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Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane

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The ADP ribosylation factor (ARF) GTP binding proteins are believed to mediate cytoskeletal remodeling and vesicular trafficking along the secretory pathway. Here we show that ARF6 is specifically associated with dense-core secretory granules in neuroendocrine PC12 cells. Stimulation with a secretagogue triggers the recruitment of secretory granules to the cell periphery and the concomitant activation of ARF6 by the plasma membrane-associated guanine nucleotide exchange factor, ARF nucleotide binding site opener (ARNO). Expression of the constitutively inactive ARF6(T27N) mutant inhibits secretagogue-dependent exocytosis from PC12 cells. Using a mutant of ARF6 specifically impaired for PLD1 stimulation, we find that ARF6 is functionally linked to phospholipase D (PLD)1 in the exocytotic machinery. Finally, we show that ARNO, ARF6, and PLD1 colocalize at sites of exocytosis, and we demonstrate direct interaction between ARF6 and PLD1 in stimulated cells. Together, these results provide the first direct evidence that ARF6 plays a role in calcium-regulated exocytosis in neuroendocrine cells, and suggest that ARF6-stimulated PLD1 activation at the plasma membrane and consequent changes in membrane phospholipid composition are critical for formation of the exocytotic fusion pore.

Introduction

Calcium-triggered release of hormones and peptides from neuroendocrine cells is mediated by the fusion of large, dense-core secretory granules with the plasma membrane, a spatially and temporally controlled process based on a complex network of interactions between regulatory molecules and mechanical components. Cytoskeletal rearrangements are a prerequisite for exocytosis. Actin filaments form a cortical network that separates secretory granules into a small release-ready granule pool and a larger reserve pool, subjecting the transit of granules to the plasma membrane to a carefully orchestrated regulation (Trifaro et al., 2000). Classically viewed as a barrier that hinders the movement of reserve granules to the plasma membrane (Aunis and Bader, 1988), it is now believed that the actin cortex provides also tracks along which the granules are pulled to the sites of exocytosis (Lang et al., 2000). Once docked at the plasma membrane, granules undergo membrane fusion through a mechanism governed by SNAREs (Jahn and Südhof, 1999; Chen and Scheller, 2001). The role of the membrane lipid composition at the site of fusion is less well understood, but several reports suggest that lipids are essential partners for proteins in the basic fusion machinery (Schmidt et al., 1999; Weigert et al., 1999; Vitale et al., 2001). A specific feature of the exocytotic release in secretory cells is its tight regulation, as the speed, length, and number of exocytotic events must be controlled. Thus, additional proteins able to link and coordinate the successive recruitment, docking, and fusion steps into the integrated secretion machinery are necessarily required but most of them remain to be identified.

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ADP ribosylation factor (ARF)* GTPases play important roles in membrane trafficking events (Moss and Vaughan, 1998; Donaldson and Jackson, 2000). ARF6, the most structurally divergent member of the family (Chavrier and Goud, 1999), is found exclusively at the plasma and/or endosomal membranes in various cells types (Peters et al., 1995; Cavenagh et al., 1996). The protein has been proposed to play a role in cell motility (Song et al., 1998; Santy and Casanova, 2001), vesicle recycling at the plasma membrane (Radhakrishna and Donaldson, 1997; D'Souza-Schorey et al., 1998), Fc-mediated phagocytosis (Zhang et al., 1999a) and insulin-regulated secretion (Yang and Muekler, 1999), and Glut-4 translocation (Millar et al., 1999). Taken together, these findings suggest that ARF6 is an ubiquitous regulator of vesicle transport at the plasma membrane.

ARF6 is unique among the six mammalian ARFs in its ability to stimulate cortical actin rearrangements (Radhakrishna et al., 1999; Boshans et al., 2000). Because ARF6 also activates phospholipase D (PLD; Massenburg et al., 1994), a lipid-modifying enzyme recently described as a key factor for exocytosis in neurons and neuroendocrine cells (Humeau et al., 2001; Vitale et al., 2001), it would be attractive to hypothesize that ARF6 functions by coordinating reorganization of the actin cytoskeleton with the lipid modifications required for neuronal exocytosis. We demonstrate here that ARF6 is the sole member of the ARF family able to exert direct control on the exocytotic machinery. We show that ARF6 is in its inactive GDP-bound state when associated with secretory granules but then switches to its active GTP-bound state at the plasma membrane upon cell stimulation. Our results indicate that the guanine nucleotide exchange factor, ARF nucleotide binding site opener (ARNO), is a regulator of ARF6 functions in this setting. Finally, we show that ARF6 participates in the regulation of secretion, most likely through the direct activation of PLD1 at the site of exocytosis.

Results

Secretagogue-evoked stimulation activates ARF6 at the plasma membrane

To probe the role of ARF proteins in dense-core granule exocytosis, we first investigated their intracellular distribution. The relatively low level of endogenous ARFs and the difficulty of generating antibodies able to discriminate between the six members of the ARF family (which share ~70% identity), prompted us instead to visualize them using overexpression of epitope-tagged isoforms. PC12 cells were cotransfected with an HA-tagged representative of each ARF class and with growth hormone (GH) to visualize dense-core secretory granules (Caumont et al., 2000; Vitale et al., 2001). ARF1, ARF3, and ARF5 are concentrated in the perinuclear region (Fig. 1), which contains the Golgi as identified with the pECFP-Golgi marker (unpublished data). Neither ARF1, ARF3, nor ARF5 colocalized with the GH-labeled secretory granules distributed in the rest of the cytoplasm (Fig. 1). In contrast, ARF6 colocalized both with GH (Fig. 1) and GFP–chromogranin A (unpublished data) throughout the cell, indicating that it represents most likely the sole ARF protein associated with mature secretory granules in PC12 cells.

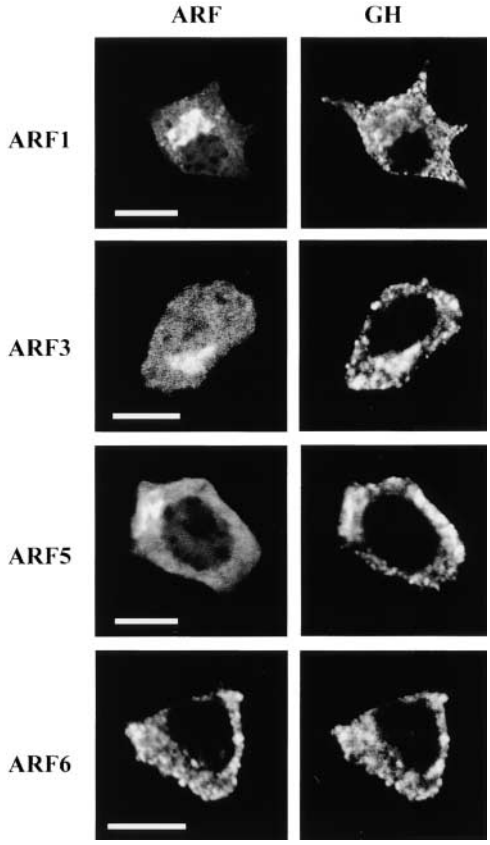

Figure 1. **Subcellular localization of overexpressed ARF1, ARF3, ARF5, and ARF6 in PC12 cells.** PC12 cells cotransfected with GH and the indicated HA-tagged ARF constructs were stained with either monoclonal anti-HA antibodies (for ARF1, ARF3, and ARF5) or rabbit anti-ARF6 antibodies. Rabbit or monoclonal anti-GH antibodies were used to stain secretory granules in transfected cells. Bars, 5 μm.

