 | 0.596 ± 0.024 |
| Untransfected YM-211387 (10 μm) (5 cells, 186 EE) | 0.155 ± 0.005 | 0.444 ± 0.010 | 0.312 ± 0.004 |

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with PIKfyve inhibitor and PIKfyve knockdown using siRNA both potentiated LDCV exocytosis. However, interfering with PIKfyve activity by overexpression of the kinase-inactive mutant of PIKfyve showed no effect on secretion. This could be related to the defects in endosomal morphology that occur upon overexpression of the mutant PIKfyve. In agreement with this, low concentrations of YM-201636 that potentiate exocytosis do not cause endosomal enlargement, whereas higher concentrations that do cause endosomal enlarging do not potentiate exocytosis.

Evidence from GLUT4 translocation would support this idea, since overexpression of a PIKfyve mutant unable to be phosphorylated by protein kinase B was shown to promote GLUT-4 vesicle exocytosis (25), whereas the PIKfyve(K1831E) kinase-inactive mutant was inhibitory for GLUT4 translocation (34, 44). This discrepancy was thought to arise as a side effect of the gross morphological defects caused by the PIKfyve kinase-inactive mutant (25). Altogether, our data point to an inhibitory role for PIKfyve catalytic activity in calcium-dependent LDCV exocytosis.

There are a number of possible explanations for the negative impact of PIKfyve on secretion. One possibility is that PIKfyve could help decrease the levels of PtdIns(3)P known to promote LDCV priming (12). In order to avoid too many LDCV reaching the primed state at a given time, ATP-dependent priming must be tightly controlled. Phosphorylation of PtdIns(3)P by PIKfyve to PtdIns(3,5)P2 would decrease PtdIns(3)P levels and as a result negatively regulate priming. PtdIns(3,5)P2 production itself might have a negative impact on secretion, potentially acting as a competitive antagonist for the phosphatidylinositol 4,5-bisphosphate effector Syt1. A recent study highlighted a role for Akt/protein kinase B in catecholamine release from chromaffin cells (45) and suggested that PIKfyve could play a role in this pathway (25). Further work is required to address these important points.

The negative effect of PIKfyve on exocytosis could also emanate from a trafficking effect. It has been proposed that the enlarged EEA1-positive structures visible in PIKfyve kinase-inactive-expressing cells at early time points are a result of an increased fusion of early endosomes, the role of endogenous PIKfyve therefore being inhibitory for early endosome fusion (22). However, RNA interference knockdown of PIKfyve, while resulting in a similar increase in size of EEA1-positive endosomes, led to the conclusion that PIKfyve positively regulates endosome-TGN trafficking where a decrease in this trafficking step leads to an accumulation of membrane in endosomes (23).

A similar conclusion was reached in a study investigating the role of protein kinase B phosphorylation of PIKfyve in 3T3-L1 adipocytes (25). A PIKfyve mutant that cannot be phosphoryl-

![FIGURE 7. Activity-dependent recruitment of PIKfyve-EGFP on LDCV.](image-url)

A, PC12 cells transiently transfected with PIKfyve-EGFP were visualized by confocal time lapse microscopy prior to and during nicotine (100 μM) stimulation. B, example of the time course of PIKfyve-EGFP recruitment on one identified organelle (the acquisition time is indicated below each image in min). The fluorescence intensity was assigned according to the indicated pixel fluorescence intensity scale. C, the time course of the PIKfyve-EGFP fluorescence intensity on identified organelles (% control) and in the cytosol (% control) were measured and analyzed (n = 6 regions of interest located either on organelles or in the cytosol; mean ± S.E.). D, peak fluorescence intensity at the maximum of nicotine effect was calculated as a percentage of control. *, p < 0.05 (Student’s t test, one-tailed). E, PC12 cells transiently transfected with PIKfyve-EGFP were stimulated with nicotine (100 μM), fixed, and processed for Syt1 immunoreactivity. F, the percentage of PIKfyve-positive organelles co-localizing with Syt1-positive LDCVs was quantified as described under “Experimental Procedures.” n = 9–10 cells examined/condition, 422–464 organelles analyzed/condition. Scale bar, 5 μm.
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FIGURE 8. Chromaffin cells were digitonin (20 μM)-permeabilized in the presence of ENTH domain (6 μg/ml) in KGEP buffer, washed, and fixed and processed for Syt1 immunostaining (A and B). A control experiment was carried out with the same concentration of preboiled ENTH (A). Confocal images of boiled (A) and unboiled (B) ENTH (green) and Syt1 (red) immunostaining of a single chromaffin cell. Partial co-localization (yellow) in the merge image can be observed (B). Co-localized pixels (>100 arbitrary units in both red and green channels) were highlighted (in white) in the optical sections highlighting the punctate nature of the co-localized area (B, bottom). Images are confocal z-sections taken every 1 μm; scale bar, 5 μm. Note that we consistently observed a nuclear ENTH staining.

PIKfyve Binding Is Sensitive to ENTH Domain

It is becoming increasingly clear that phosphoinositides can function in a number of different processes within the cell, depending on the site of local synthesis. For example, there is evidence that phosphatidylinositol 4,5-bisphosphate plays a role in coordinating both exocytosis and endocytosis at the synapse (10, 11, 13, 47), whereas PtdIns(3)P, a lipid best characterized for its role in early endosome function (48), has also been shown to be involved in lyso-phosphatidic acid-stimulated cell migration (49), ATP-dependent priming in neurosecretory cells (12), and translocation of GLUT4-containing vesicles to the plasma membrane (50, 51).

An important point was therefore to investigate whether PtdIns(3,5)P_2 is produced upon stimulation of exocytosis. We attempted to detect changes in global PtdIns(3,5)P_2 levels upon stimulation of exocytosis in ^32P-labeled cells by HPLC analysis. However, due to the relatively low levels of PtdIns(3,5)P_2, this analysis was unsuccessful. Possible reasons for this include the presence of phosphatases causing the turnover of PtdIns(3,5)P_2 or that PtdIns(3,5)P_2 may only be generated at a very restricted location with very little change in whole cell PtdIns(3,5)P_2 levels. Using the PtdIns(3,5)P_2-selective probe ENTH (from the yeast Ent3p), we show a selective binding to LDCV, suggesting that PtdIns(3,5)P_2 might indeed localize on a discrete subpopulation of chromaffin granules. Further study should address the dynamic role played by this pool of PtdIns(3,5)P_2 in LDCV exocytosis.

Our results indicate that PIKfyve may function at multiple trafficking steps in neurosecretory cells. As seen in nonneuronal cells, our results support an important role of PIKfyve in endosomal dynamics. However, PIKfyve also clearly localizes to a subpopulation of LDCV, a dynamic process that seems to be increased by stimulation of exocytosis. The combination of inhibitor and overexpression studies here demonstrates that PIKfyve also functions in the regulated exocytic pathway. Finally, many of the proteins and lipids involved in calcium-dependent exocytosis in neurosecretory cells also play a role in neurotransmitter release from small synaptic vesicles. The possibility that PtdIns(3)P, PtdIns(3,5)P_2, and their respective kinases may also be important for synaptic vesicle and/or dense core vesicle exocytosis in neurons remains to be addressed.

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