ARF6 Regulates the Synthesis of Fusogenic Lipids for Calcium-regulated Exocytosis in Neuroendocrine Cells*#S

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An important role for specific lipids in membrane fusion has recently been established, but regulation of their biosynthesis remains poorly understood. Among fusogenic lipids, phosphatidic acid and phosphoinositol 4,5-bisphosphate (PIP2) have been proposed to act at various steps of neurotransmitter and hormone exocytosis. Using real time FRET (fluorescence resonance energy transfer) measurements, we show here that the GTPase ARF6, potentially involved in the synthesis of these lipids, is activated at the exocytotic sites in PC12 cells stimulated for secretion. Depletion of endogenous ARF6 by siRNA dramatically inhibited secretagogue-evoked exocytosis. ARF6-siRNA greatly reduced secretagogue-evoked phospholipase D (PLD) activation and phosphatidic acid formation at the plasma membrane and moderately reduced constitutive levels of PIP2 present at the plasma membrane in resting cells. Expression of an ARF6-insensitive short interference RNA (siRNA) fully rescued secretion in ARF6-depleted cells. However, a mutated ARF6 protein specifically impaired in its ability to stimulate PLD had no effect. Finally, we show that the ARF6-siRNA-mediated inhibition of exocytosis could be rescued by an exogenous addition of lysophosphatidylcholine, a lipid that favors negative curvature on the inner leaflet of the plasma membrane. Altogether these data indicate that ARF6 is a critical upstream signaling element in the activation of PLD necessary to produce the fusogenic lipids required for exocytosis.

Release of neurotransmitters and hormones occurs through exocytosis, a highly regulated process that culminates with the fusion of secretory vesicles/granules and the plasma membrane. In the exocytotic process key proteins such as SNAREs have been shown to be fundamental players by providing the energy required to fuse membranes through the formation of high affinity, parallel, four-α-helix bundles. The role of lipids is less well understood, but recent findings indicate that the shape of the lipids (determined by the size of their head group) and their charge might also be important for the fusion process. In vitro cone-shaped lipids that spontaneously form negative membrane curvatures favor the formation of hemifusion intermediates and fusion when present in the contacting leaflets of apposed membranes. Accordingly, the lipid-modifying enzyme PLD, which produces cone-shaped PA, emerges as a major actor in various cellular processes that have in common membrane fusion. ARF6 in regulated exocytosis, using FRET measurements and ARF6 mutants has been reported to induce a profound redistribution of intracellular proteins and lipids, a finding that questions the physiological relevance of studies based on the use of mutated ARF6 proteins.

The present work was undertaken to assess the actual role of ARF6 in regulated exocytosis, using FRET measurements and RNA interference to reduce the level of the endogenous protein. We demonstrate that ARF6 is an essential element of the calcium-regulated exocytotic machinery and show that its

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2 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; PLD, phospholipase D; PA, phosphatidic acid; PABD, PA-binding domain; PIP2, phosphoinositol 4,5-bisphosphate; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; FRET, fluorescence resonance energy transfer; GH, growth hormone; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; siRNA, short
MATERIALS AND METHODS

Reagents, Antibodies, Plasmid, and Short Interference RNA—Palmitoyllyso phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). The following antibodies were used: monoclonal anti-ARF6 (Santa Cruz Biotechnology), anti-actin antibodies (Sigma), monoclonal anti-Ha (Covance), anti-GIT1 (28), anti-ARNO (29), rabbit polyclonal anti-human growth hormone (GH) antibodies (Dr. A. F. Parlow, National Institutes of Health, NIDDK, National Hormone and Pituitary Program, Torrance, CA), monoclonal anti-SNAP25 antibodies (Sterneberger), and monoclonal anti-PIP2 (Abcam). Alexa-label- ed secondary anti-mouse and anti-rabbit antibodies were obtained from Molecular Probes.

ARF6 was fused to the N-terminal part of ECFP into Xhol and KpnI sites of pECFPN1. MT2 was fused to the N-terminal part of EGFp or EYFP into EcoRI and Sall sites of pEGFPN1 and pEGFpN1. The PA-binding domain (PABD) of the yeast soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein Spo20p was fused to EGFP as described previously (12).