We then compared the distribution of overexpressed WT and mutant ARF6 in resting and stimulated PC12 cells using in addition anti-SNAP25 antibodies to visualize the plasma membrane. In resting cells, WT ARF6, the inactive ARF6(T27N) mutant unable to bind GTP, and the constitutively active ARF6(Q67L) mutant unable to hydrolyze GTP (Peters et al., 1995) associated with GH-containing secretory granules (Fig. 2). Membrane depolarization by high potassium concentrations initiates calcium-dependent exocytosis in PC12 cells. Accordingly, a 5-min stimulation with 59 mM K^+ triggered a partial movement of secretory granules to the cell periphery and the docking of a portion of the granules at the plasma membrane, as indicated by the increased colocalization of GH with SNAP-25 (Fig. 2, mask GH/SNAP-25). WT and mutant ARF6 displayed a staining pattern very similar to GH in K^+-treated cells, indicating that ARF6 accompanied the secretory granules to the periphery (Fig. 2, mask ARF6/SNAP-25). Immunoelectron microscopy confirmed the association of ARF6 with the plasma

*Abbreviations used in this paper: ARF, ADP ribosylation factor; ARNO, ARF nucleotide binding site opener; GH, growth hormone; PA, phosphatidic acid; PLD, phospholipase D.

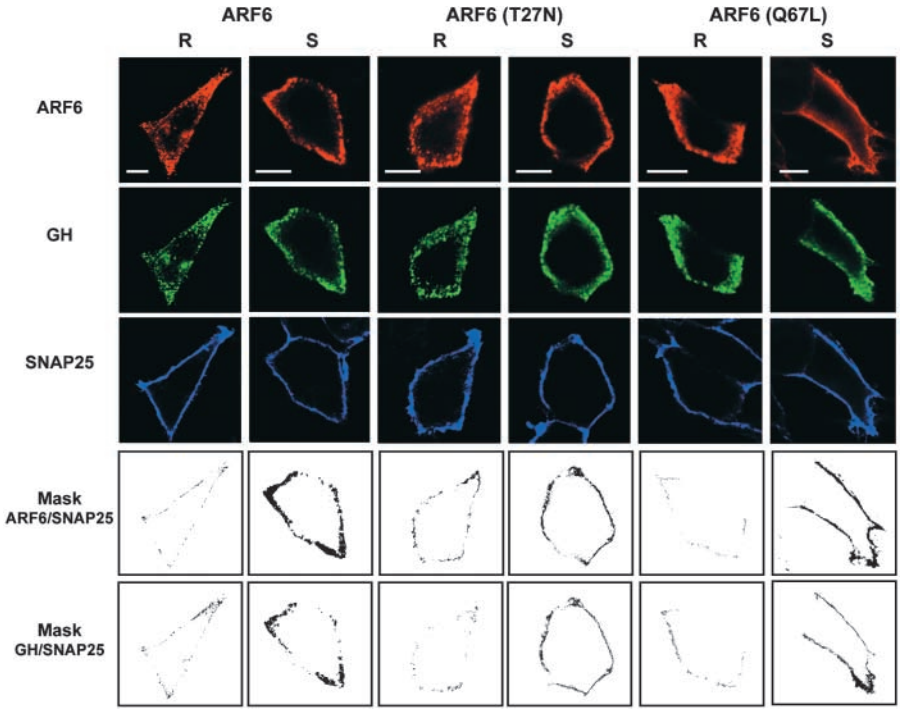

Figure 2. **Distribution of WT and mutated ARF6 proteins in resting and stimulated PC12 cells.** PC12 cells were transfected with pXS-ARF6, pXS-ARF6(T27N), or pXS-ARF6(Q67L) in combination with the GH expression plasmid. 48 h after transfection, cells were stimulated for 5 min with 59 mM K^+ (S) or maintained under resting conditions (R) and then processed for immunocytochemistry. Cells were stained with monoclonal anti-GH and rabbit polyclonal anti-ARF6 antibodies, postfixed, and then stained with monoclonal anti-SNAP25 antibodies. Confocal images were recorded in the same optical section by a triple exposure procedure. Masks representing the regions of colocalization (ARF6/SNAP-25 or GH/SNAP-25) were generated by selecting the double-labeled pixels. Bars, 5 μm.

membrane after stimulation with elevated K^+ (Fig. 3). ARF6 was detected not only on secretory granules within the cytoplasm and on granules docked at the plasma membrane, but also on portions of the plasma membrane seemingly free of granules. However, coated pits and vesicles were not decorated by gold particles, suggesting that ARF6 does not undergo clathrin-dependent endocytosis in this cell type.

To correlate the intracellular distribution of ARF6 with its nucleotide status, we used a biochemical approach that in the first phase offers an alternative method to probe ARF6 subcellular distribution: PC12 cells expressing WT or mutated ARF6 were incubated with [^{32}P]orthophosphate to label the ARF6-bound nucleotide, subjected to subcellular fractionation, and assayed for ARF6 distribution using immunoprecipitation and quantitation of the coprecipitated labeled nucleotide. As illustrated in Fig. 4 B, the labeled nucleotide was not recovered from the cytosol, signifying, as expected, that ARF6 is membrane associated. Instead, under resting conditions, most of the labeled guanine nucleotides were immunoprecipitated from the secretory granule fractions (Fig. 4 B), in agreement with the granule association of ARF6 observed by confocal and electron microscopy. However, stimulation with elevated K^+ increased the amount of labeled guanine nucleotides detected in the plasma membrane fraction at the expense of the granule fraction (Fig. 4 B), consistent with the immunofluorescent results shown in Fig. 2 and the Western blot results in Fig. 4 A. This redistribution was exaggerated by ARF6(Q67L) and blunted by ARF6(T27N). Note that endogenous ARF6 similarly translocated from secretory granules to the plasma membrane upon cell stimulation (Fig. 4 A). The observation of ARF6 at the plasma membrane in stimulated cells could result from the fusion of granules into the plasma membrane at the sites of exocytosis. In support of this hypothesis, dopamine–β-hydroxylase, a granule membrane protein, could be detected in the plasma membrane-containing fraction when the membrane fractions were prepared from stimulated cells (Fig. 4 A). However, note the low level of GH in this fraction (Fig. 4 A), suggesting that most of the granule membrane recovered with the plasma membrane originated from granules that had already released their content. ARF6 could also directly interact with a plasma membrane–bound protein after granule docking, and thereby be retained at the plasma membrane upon cell fractionation.

In a second phase, this method permits the nucleotide status of ARF6 in resting and stimulated cells to be assessed by analyzing on chromatography plates the guanine nucleotides bound to the immunoprecipitated WT and mutant ARFs. As shown in Fig. 5, in resting cells, only GDP was found to be associated with the ARF6 on secretory granules, and this was true even for ARF6(Q67L) which exhibits a dramatic preference for GTP. Some ARF6(Q67L) was also detected on the plasma membrane in resting cells (Fig. 4 B), but it was found to be loaded both with GDP and GTP (Fig. 5). Stimulation with K^+ triggered a partial translocation of the ARF6s to the plasma membrane, but only WT ARF6 and ARF6(Q67L) switched to their active GTP-bound state. 97% of the ARF6(T27N) remained in the GDP-bound state despite its association with the plasma membrane (Fig. 5). Taken together, these results indicate that the secretory granule–associated ARF6 protein is in its inactive GDP-bound form. Stimulation with a secretagogue triggers the activation of ARF6 but the GDP to GTP exchange occurs only at or after arrival to the plasma membrane.

ARNO activates ARF6 at the plasma membrane in stimulated PC12 cells

In chromaffin cells, ARNO, a nucleotide exchange factor for ARF proteins (Chardin et al., 1996; Frank et al., 1998b), is localized at the plasma membrane (Caumont et al., 2000).

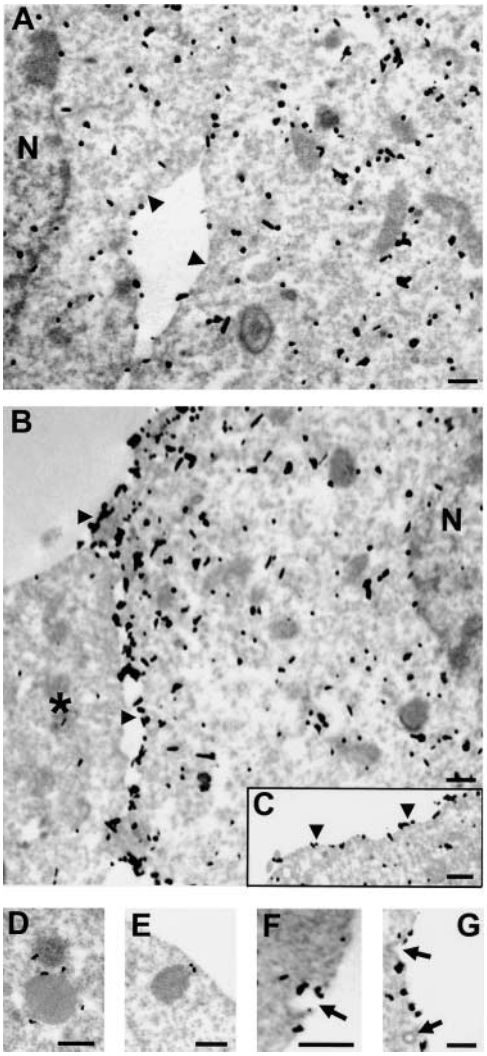

Figure 3. **Ultrastructural immunolocalization of HA-ARF6 in resting and stimulated PC12 cells.** PC12 cells expressing HA-ARF6 were maintained in Locke's solution (A) or stimulated with 59 mM K^+ (B–G), fixed, and stained with anti-HA antibodies. In stimulated cells, silver-intensified immunogold particles concentrate at the plasma membrane (compare A and B, arrowheads). Immunogold particles label the membrane plasma (C), the periphery of the secretory granules present in the cytoplasm (D), the docking site of secretory granules with the plasma membrane (E), and omega structures found at the plasma membrane most likely corresponding to granules having released their content (F). Coated pits at the plasma membrane and intracellular coated vesicles (G, arrows) are devoid of HA-ARF6 immunogold labeling. N, nucleus; asterisk, nontransfected cell; arrowheads in A–C, plasma membrane. Bars, 250 nm.

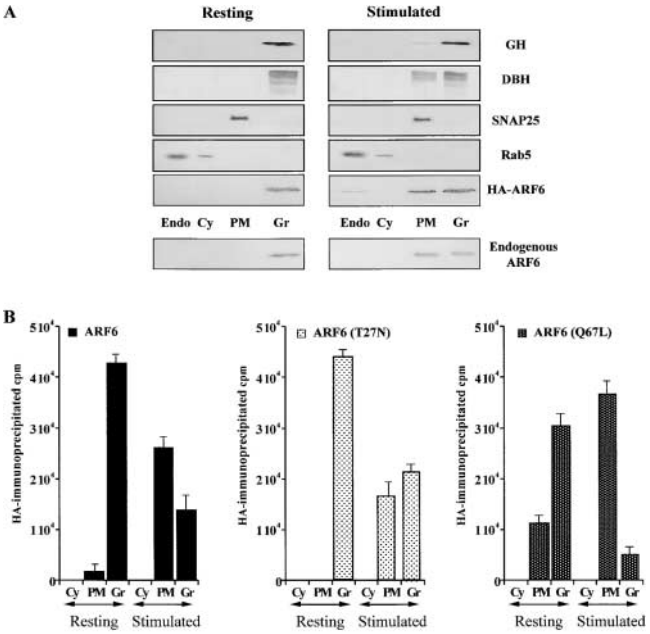

Figure 4. **Subcellular distribution of radiolabeled HA-ARF6 proteins in resting and stimulated PC12 cells.** PC12 cells expressing HA-ARF6, HA-ARF6(T27N), or HA-ARF6(Q67L) were transferred to phosphate- and serum-free medium containing 175 μCi [^{32}P]orthophosphate for 24 h. Cells were washed twice and then stimulated with 59 mM K^+ (Stimulated) or maintained in Locke's solution (Resting) for 15 min. Cells were immediately lysed and processed for subcellular fractionation on sucrose gradients. Fractions containing endosomes (Endo), cytosol (Cy), plasma membrane (PM), and secretory granules (Gr) were collected. (A) Fractions were separated by gel electrophoresis and transferred to nitrocellulose for immunoblotting analysis using anti-GH (granule content), anti-dopamine-β-hydroxylase (DβH; granule membrane), anti–SNAP-25 (plasma membrane), anti-Rab5 (endosomes), and anti-HA antibodies. Endogenous ARF6 was detected in parallel fractions prepared from nontransfected PC12 cells using anti-ARF6 antibodies. (B) HA-tagged ARF6 proteins were immunoprecipitated from the indicated fractions and labeled guanine nucleotides bound to the HA-ARF6 proteins were counted. Similar results were obtained from three different cell preparations.

To establish whether ARNO is the activator of ARF6 at the cell surface in stimulated PC12 cells, we compared the intracellular distribution of ARNO and ARF6. In resting PC12 cells, ARF6 associates with secretory granules as described earlier, whereas ARNO is restricted to the plasmalemma (Fig. 6 A). In contrast, in K^+-stimulated cells, ARF6 colocalized with ARNO at the plasma membrane (Fig. 6 A). To investigate directly whether ARNO triggers nucleotide exchange on the plasma membrane–associated ARF6, we determined the nature of the guanine nucleotide bound to immunoprecipitated ARF6 in cells cotransfected with ARNO or the catalytically inactive ARNO(E156K) mutant that lacks nucleotide exchange activity (Frank et al., 1998a). Overexpression of ARNO or ARNO(E156K) modified neither the level of expression of ARF6 (unpublished data) nor its subcellular distribution in resting and stimulated PC12 cells (Fig. 6 B, top); as was shown earlier, ARF6 was detected exclusively in the fraction containing secretory granules and it was in its GDP-bound state (Fig. 6 B, bottom). Stimulation with potassium triggered ARF6 translocation to the plasma membrane to a similar extent in all of the cells (Fig. 6 B). However, in cells overexpressing ARNO, all of the plasma membrane–associated ARF6 was in its GTP-bound state, whereas in cells expressing ARNO(E156K) the plasma membrane–bound ARF6 remained in its GDP-bound form (Fig. 6 B). Thus, ARNO stimulates GDP/GTP exchange on ARF6 in PC12 cells. Critically, overexpression of ARNO did not induce GTP loading of the secretory granule-associated ARF6 in resting or stimulated cells, indicating that ARNO can activate ARF6 only at or near the plasma membrane.

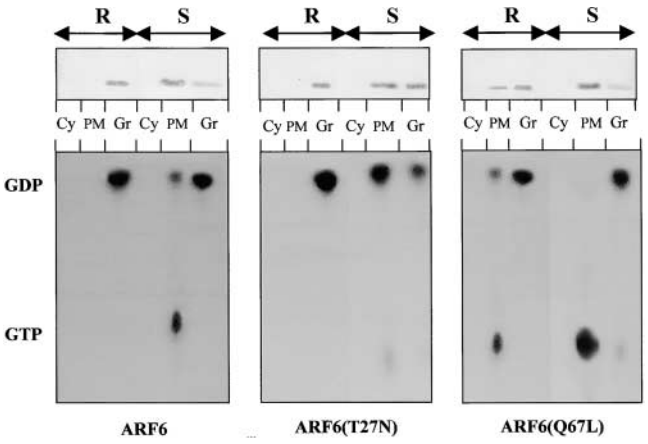

Figure 5. **Nucleotide status of ARF6 in resting and stimulated PC12 cells.** PC12 cells expressing HA-ARF6 or HA-ARF6 mutants were labeled with [^{32}P]orthophosphate and then incubated in Locke (R) or 59 mM K^+ (S) for 15 min. Cells were processed for subcellular fractionation and HA-tagged ARF6 proteins were immunoprecipitated from the cytosol (Cy), plasma membrane (PM), and secretory granules (Gr). (Top) 5 μl aliquots were analyzed by electrophoresis and Western blotting using anti-ARF6 antibodies. (Bottom) Guanine nucleotides eluted from the HA-immunoprecipitated ARF6 proteins were separated on chromatography plates and subjected to autoradiography. Similar results were obtained from three different cell preparations.