For short interference RNA (siRNA) targeting, rat ARF6 cDNA fragments encoding a 19-nucleotide siRNA sequence (siRNA#1, AATCTCATATCTGCACACAA; siRNA#2, gcgtgacgctattataatg; siRNA#3, GCACCGCATATCATATGACCG) 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 N-terminus of EGFP (siRNA#1, AATCCTCATCTTCGCCAACAA; siRNA#2, GACCG), derived from the target transcript and separated from its reverse complement by a short spacer, were annealed and cloned in the BglII and HindIII sites in front of the N-terminus of EGFP.

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to distinguish donor and acceptor fluorescence intensities as described previously (32).

Determination of PLD Activity—72 h after transfection, PC12 cells were washed four times with Locke’s solution and then incubated for 10 min in calcium-free Locke’s solution (basal PLD activity) or stimulated in Locke’s solution containing a depolarizing concentration of K⁺. Medium was then replaced by 100 µl of ice-cold 50 mM Tris, pH 8.0, and the cells were broken by three freeze/thaw cycles. Samples were collected and mixed with an equal amount of Amplex Red reaction buffer (Amplex Red phospholipase D assay kit, Molecular Probes), and the PLD activity was estimated after a 1-h incubation at 37 °C with a Mithras fluorometer (Berthold). A standard curve was established with purified PLD from Streptomyces chromofuscus (Sigma).

Immunofluorescence, Confocal Microscopy, and Image Analysis—Transfected PC12 were washed four times with Locke’s solution and then incubated for 10 min in Locke’s solution (resting conditions) or in Locke’s solution containing a depolarizing concentration of potassium (stimulation) before the fixation step and further processed for immunofluorescence as described previously (33, 34) except for PIP2 labeling. Briefly, for PIP2 staining cells were fixed on ice for 3 h in 4% paraormaldehyde-Dulbecco’s modified Eagle’s medium with 2 mM EGTA, washed three times in 50 mM NH₄Cl-phosphate-buffered saline, and permeabilized for 4 h at 4 °C in permeabilization buffer (1 mM MgCl₂, 0.2% saponin, 1% fetal calf serum, 0.1% bovine serum albumin, 50 mM glycine, 0.05% NaN₃ in phosphate-buffered saline). Incubation with anti-PIP2 antibody (1/50) in permeabilization buffer was performed overnight at 4 °C and then with the secondary antibody for 2 h at 4 °C. Stained cells were visualized using a Zeiss LSM 510 confocal microscope. Quantification was performed using Zeiss CLSM instrument software, version 3.2. The percentage of the EGFP/PABD binding probe co-localizing at the plasma membrane with SNAP25 was measured by determining the double-labeled pixels, expressed as average fluorescence intensity, and then converted to a percentage of the total fluorescence obtained from the two labels in each cell.

The amount of PIP2 staining was measured and expressed as the average fluorescence intensity (mean pixel intensity multiplied by the pixel number) normalized to the corresponding surface area by dividing by the total plasma membrane surface of each cell. We performed a quantitative cell-to-cell comparison in the same field (fluorescence from nontransfected cells compared with that of transfected cells expressing GFP). The PIP2 level in the nontransfected cells was fixed at 100%. To validate the specificity of the anti-PIP2 antibody and our analysis, we used the recently described drug-inducible type IV 5-phosphatase to reduce PIP2 levels in PC12 cells (35). Quantification revealed that PIP2 staining was almost completely eliminated (supplemental Fig. 1). On the other hand, overexpression of PIP5K induced an increase in PIP2 staining of up to 6-fold (supplemental Fig. 1). In conclusion, these data confirm that we were able to detect variation in PIP2 staining over a large scale and that the PIP2 antibody used was highly specific.

Data Analysis—In all figures, data are given as the mean values ± S.E. obtained from at least three independent experiments performed on different cell cultures, where n represents the number of experiments. Data were analyzed with Minitab® statistical software. Statistical significance was established using Student’s test, and data were considered significantly different when the p value was less than 0.05. Gaussian distributions of the data were verified.