ARF6 is a functional element of the exocytotic machinery in PC12 cells

To establish whether ARF6 plays a role in exocytosis, we examined the effect of overexpressing the various ARF isoforms using GH as a secretory reporter in PC12 cells (Vitale et al., 2001). As illustrated in Fig. 1, overexpression of ARF1, ARF3, ARF5, or ARF6 did not apparently modify the distribution of GH. However, expression of the constitutively active ARF1(Q71L) and ARF3(Q71L) proteins and the dominant-negative ARF1(T31N) and ARF3(T31N) slightly but significantly reduced the total cellular GH measured by radioimmunoassay (Fig. 7 B), in agreement with the participation of ARF1 and ARF3 in the biosynthetic pathway of secretory granules (Peters et al., 1995). Of the ARFs tested, only ARF6 mutants modified the GH secretion in response to elevated K^+ (Fig. 7 C). Expression of the inactive ARF6(T27N) strongly inhibited GH secretion, whereas ARF6(Q67L) significantly stimulated it (Fig. 7 C). The degree of inhibition observed is consistent with other studies using this assay to characterize molecular components of regulated exocytosis (Holz et al., 1994; Sugita et al., 1999). It is also noteworthy that expression of the catalytically inactive ARNO(E156K) mutant resulted in a comparable inhibition of K^+-evoked GH secretion in PC12 cells (Caumont et al., 2000), most likely because ARNO(E156K) forms an abortive ternary complex with endogenous ARF6 in which GDP remains bound to the nucleotide binding site (Béraud-Dufour et al., 1998).

To confirm that ARF6 plays a role in the exocytotic machinery, we examined the effect of the ARF6 mutants on exocytotic activity as visualized by the binding of fluorescent annexin 5 to patches of phosphatidylserine exposed on the cell surface by secretory granule fusion (Demo et al., 1998;

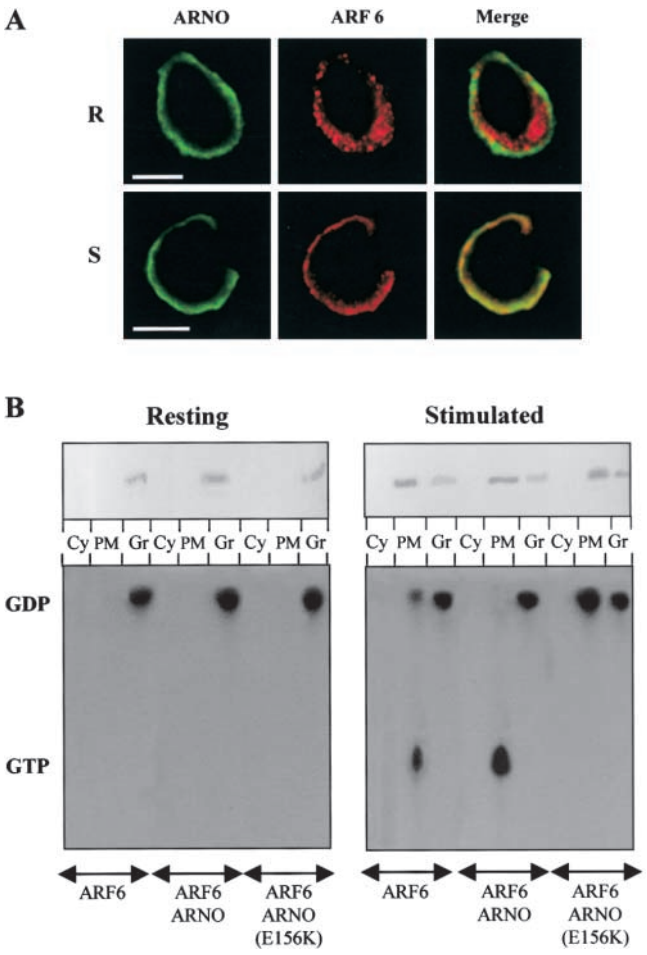

Figure 6. **ARF6 is activated by ARNO at the plasma membrane in stimulated PC12 cells.** (A) PC12 cells transfected with pXS-ARF6 in combination with the pCB7-myc-ARNO expression plasmid were incubated in Locke (R) or stimulated with 59 mM K^+ (S) for 5 min. After fixation, cells were stained with monoclonal anti-myc (ARNO) and rabbit polyclonal anti-ARF6 antibodies. In the merge images, the yellow-orange staining indicates the areas of colocalization between ARNO and ARF6. Bars, 5 μm. (B) PC12 cells transfected with pXS-ARF6 in combination with pCB7, pCB7-myc-ARNO, or pCB7-myc-ARNO(E156K) were labeled with [^{32}P]orthophosphate and then incubated in Locke (Resting) or stimulated with 59 mM K^+ (Stimulated). Cells were subsequently processed for subcellular fractionation and HA-ARF6 was immunoprecipitated from the cytosol (Cy), the plasma membrane (PM), and the secretory granules (Gr). (Top) 5 μl aliquots were analyzed by Western blotting using anti-ARF6 antibodies. (Bottom) guanine nucleotides bound to HA-ARF6 were analyzed by chromatography. Identical results were obtained with two independent cell preparations.

Vitale et al., 2001). Resting PC12 cells showed no fluorescent annexin 5 patches (Fig. 7 D), indicating that the annexin 5 binding assay does not detect baseline activity in the absence of secretagogue. Stimulation with elevated K^+ triggered the appearance of a patchy pattern of surface annexin 5 binding in cells expressing ARF6 or ARF6(Q67L), but not in cells expressing the inactive ARF6(T27N) mutant (Fig. 7 D). In line with the finding that ARF6(T27N) inhibits GH secretion from PC12 cells, this observation suggests that activation of ARF6 is critically important for calcium-evoked exocytosis of dense-core secretory granules.