RESULTS

Endogenous ARF6 Is Activated during Calcium-regulated Exocytosis—To visualize the activated form of ARF6 in cells, we adapted a recently described probe shown to interact specifically with GTP-bound ARF6 in vitro and in a yeast two-hybrid assay (36). MT2 (metallothionein-2), a ubiquitous 6–7-kDa metal-containing protein, was cloned from PC12 cells and expressed as MT2-GFP fusion protein. In resting PC12 cells, this ARF6-GFP sensor was accumulated in the nucleus and also moderately in the cytosol (Fig. 1A). Stimulation with a depolarizing concentration of potassium led to a reduction of cytosolic...
MT2-GFP and a concomitant recruitment of the probe to the cell periphery where it co-localized with the plasma membrane marker SNAP25 (Fig. 1A). Quantification revealed that more than 10 ± 1.2% of the MT2-GFP signal co-localized with SNAP25 in stimulated PC12 cells, compared with less than 1% under resting conditions. Accumulation of the ARF6-GTP sensor at the cell periphery was transient and rapidly became undetectable when the cells were placed in a resting condition (data not shown). Similar observations were made in chromaffin and PC12 cells stimulated with various secretagogues, such as nicotine, ATP, or barium (data not shown). These data suggest that endogenous ARF6 is activated transiently at the plasma membrane in cells stimulated for exocytosis.

To assess the dynamics of ARF6 activation in living cells, next we decided to use FRET measurements. For this purpose the fluorophore pair ECFP-EYFP, which has been shown previously in several applications to act as a donor/acceptor pair with a Förster radius of ~50 Å, was chosen. PC12 cells were cotransfected to express ARF6-ECFP and MT2-EYFP as donor and acceptor fluorophores. In resting cells, excitation at 430 nm (the excitation wavelength of ECFP) induced an emission spectrum displaying two fluorescence intensity peaks at 495 and 525 nm characteristic of ARF6-ECFP and MT2-EYFP, respectively (Fig. 1B). Bleaching of each fluorophore was estimated in monotransfected cells, and these values were used to correct for bleaching in cotransfected cells. Following cell stimulation with high potassium, ARF6-ECFP fluorescence decreased and MT2-EYFP increased concomitantly (Fig. 1B). We quantified this effect after spectral deconvolution in cells stimulated for different periods of time. As illustrated in Fig. 1C, FRET occurred between ARF6-ECFP and MT2-EYFP in stimulated PC12 cells. Accordingly, immunofluorescence images confirmed that ARF6-ECFP and MT2-EYFP co-localized at the cell periphery in stimulated cells (data not shown). An optimal FRET signal was detected between 45 s and 2 min of stimulation, which correlates well with the time course of GH secretion elicited by high potassium stimulation (compare Figs. 1C and 3A). Taken together, these data indicate that MT2 interacts with ARF6 in the cell periphery of secretagogue-stimulated cells and reveal that activation of ARF6 occurs at the plasma membrane prior to exocytosis and hormone secretion.

Characterization of ARF6-siRNA and Effect on Exocytosis in PC12 Cells—To probe the functional importance of ARF6 in regulated exocytosis, we used a siRNA approach to knock down endogenous ARF6 expression in PC12 cells. To accomplish this and simultaneously enable a quantitative assessment of exocytosis, we engineered plasmids that express both full-length human GH and various siRNA targeted against the sequence of ARF6. Because GH is stored in secretory granules and released by exocytosis, they allow a direct measurement of the secretory activity of siRNA-expressing cells (28, 30). These plasmids also allow the identification of the subpopulation of cells that transiently express siRNAs through the immunostaining of GH. Western blot analysis revealed that transient expression of different ARF6-siRNAs reduced the level of endogenous ARF6, whereas actin levels were constant (Fig. 2A). Densitometry scans from three independent experiments indicated that the level of ARF6 was reduced by about 55 ± 5%, whereas a mutated ARF6-siRNA had no effect. When normalized to the transfection efficiency as evaluated by GH immunostaining, the level of ARF6 in PC12 cells expressing the siRNAs was reduced by up to 91 ± 4% (Fig. 2B). In agreement with their specificity, the three different ARF6-siRNAs did not affect the level of expression of the related isoform ARF1 (Fig. 2A). We also verified that the different ARF6-siRNAs did not affect the expression of levels of the guanine nucleotide exchange factor ARNO and GTPase-activating protein GIT1 for ARF6 in PC12 cells (28, 29).