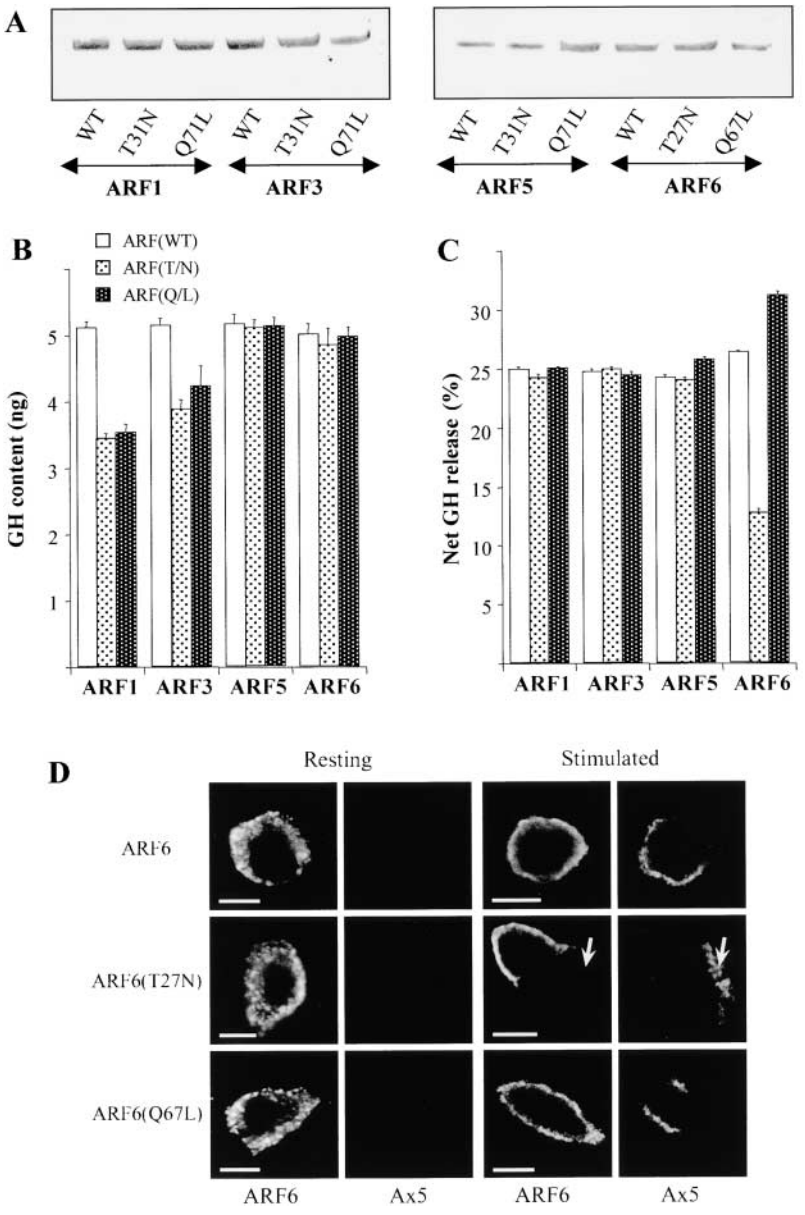

Figure 7. **ARF6 plays a role in dense-core granule exocytosis in PC12 cells.** PC12 cells were cotransfected with GH and the indicated HA-tagged ARF6 constructs. (A) 48 h after transfection, the expressed ARF proteins were detected by Western blots using anti-HA antibodies. (B) The total GH content in cells expressing WT ARFs, constitutively inactive ARFs(T/N), or active ARFs(Q/L) was measured by radioimmunoassay. In control experiments with the empty pXS vector, the GH content per well was 5.21 ng ± 0.12. (C) Transfected cells were incubated for 10 min in calcium-free Locke's solution or in 59 mM K^+ solution. The net K^+-evoked GH release was obtained by subtracting the basal release from the release measured in the presence of 59 mM K^+. In the different condition tested, basal release ranged from 5.2 to 7.1%. The net K^+-evoked GH release from cells transfected with empty pXS was 24.8% ± 0.4. Data are given as the mean values ± SE ($n = 3$). Similar results were obtained in three independent experiments performed with different cell cultures. (D) PC12 cells expressing ARF6, ARF6(T27N), or ARF6(Q67L) were stimulated with 59 mM K^+ in the presence of FITC-conjugated annexin 5 (Ax5) to reveal exocytotic activity. Cells were subsequently fixed and stained with rabbit anti-ARF6 antibodies. The arrow points to a nontransfected cell displaying exocytotic patches in response to 59 mM K^+ while the adjacent transfected cell expressing ARF6(T27N) exhibits no exocytotic activity. Bars, 5 μm.

PLD1 is a molecular partner of ARF6 in the exocytotic machinery

ARF proteins including ARF6 are established activators of PLD. In neurons and neuroendocrine cells, we previously described that a plasma membrane–associated PLD1 plays an important function in a late postdocking step of exocytosis (Humeau et al., 2001; Vitale et al., 2001). In order to test whether PLD1 might be an effector of ARF6 in the exocytotic process, we generated a mutant of ARF6 unable to stimulate PLD activity, namely ARF6(N48I), based on analogy with a similar mutant generated for ARF1 by Jones et al. (1999). As illustrated in Fig. 8 A, GTPγS binding to recombinant myrARF6(N48I) was indistinguishable from that of WT myrARF6. Dissociation of GDPβS from myrARF6(N48I) and myrARF6 was also very similar (unpublished data). ARNO stimulated GTPγS binding to myrARF6(N48I) and to myrARF6 to a similar extent (Fig. 8 A). Additionally, GIT1, a GAP protein for ARF6 (Vitale et al., 2000a), stimulated GTP hydrolysis on myrARF6(N48I) (Fig. 8 B). Together, these experiments indicate that the N48I mutation does not affect the activation/inactivation cycle of ARF6 and its regulation by endogenous accessory proteins. We next tested the ability of myrARF6(N48I) to stimulate known ARF effectors. In presence of GTP, cholera toxin ADP-ribosyltransferase activity was stimulated similarly by myrARF6(N48I) and myrARF6 (Fig. 8 C). However, as intended, the mutation N48I abolished almost completely the ability of ARF6 to stimulate PLD1 (Fig. 8 D), making ARF6(N48I) an ideal tool to test the involvement of the ARF6-PLD pathway in a cellular function.

We next examined whether ARF6 and PLD1 colocalize, and could thereby physically interact in stimulated PC12 cells. In agreement with the presence of endogenous PLD1 at the plasma membrane in chromaffin cells (Vitale et al., 2001), GFP-PLD1 was found at the plasma membrane in both resting and stimulated PC12 cells (Fig. 9 A). Little ARF6 colocalized with PLD1 in resting cells. In contrast, stimulation with K^+ triggered the colocalization of a fraction of ARF6 with GFP-PLD1 at the plasma membrane (Fig. 9

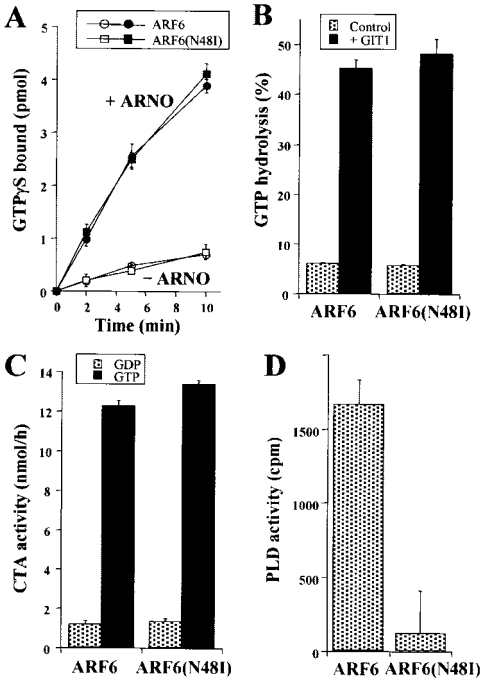

Figure 8. **Characterization of the PLD1-nonresponsive ARF6(N48I) mutant.** (A) GTPγS binding to 0.5 μg myrARF6 (circles) or myrARF6(N48I) (squares) was estimated for the indicated periods of time in the absence (open symbols) or in the presence (closed symbols) of 0.5 μg ARNO. (B) 0.5 μg myrARF6 or myrARF6(N48I) with [α-^{32}P]GTP bound were incubated for 10 min with buffer (control) or 250 ng GIT1 before separation of the bound nucleotide by TLC. (C) 1 μg myrARF6 or myrARF6(N48I) together with 100 μM of GDP or GTP were incubated with components needed for cholera toxin A subunit-catalyzed ADP-ribosylagmatine formation for 1 h Basal activity in the absence of guanine nucleotide has been subtracted. (D) The activity exhibited by partially purified recombinant PLD1 as stimulated by 1 μg of GTP-loaded myrARF6 or myrARF6(N48I) was quantitated using the standard in vitro headgroup release assay. Averaged over three separate experiments, ARF6(N48I) demonstrated 7% of the activity of wt ARF6. Data are mean ± one half the range of values from triplicate assays in one experiment representative of two/three experiments conducted using different protein preparations.