Because the amount of activated ARF6 may be relatively low even in stimulated cells (37), the residual endogenous ARF6 expressed in siRNA-transfected cells may be sufficient to provide levels of active ARF6 similar to that found in control cells. To check this possibility, the MT2 probe fused to GST was used to pull down the GTP-bound form of HA-tagged ARF6 (expressed at low level) in cells expressing the various ARF6-siRNAs. As observed for the endogenous ARF6 protein, HA-ARF6 was significantly activated in control cells stimulated with high K+ (Fig. 2C). Expression of the ARF6-siRNAs strongly reduced the amount of GTP-loaded HA-ARF6 in stimulated cells by up to 90% as revealed by Western blot quantification (Fig. 2D). Thus, ARF6-siRNAs not only reduced the total level of endogenous ARF6 but also the amount of activated ARF6 found in stimulated cells. To further substantiate these findings, we measured the recruitment of the MT2-GFP probe to the cell periphery and used it as an index for ARF6 activation at the plasma membrane. By quantifying the co-localization of MT2-GFP with SNAP25, we found that expression of the ARF6-siRNA#1, but not the mutated siRNA, strongly reduced the translocation of MT2 to the plasma membrane (Fig. 2, E and F), confirming that ARF6-siRNA was able to prevent the formation of active ARF6 at the plasma membrane in cells stimulated with a secretagogue.

Expression of the ARF6-siRNA#1 silencer in PC12 cells inhibited GH release evoked by increasing periods of stimulation (Fig. 3A). ARF6 silencing did not modify basal GH release, but it resulted in a strong decrease (~75% inhibition) in the amount of GH secreted during a 10-min exposure to 59 mM K+ (Fig. 3A). Similar observations were made with the two other ARF6-siRNAs (data not shown), whereas the mutated siRNA sequence that did not affect endogenous ARF6 levels also had no effect on secretion (Fig. 3A). In parallel we verified that the expression of ARF6-siRNAs neither reduced the expression level of GH nor affected the distribution of GH-containing secretory vesicles (supplemental Fig. 2).

The signaling pathway that leads to secretagogue-evoked rise of cytosolic calcium can be bypassed by permeabilizing the plasma membrane and directly controlling calcium concentration (38). GH secretion was triggered by the presence of 20 μM free calcium in the incubation medium of digitonin-permeabilized PC12 cells (Fig. 3B). The three ARF6-siRNAs potently inhibited GH release from permeabilized cells (Fig. 3B), indicating that their inhibitory effect on secretion was not the consequence of a reduction in cytosolic calcium rise but was linked directly to a defect in the exocytotic machinery. These results are in line with the idea that ARF6 is a major positive regulator of calcium-regulated exocytosis.
Depletion of Endogenous ARF6 Moderately Affects PIP₂ Level but Strongly Inhibits PLD Activation and PA Production in PC12 Cells Undergoing Exocytosis—In light of the known regulation of PI5PK activity by ARF6, we examined whether PIP₂ levels were affected in PC12 cells expressing ARF6-siRNA using either the GFP-PH-PLC/H9254 probe (data not shown) or specific anti-PIP₂ antibodies to detect endogenous PIP₂. In line with previous reports, PIP₂ essentially was detected at the plasma membrane in PC12 cells (Fig. 4A), and stimulation with a secretagogue apparently did not modify its cellular level and distribution (data not shown). We found that depletion of ARF6 resulted in a moderate −10–30% decrease of PIP₂ levels present at the plasma membrane in resting PC12 cells (Fig. 4). However, only the effect of the ARF6-siRNA#3 was found to be statistically significant (Fig. 4B).

ARF6 is also a well known activator of PLD1 (17, 18), an essential lipid-modifying enzyme for the exocytotic fusion reaction (6, 12). On the basis of the results obtained using mutated ARF6 proteins impaired in their ability to activate selected effectors, we had proposed previously that the main function of ARF6 during exocytosis is linked to the regulation of PLD1 activity (26). This hypothesis predicts that a reduction of endogenous ARF6 levels should affect secretagogue-evoked PLD activation. To probe this possibility, PLD activity was measured in homogenates prepared from resting and K⁺-stimulated PC12 cells expressing the various ARF6-siRNAs. In line with our previous reports (12), stimulation with high K⁺ triggered a marked activation of PLD (Fig. 5A). Down-regulation of endogenous ARF6 by siRNA expression resulted in a 60–70% inhibition of K⁺-induced PLD activity in cell homogenates. In contrast, the mutated ARF6-siRNA had no effect on PLD activity (Fig. 5A). In support of the specific involvement of ARF6 in PLD activation in stimulated neuroendocrine cells, depletion of ARF1 by
siRNA did not significantly modify the secretagogue-induced PLD activity (supplemental Fig. 3).

Activated PLD produces PA that can be visualized by using the PA-binding domain of the yeast homologue of SNAP25, Spo20p, fused to GFP (12). In secretagogue-stimulated PC12 cells, this PA sensor is recruited to the plasma membrane and reveals the PLD1-mediated formation of PA at the sites of exocytosis (12). To further assess the importance of ARF6 in the upstream signaling pathway of PLD1, we examined the distribution of the PABD-GFP PA sensor in cells expressing ARF6-siRNAs. In these experiments, PC12 cells were first transfected with a plasmid driving the expression of GH alone or GH and the ARF6-siRNA and then, 48 h later, transfected with the PABD plasmid. As shown in Fig. 4B, the PA sensor was recruited to the plasma membrane in stimulated cells where it co-localized with SNAP25, in agreement with our previous results (12). Expression of ARF6-siRNAs, revealed by the presence of GH, largely inhibited the recruitment of PABD at the cell periphery (Fig. 5B). We quantified the percentage of PABD co-localizing with SNAP25 under resting and stimulated conditions and found that ARF6-siRNA was almost as efficient as PLD1-siRNA in inhibiting the recruitment of PABD to the plasma membrane in K\(^+\)/H11001-stimulated PC12 cells (Fig. 4C). Note that PLD2-siRNA did not affect the translocation of PABD to the cell periphery (Fig. 4C). These results confirm that PLD1 is the major source of PA synthesis at the plasma membrane in cells stimulated for exocytosis. They also suggest an absolute requirement for ARF6 activation, as PA is not formed at the sites of exocytosis in cells expressing PLD1 but is depleted in ARF6.

A Mutant of ARF6 That Is Unable to Activate PLD Does Not Rescue GH Secretion from ARF6-depleted PC12 Cells—We previously characterized the ARF6(N48I) mutant, which is selectively unable to activate PLD while retaining its GTP-binding and GDP-dissociation characteristics, and its ability to be activated by ARNO and inactivated by GIT1 (26). ARF6(N48I) is still able to activate in vitro any known ARF6 effectors such as cholera toxin, and based on the analogous ARF1 mutant, one...
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FIGURE 5. siRNA-mediated ARF6 knockdown specifically inhibits secretagogue-evoked PLD1 activation and PA synthesis at the plasma membrane in PC12 cells. A, PC12 cells were transfected with the pGHsuper, the mutated (Mut) siRNA#1, or the pGHsuper-ARF6-siRNA (siRNA#1–3) vectors. 72 h after transfection, cells were stimulated for 10 min with 59 mM K+/H11001 or maintained under resting condition and processed for PLD activity assay. K+-dependent PLD activity is obtained by subtracting the PLD activity detected in cells maintained in calcium-free Locke’s solution. Data are given as the mean values ± S.E. obtained in three experiments performed on three different cell cultures *, p ≤ 0.001 (n = 3). B, PC12 cells cotransfected with pEGFP-PABD and the pGHsuper, mutated siRNA#1, or pGHsuper-ARF6-siRNA (siRNA#1–3) vector were stimulated for 10 min with 59 mM K+ and processed for GH and SNAP25 staining. Mask images obtained by selecting double-labeled pixels show the areas of co-localization of PABD-GFP with SNAP25. Bar, 5 μm. The arrows highlight cells that do not express ARF6-siRNA#1, as seen by the absence of GH in which PABD is recruited to the plasma membrane.

C, histogram presenting a semiquantitative analysis of the percentage of PABD that co-localizes with SNAP25 in resting and stimulated cells expressing ARF6-siRNA, PLD1-siRNA, or PLD2-siRNA. Data are presented as the mean values ± S.E. obtained in three independent experiments. *, p ≤ 0.001 (n = 3 with more than 75 cells analyzed per condition).

can assume that it also stimulates PIP5K (39, 40). Expression of a wobble-mutated form of ARF6 insensitive to the ARF6-siRNA was able to completely rescue K+-evoked GH release in ARF6-depleted cells (Fig. 6). However, expression of a siRNA-resistant ARF6(N48I) construct failed to rescue GH secretion resulting from ARF6 depletion (Fig. 6). These results indicate that the ability of ARF6 to activate PLD is essential for secretion rescue in ARF6-depleted cells, reinforcing the idea that the main function of ARF6 in the exocytotic pathway is the activation of PLD1 at the plasma membrane.