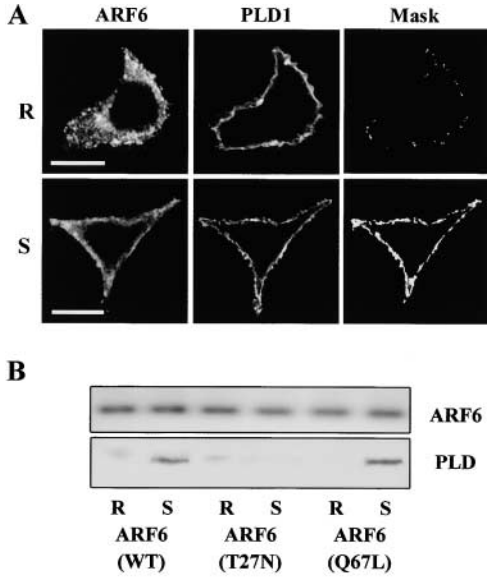

Figure 9. **ARF6 interacts with PLD1 in stimulated PC12 cells.** (A) PC12 cells cotransfected with pEGFP-PLD1 and pXS-ARF6 were incubated for 5 min in either Locke's solution (R) or in 59 mM K^+ (S). Cells were subsequently stained with rabbit anti-ARF6 antibodies. Masks representing the regions of colocalization were generated by selecting the double-labeled pixels. Bars, 10 μm. (B) PC12 cells expressing HA-ARF6 or HA-ARF6 mutants were incubated for 10 min in either Locke's solution (R) or in 59 mM K^+ (S). Cells were scrapped and HA-tagged ARF6 proteins were immunoprecipitated. Samples were analyzed by electrophoresis and Western blotting using anti-ARF6 and anti-PLD1 antibodies. Similar results were obtained from three different cell preparations.

A), thereby making feasible a potential interaction of ARF6 with PLD1 near the site of exocytosis. Direct interaction of ARF6 and PLD1 in secretagogue-activated cells was assessed by immunoprecipitation. As illustrated in Fig. 9 B, immunoprecipitation of WT ARF6 or the active ARF6(Q67L), but not the inactive ARF6(T27N), coprecipitated PLD1 from lysates of K^+-stimulated PC12 cell but not from lysates of resting cells (Fig. 9 B), confirming the binding of activated ARF6 to PLD1 upon cell activation.

Next we examined the effects of ARF6(N48I) and ARF6 (N48I/Q67L) on K^+-evoked GH secretion. ARF6(N48I) expressed in PC12 cells associated with secretory granules (Fig. 10 A) and, similar to WT ARF6, became activated at the plasma membrane upon cell stimulation (Fig. 10 B). However, as shown in Fig. 10 C, expression of ARF6(N48I) in PC12 cells clearly decreased GH secretion by ~50%. Furthermore, superimposing the N48I mutation onto the constitutively active ARF6(Q67L) caused it to lose its ability to stimulate exocytosis; instead, ARF6(N48I/Q67L) behaved as a dominant negative, inhibiting secretion to an extent similar to the PLD1 nonstimulating ARF6(N48I) mutant (Fig. 10 C) and the inactive GDP-bound ARF6(T27N) mutant (Fig. 7 C). Immunoprecipitation experiments indicate that ARF6(N48I) still binds to PLD1 upon cell stimulation (unpublished data), suggesting that the inhibition of secretion after ARF6(N48I) expression is likely to be due to the partial sequestration and inactivation of endogenous PLD1. These results argue for a major contribution of PLD1 in the pathway by which ARF6 regulates exocytosis in neuroendocrine cells.

Discussion

ARF proteins have been proposed as ubiquitous regulators of vesicle transport within cells. Here, we investigated their possible function in calcium-regulated exocytosis of dense-core secretory granules, using PC12 cells which have proved to be a good model for the study of neuroendocrine secretion. We find that ARF6 is the sole member of the ARF family specifically associated with secretory granules and that it is activated upon secretagogue-evoked stimulation, most likely after targeting and docking of granules onto the plasma membrane. Transfection experiments with ARF6 mutants confirm the importance of ARF6 in the exocytotic reaction in PC12 cells. Our results suggest an intimate connection between ARF6 and the plasma membrane-bound PLD1 that we recently described as a key factor in the late fusion reaction in neurons (Humeau et al., 2001) and neuroendocrine cells (Vitale et al., 2001).

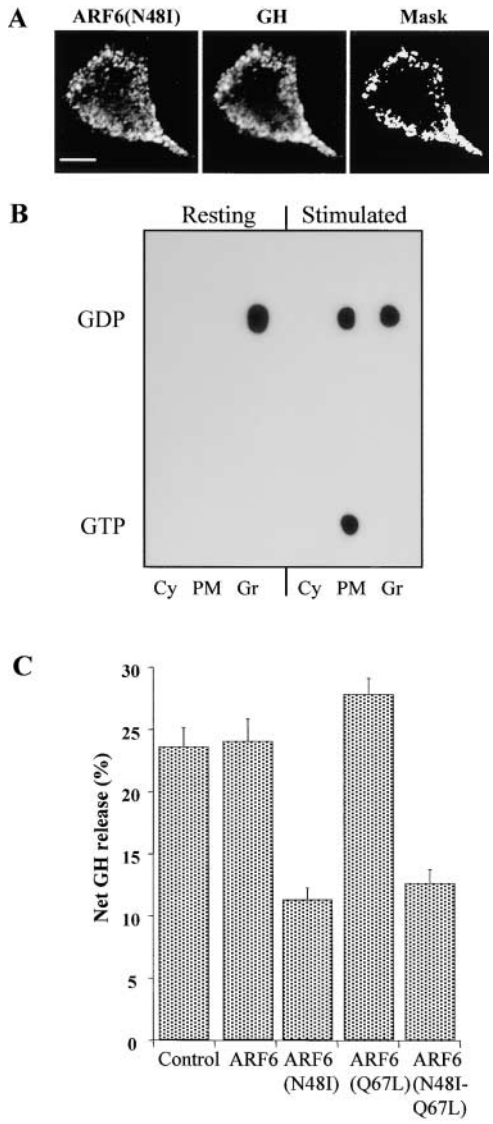

Figure 10. **ARF6 regulates exocytosis by stimulating the plasma membrane-associated PLD1 in PC12 cells.** (A) PC12 cells transfected with pXS-ARF6(N48I) in combination with the GH expression plasmid were stained with monoclonal anti-GH and rabbit polyclonal anti-ARF6 antibodies. Mask represents the regions of colocalization. Bar, 5 μm. (B) PC12 cells expressing HA-ARF6(N48I) were labeled with [^{32}P]orthophosphate. Cells were then incubated in Locke (Resting) or stimulated with 59 mM K^+ (Stimulated) and subsequently processed for subcellular fractionation. HA-ARF6(N48I) was immunoprecipitated from the cytosol (Cy), the plasma membrane (PM), and the secretory granules (Gr) and bound guanine nucleotides were analyzed by chromatography. (C) PC12 cells cotransfected with GH and the indicated ARF6 constructs were incubated for 10 min in calcium-free Locke's solution or in 59 mM K^+. The net K^+-evoked secretory response was obtained by subtracting the basal release from the release evoked by 59 mM K^+. Similar results were obtained in two independent experiments performed with different cell cultures.

The granule-associated ARF6 is activated by ARNO at the plasma membrane upon PC12 cell stimulation

We previously compared the distribution of ARF6 in subcellular fractions obtained from resting and stimulated chromaffin cells and found that ARF6 apparently dissociates from secretory granules and binds to the plasma membrane upon cell stimulation (Caumont et al., 1998). Similarly, we found here that overexpressed ARF6 proteins translocate from secretory granule– to plasma membrane–containing fractions in stimulated PC12 cells. Immunofluorescence and ultrastructural analysis confirm the association of ARF6 with secretory granules and its relocalization at the plasma membrane upon cell stimulation. A reasonable explanation is that the plasma membrane location of ARF6 results from the docking of secretory granules to sites of exocytosis. The observation that ARF6 remains attached to the plasma membrane upon subcellular fractionation of stimulated cells may reflect the direct interaction of ARF6 with a plasma membrane–bound protein. The nucleotide exchange factor ARNO is an attractive candidate to be the plasma membrane-bound partner of ARF6 (Frank et al., 1998b). Indeed, ARNO colocalizes with ARF6 in stimulated PC12 cells. Furthermore, we show here that ARF6 is in its inactive GDP-bound state when associated with secretory granules, but becomes activated when recruited to the plasma membrane. Conversely, overexpression of ARNO increases GTP binding to ARF6 at the plasma membrane whereas a catalytically inactive ARNO mutant dramatically inhibits it. It is interesting to note that the constitutively GDP-bound ARF6(T27N) could also be found associated to the plasma membrane in stimulated cells, suggesting that GDP/GTP exchange is not required for the ARF6 association with plasma membrane. This observation is in line with the idea that granules bring ARF6 to the plasma membrane, making the protein transiently available for ARNO upon docking at the exocytotic sites.

GDP-bound ARFs interact weakly with membranes through hydrophobic interactions of the myristate and electrostatic interactions of cationic residues with anionic lipids (Antonny et al., 1997). Binding of GTP releases the NH_2-terminal helix from the protein core allowing a stronger interaction with membranes through the NH_2-terminal hydrophobic residues (Antonny et al., 1997). Using purified chromaffin granules, we previously described that ARF6 coimmunoprecipitates with trimeric G protein β subunits (Galas et al., 1997), suggesting that βγ subunits could serve as anchors for GDP-bound ARF6 in the secretory granule membrane. An appealing speculation is that the ARF6/ARNO interaction is in some way under the control of the granule-associated G protein βγ subunits. Thus, activation of ARF6 would require the docking of granules to appropriate ARNO-containing domains on the plasma membrane and concomitant changes in the interactions of ARF6 with βγ subunits. In favor of this idea, several recent reports describe βγ subunit regulation of the exocytotic fusion machinery, downstream of calcium entry, in secretory cells (Pinxteren et al., 1998; Gensse et al., 2000) and as well as in neuronal presynaptic terminals (Blackmer et al., 2001).

ARNO-induced ARF6 activation is critical for exocytosis in PC12 cells

We previously reported that a synthetic peptide, corresponding in sequence to the myristoylated NH_2-terminal domain of ARF6 inhibited, in a dose-dependent manner, calcium-evoked catecholamine secretion from permeabilized chromaffin cells (Galas et al., 1997; Caumont et al., 1998). More recently, we described the presence of ARNO at the plasma

membrane in chromaffin cells and demonstrated that overexpression of WT ARNO enhanced secretagogue-evoked secretion from PC12 cells whereas the catalytically inactive ARNO(E156K) mutant inhibited it (Caumont et al., 2000). These results suggested, but did not prove, that an ARNO-regulated ARF6 plays an important role in exocytosis. Indeed, ARNO is exchange factor for both ARF1 and ARF6, and both ARFs can be detected at the plasma membrane in chromaffin cells (Galas et al., 1997). Moreover, although ARF peptides have been described to block ARF activities in various cellular processes (Barr and Huttner, 1996; Le Stunff et al., 2000; Mukherjee et al., 2000), their specificity has also been questioned (Fensome et al., 1994). Using direct means, we demonstrate here for the first time that ARF6 is the sole member of the ARF family involved in the molecular pathway underlying calcium-regulated exocytosis. Although the involvement of ARF6 in peripheral vesicle trafficking has been reported previously in various cell types, this specific requirement for ARF6 in secretion is surprising and may be linked to its granular location and its specific activation at granule targeting sites.

PLD1 is a major downstream effector of ARF6 in the exocytotic process

One of the proposed functions of ARF6 is to mediate actin rearrangements (Chavrier and Goud, 1999). Activation of ARF6 can also lead to significant changes in the lipid composition of specific membrane domains, as the protein is known to directly activate two lipid-modifying enzymes, PLD and phosphatidylinositol-4-phosphate 5-kinase (Honda et al., 1999). Thus, activation of ARF6 at the exocytotic sites may provide a mechanism whereby secretory cells can engineer a localized remodeling of the actin cytoskeleton with phospholipid modifications, so that the fusion machinery can negotiate the exocytotic event. Using rhodamine-conjugated phalloidin to visualize actin filaments, we have investigated whether ARF6 mutants affect the depolymerization of cortical actin observed in stimulated cells. However, we could not correlate the strong inhibition of secretion induced by ARF6(T27N) to a stabilization of the cortical actin barrier (unpublished data). Accordingly, we found that the secretagogue-dependent movement of secretory granules to the cell periphery was similar in control and in ARF6(T27N)-expressing cells. Thus, ARF6 is probably not involved in the partial depolymerization of the cortical actin cytoskeleton that enables recruitment of the reserve pool of secretory granules to the plasma membrane. This finding is in line with the idea that ARF6 is activated after docking of secretory granules to the sites of exocytosis. Whether ARF6 mediates some other subtle modifications of the actin cytoskeleton functionally required in late stages of the exocytotic machinery cannot be excluded and will be an interesting future issue.

We recently demonstrated that PLD1 constitutes a critical factor for regulated exocytosis in neuroendocrine cells (Vitale et al., 2001) and neurons (Humeau et al., 2001), operating subsequent to the cytoskeletal-mediated recruitment of secretory granules to exocytotic sites (Vitale et al., 2001). To directly probe the idea that ARF6 is the upstream activator of the plasma membrane-bound PLD1 in the exocytotic pathway, we generated a novel ARF6(N48I) mutant that specifically lost its ability to stimulate PLD1, based on analogy with a similar ARF1 mutant defective in PLD activation but still able to activate phosphatidylinositol-4-phosphate 5-kinase (Jones et al., 1999; Skippen et al., 2002). Expression of ARF6(N48I) inhibited secretagogue-evoked GH secretion to an extent similar to the constitutively inactive ARF6(T27N), suggesting that the stimulation of PLD1 is the major function ARF6 undertakes in regulated exocytosis. Accordingly, ARF6 interacts directly with PLD1 at the plasma membrane in stimulated cells. Our previous observation that the introduction of specific anti-ARNO antibodies into permeabilized chromaffin cells inhibited in a similar manner catecholamine secretion and PLD activation (Caumont et al., 2000) further corroborates the ARNO–ARF6–PLD1 cascade in the exocytotic process.

How does PLD1 relate to our current understanding of the protein machinery responsible for regulated exocytosis? PLD1 catalyzes the hydrolysis of phosphatidylcholine to produce membrane-localized phosphatidic acid (PA). An exciting speculation relates to the changes in the lipid bilayer properties created by the activation of PLD. PA is a cone-shaped lipid which promotes membrane curvature and the formation of hemifusion intermediates required for the fusion of two membranes (Monck and Fernandez, 1994). Thus, the predicted effect of a local elevation of PA due to the activation of PLD1 at exocytotic sites would be to promote membrane bending, particularly in the presence of calcium, thereby facilitating membrane breakdown and subsequent formation of the exocytotic fusion pore. Accordingly, data obtained by amperometry on isolated chromaffin cells suggest that PLD1 affects the kinetics of the initial fusion event and/or the rate of the fusion pore opening (Vitale et al., 2001). Similarly, electrophysiological analysis suggest that the role played by PLD1 in neurotransmission is related to the fusogenic status of the presynaptic release sites (Humeau et al., 2001). Hence, the ARF6–PLD1 complex may provide to the protein scaffold pulling membrane together the lipid counterparts required to open and/or expand the fusion pore.