Exocytosis in ARF6-depleted Cells Can Be Rescued by the Addition of an Inverted Cone-shaped Lipid to the Outer Plasma Membrane Leaflet—Lipids have been proposed to play a decisive role in the late post-docking stages of exocytosis. It has been suggested that once a secretary granule is juxtaposed to the plasma membrane through the formation of SNARE complexes, pore formation, and eventually expansion, proceeds through the progressive formation of a granule/plasma membrane stalk and a lipid zipping process that generates hemifusion intermediates (4). Cone-shaped lipids like PA at the juxtaposed membrane leaflets reduce the energy requirements for the curvature process and promote the formation of the hemifusion intermediates. Conversely, inverted cone-shaped lipids present in the outer leaflet of the plasma membrane should bend the outer membrane inward and similarly promote hemifusion. In light of this assumption, we attempted to rescue the secretory activity in ARF6-depleted cells, by challenging them with external application of the inverted cone-shaped lipid LPC. Control PC12 cells or cells expressing various siRNA were incubated in the presence of 1 μM LPC and then stimulated with high K+. Under our experimental conditions, LPC did not modify basal or K+-stimulated GH release from control cells (Fig. 7). In line with our previous report (12), the addition of LPC rescued K+-evoked GH secretion from PLD1-depleted cells, indicating that exogenous LPC was able to compensate for the decreased production of PA on the inner leaflet of the plasma membrane because of PLD1 knockdown. In other words, exogenous LPC is able to mimic the biophysical modifications of the plasma membrane induced by the PLD1-dependent production of PA. The addition of LPC also rescued K+-evoked GH secretion in cells expressing ARF6-siRNA (Fig. 7). Thus, external LPC promotes exocytosis in ARF6-depleted cells, supporting the idea that endogenous ARF6 is an activator for the plasma membrane-bound PLD1 to produce fusogenic PA at the exocytotic sites.

DISCUSSION

Over the last decade, accumulating evidence has suggested the implication of ARF6 in various membrane trafficking events including endocrine and neuroendocrine exocytosis (41). The downstream effector(s) of ARF6 in these processes remain, however, uncertain, albeit linked to the regulation of lipid synthesis. Because most of the previous studies have employed ARF6 mutants, which are now recognized to artificially disturb the distribution of cellular lipids and SNARE proteins (27), we decided to use an RNA interference strategy to investigate the function of ARF6 in calcium-regulated exocytosis. We found that depletion of endogenous ARF6 profoundly reduces the
Perfused on different cell cultures, *, translated for 10 min with 59 mM K

Cells were incubated for 10 min in Locke’s solution ([H11006]), or pGSuper-ARF6-siRNA#1 were cotransfected with pXS, pXS-ARF6-rescue (ResARF6-WT), or pXS-ARF6-rescue(N48I) (ResARF6-N48I), 72 h after transfection, cells were incubated for 10 min in Locke’s solution (Resting) or stimulated for 10 min with 59 mM K⁺ in the presence or absence of 1 µM LPC. GH release is expressed as the percentage of total GH present in the cells before stimulation. Data are presented as the mean values ± S.E. obtained from four independent experiments performed on different cell cultures (*, p = 0.005 (n = 3).

Exocytotic activity of PC12 cells at a step distal to calcium entry. This inhibition of secretion appears to be correlated with a strong reduction of PLD activity in stimulated cells and more specifically to the absence of PA synthesis at the plasma membrane. Moreover, we show that external agents that promote membrane bending are able to compensate for the inhibition of secretion resulting from ARF6 deficiency.