Materials and methods

Antibodies, DNA constructs, and proteins

The following antibodies were used: rabbit polyclonal anti-ARF6 antibodies, provided by J. Donaldson (National Institutes of Health, Bethesda, MD) (Radhakrishna and Donaldson, 1997); rabbit polyclonal anti–human GH antibodies, provided by Dr. A.F. Parlow (Los Angeles County Harbor–UCLA Medical Center, Torrance, CA) and the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program; polyclonal anti-Rab5 antibodies, provided by M. Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany); monoclonal anti–human GH antibodies (Nichols Institute); monoclonal anti–SNAP-25 antibodies (Sternberger Monoclonals, Inc.); monoclonal anti-HA antibodies (BabCO); monoclonal anti-myc antibodies (Novocastra Laboratories). Cy2-anti–mouse, Cy3-anti–rabbit, and Cy5-anti–mouse were obtained from Amersham Biosciences. pECFP-Golgi was purchased from Stratagene.

The ARF and ARNO constructs, provided by J.E. Casanova (University of Virginia Health System, Charlottesville, VA), were described previously (Frank et al., 1998a; Morinaga et al., 2001). pXS-ARF6(N48I) and pXS-ARF6(N48I-Q67L) were generated by site-directed mutagenesis with a Quickchange mutagenesis kit from Stratagene (Vitale et al., 2000a).

Recombinant myrARF6 and myrARF6(N48I) were expressed and puri-

fied as described previously (Vitale et al., 2000b). Recombinant ARNO was expressed as described (Pacheco-Rodriguez et al., 1998).

Culture, transfection, and assay of GH release from PC12 cells

PC12 cells were grown in DME supplemented with glucose (4,500 mg/L) and containing 30 mM $NaHCO_3$, 5% fetal bovine serum, 10% horse serum and 100 U/ml penicillin/streptomycin. Mammalian expression vectors were introduced into PC12 cells together with the GH plasmid pXGH5 (6-well dishes, 80% confluent, 0.4 μg/well of each plasmid) using GenePorter (Gene Therapy Systems) according to the manufacturer's instruction. After 5 h of incubation at 37°C, 2 ml of culture medium containing fetal bovine serum, horse serum, and antibiotics was added.

GH release experiments were performed 48 h after transfection. PC12 cells were washed twice with Locke's solution and then incubated for 10 min with calcium-free Locke's solution (basal release) or stimulated with an elevated K^+ solution (Locke's containing 59 mM KCl and 85 mM NaCl). The supernatant was collected and the cells harvested by scraping in 10 mM phosphate buffered saline. The amounts of GH secreted into the medium and retained in the cells were measured using a radioimmunoassay kit (Nichols Institute). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

Distribution and nucleotide status of ARF6 in PC12 cell subcellular fractions

PC12 cells grown in 100-mm plates were transfected with various plasmids (for each 10 μg per plate) using GenePorter. 36 h after transfection, cells were incubated in 2 ml/plate phosphate- and serum-free DME medium, supplemented with 25 mM Hepes, pH 7.2, 2 mM pyruvate and 175 μci of [^{32}P]orthophosphate for 24 h. PC12 cells were washed twice with Locke's solution and then incubated for 15 min with Locke's solution (basal release) or stimulated with an elevated K^+ solution. Medium was removed and cells immediately scrapped in 1 ml of sucrose 0.32 M (20 mM Tris, pH 8.0). Cells were broken in a Dounce homogenizer and centrifuged at 800 *g* for 15 min. The supernatant was further centrifuged at 20,000 *g* for 20 min. The resulting supernatant was further centrifuged for 60 min at 100,000 *g* to obtain the cytosol (supernatant) and microsomes (pellet enriched in endosomes). The 20,000 *g* pellet containing the crude membrane fraction was resuspended in sucrose 0.32 M (20 mM Tris, pH 8.0), layered on a cushion sucrose density gradient (sucrose 1–1.6 M, 20 mM Tris, pH 8.0) and centrifuged for 90 min at 100,000 *g* to separate the plasma membrane from secretory granules (Caumont et al., 2000). The upper fractions containing SNAP-25 (plasma membrane marker) and the pellet containing GH and dopamine–β-hydroxylase (secretory granule markers) were collected and resuspended in buffer A (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 0.05% cholate, 0.005% SDS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 1 mM vanadate, 0.32 M sucrose). To immunoprecipitate ARF6 proteins, 5 μl anti-HA antiserum and 10 μl of protein A Sepharose beads were added to 500 μl of cytosol, plasma membrane, and secretory granule fractions. Immunoprecipitation was performed for 3 h at 4°C, followed by extensive washing of the protein A beads with ice cold buffer B (50 mM Hepes, pH 7.4, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.05% cholate, 0.005% SDS). Elution was carried out in Laemmli buffer for samples subjected to SDS-PAGE and in 40 μl of 2 M formic acid (heated at 70°C for 3 min) for samples analyzed by thin layer chromatography (Vitale et al., 2000b).

Immunoblotting, immunofluorescence, and confocal microscopy

One-dimensional SDS gel electrophoresis was performed on 10% acrylamide gels in Tris-Glycine buffer. The proteins were transferred to nitrocellulose sheets at a constant current of 120 mA for 1 h. Blots were developed using secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences) and the immunoreactive bands detected using the ECL system (Amersham Biosciences).

For immunocytochemistry, PC12 cells grown on poly-D-lysine–coated glass coverslips were maintained in Locke's solution or stimulated with elevated K^+. The cells were then fixed for 20 min in 4% paraformaldehyde in 0.12 M sodium/phosphate, pH 7.0, and for a further 10 min in fixative containing 0.1% Triton X-100. Immunostaining was performed as described previously (Vitale et al., 2001) and stained cells were visualized using a Zeiss confocal microscope LSM 510.

Preembedding immunoelectron microscopy

PC12 cells transfected with pXS-ARF6 were stimulated with 59 mM K^+ and fixed for 45 min with 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium/phosphate, pH 7.2. Immunostaining was performed using anti-HA antibodies according to the immunogold silver labeling procedure described by Yi et al. (2001).

Molecular characterization of myrARF6(N48I)

Time-course GTPγS binding assays were performed with 0.5 μg of myrARF6 or myrARF6(N48I) essentially as described (Vitale et al., 1997). In the presence or absence of GIT1, provided by R.T. Premont (Duke University Medical Center, Durham, NC), GTPase activity of 0.5 μg of myrARF6 or myrARF6(N48I) was measured for 10 min at 30°C as described previously (Vitale et al., 2000b). ARF stimulation of cholera toxin-catalyzed ADP-ribosylagmatine formation was performed with 0.5 μg of myrARF6 or myrARF6(N48I) as described (Vitale et al., 1997). PLD activity assays were carried out using the in vitro head-group release assay for a 30-min time period as previously described (Zhang et al., 1999b). Recombinant WT and mutant ARF6 were loaded using 50 μM GTPγS as previously described (Frohman et al., 2000) and were used at 0.5 μM (1 μg/100 μl reaction). Assay blanks were subtracted. The source of PLD1 protein was a tet-regulated stable CHO cell line that had been induced for 24 h in a 35-mm dish before harvesting and sonication. The sonicated lysate was centrifuged for 30 min at 4°C and 20,000 *g* to pellet the PLD1, and the supernatant containing the Rho and ARF small GTP proteins removed. 100 μl of PBS containing protease inhibitors was added to the pellet which was then sonicated briefly to evenly resuspend it. 1 μl of the resulting PLD1 source was used for each assay sample.

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