The calcium-triggered merger of two apposed membranes is the defining step of regulated exocytosis. To date, fusion of two lipid bilayers has been modeled as a series of membrane intermediates (4, 42). Firstly membranes are brought into close proximity to establish a region of dehydrated contact at the initial contact point. A fusion stalk forms and then radial expands to yield a hemifusion diaphragm, while distal membrane leaflets remain separate. Different lipids such as negative curvature-forming lipids greatly reduce the energy required to overcome the hydration layer, and promote stalk formation and hemifusion when present in the contacting leaflets (3). PA under physiological conditions has been shown to generate spontaneous negative membrane curvature (43). In neuroendocrine cells, morphometric ultrastructural analysis reveals that PA accumulates at the plasma membrane granule docking sites upon cell stimulation with a secretagogue (12). Interestingly, SNARE syntaxin 1A recently has been reported to bind PA (44). Neutralization of the juxtamembrane PA-binding region of syntaxin 1A results in a delay in fusion pore expansion and a decrease in fusion pore diameter (44), in line with the idea that PA is an essential lipid for membrane fusion. Additionally, these findings suggest that syntaxin 1A may to some extent contribute to localizing PA at the fusion site (i.e. where the granules dock at the plasma membrane) (44).

We demonstrated previously that PLD1 is the only source for secretagogue-dependent PA formation at the plasma membrane (12). We show here that depletion of endogenous ARF6 prevents normal PLD1 activation and inhibits exocytosis, but the addition of an inverted cone-shaped lipid, such as LPC, on the outer leaflet of the plasma membrane compensates for the absence of ARF6 and restores exocytosis. In other words, knockdown of ARF6 produces a phenotype that is similar to the previously described PLD1 knockdown, which is also rescued by an exogenous supply of inverted-cone shaped lipids (12). These data support the idea that the reduced exocytotic activity observed in ARF6-depleted cells is primarily the consequence of the lack of synthesis of negative curvature-forming lipids at the inner leaflet of the plasma membrane. Accordingly, exocytosis in ARF6-depleted cells is not restored by ARF6(N48I), a mutant that is unable to overcome the hydration layer, and promote stalk formation and hemifusion when present in the contacting leaflets (3). PA under physiological conditions has been shown to generate spontaneous negative membrane curvature (43). In neuroendocrine cells, morphometric ultrastructural analysis reveals that PA accumulates at the plasma membrane granule docking sites upon cell stimulation with a secretagogue (12). Interestingly, SNARE syntaxin 1A recently has been reported to bind PA (44). Neutralization of the juxtamembrane PA-binding region of syntaxin 1A results in a delay in fusion pore expansion and a decrease in fusion pore diameter (44), in line with the idea that PA is an essential lipid for membrane fusion. Additionally, these findings suggest that syntaxin 1A may to some extent contribute to localizing PA at the fusion site (i.e. where the granules dock at the plasma membrane) (44).

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PPIP2 is required for secretion in neuroendocrine cells (27, 46, 47), even though PIP2 levels at the plasma membrane do not seem to increase upon stimulation with secretagogues. It has been proposed that PIP2 regulates the size of the releas-
able pool of secretory granules in chromaffin cells, because a reduction in PIP₂ levels affects directly and dynamically secretion (47). However, inhibition of the secretory response was observed only in cells that had nearly completely lost their constitutive levels of PIP₂ at the plasma membrane (47). Whether the mild reduction of PIP₂ observed in ARF6-depleted cells significantly contributes to the inhibition of exocytosis remains difficult to estimate. Effectively, a small reduction in PIP₂ may affect the number of granules docked at the plasma membrane or the distribution of the numerous PIP₂-binding proteins involved in regulated exocytosis. PLD1 itself requires PIP₂ for its association with the plasma membrane in stimulated cells (48), but we observed no apparent difference in the distribution of PLD1 in cells having reduced levels of ARF6 (data not shown). Finally, the observation that secretion can be almost fully restored in ARF6-depleted PC12 cells by the exogenous addition of inverted cone-shaped lipids is a strong argument for a late post-docking role for ARF6 in exocytosis. PIP₂ has been described recently as an essential lipid that recruits protein-priming factors with secretory granules inchromaffin cells, because a critical reading of the manuscript. We thank Dr. P. Chavrier for technical assistance and Drs. N. Grant and S. Chasserot-Golaz for critical reading of the manuscript. We thank Dr. P. Chavrier for sharing valuable preliminary information on ARF6-siRNA and Dr. Tamas Balla for the 5-phosphatase plasmid. We also acknowledge the confocal microscopy facilities of la plateforme “Imagerie in Vitro” of IFR 37.

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