ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis

Yoshikatsu Aikawa and Thomas F.J. Martin

Department of Biochemistry, University of Wisconsin, Madison, WI 53706

A DP-ribosylation factor (ARF) 6 regulates endosomal plasma membrane trafficking in many cell types, but is also suggested to play a role in Ca\(^{2+}\)-dependent dense-core vesicle (DCV) exocytosis in neuroendocrine cells. In the present work, expression of the constitutively active GTPase-defective ARF6\(^{Q67L}\) mutant in PC12 cells was found to inhibit Ca\(^{2+}\)-dependent DCV exocytosis. The inhibition of exocytosis was accompanied by accumulation of ARF6\(^{Q67L}\), phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), and the phosphatidylinositol 4-phosphate 5-kinase type I (PIP5K1) on endosomal membranes with their corresponding depletion from the plasma membrane. That the depletion of PIP\(_2\) and PIP5K from the plasma membrane caused the inhibition of DCV exocytosis was demonstrated directly in permeable cell reconstitution studies in which overexpression or addition of PIP5K1 restored Ca\(^{2+}\)-dependent exocytosis. The restoration of exocytosis in ARF6\(^{Q67L}\)-expressing permeable cells unexpectedly exhibited a Ca\(^{2+}\) dependence, which was attributed to the dephosphorylation and activation of PIP5K. Increased Ca\(^{2+}\) and dephosphorylation stimulated the association of PIP5K1 with ARF6. The results reveal a mechanism by which Ca\(^{2+}\) influx promotes increased ARF6-dependent synthesis of PIP\(_2\). We conclude that ARF6 plays a role in Ca\(^{2+}\)-dependent DCV exocytosis by regulating the activity of PIP5K for the synthesis of an essential plasma membrane pool of PIP\(_2\).

Introduction

The release of neurotransmitters from neural and neuroendocrine cells is mediated by the exocytotic fusion of synaptic vesicles and dense-core vesicles (DCVs) with the plasma membrane in a process activated by cytoplasmic Ca\(^{2+}\) elevations (Rettig and Neher, 2002). Secretory vesicles dock at the plasma membrane and undergo priming, which confers competence for Ca\(^{2+}\)-dependent DCV exocytosis. The inhibition of exocytosis was accompanied by accumulation of ARF6\(^{Q67L}\), phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), and the phosphatidylinositol 4-phosphate 5-kinase type I (PIP5K1) on endosomal membranes with their corresponding depletion from the plasma membrane. That the depletion of PIP\(_2\) and PIP5K from the plasma membrane caused the inhibition of DCV exocytosis was demonstrated directly in permeable cell reconstitution studies in which overexpression or addition of PIP5K1 restored Ca\(^{2+}\)-dependent exocytosis. The restoration of exocytosis in ARF6\(^{Q67L}\)-expressing permeable cells unexpectedly exhibited a Ca\(^{2+}\) dependence, which was attributed to the dephosphorylation and activation of PIP5K. Increased Ca\(^{2+}\) and dephosphorylation stimulated the association of PIP5K1 with ARF6. The results reveal a mechanism by which Ca\(^{2+}\) influx promotes increased ARF6-dependent synthesis of PIP\(_2\). We conclude that ARF6 plays a role in Ca\(^{2+}\)-dependent DCV exocytosis by regulating the activity of PIP5K for the synthesis of an essential plasma membrane pool of PIP\(_2\).

Plasma membrane PIP\(_2\) is also essential for mediating the sequential recruitment of adaptor and accessory proteins to sites of clathrin-dependent endocytosis (Cremona and DeCamilli, 2001). The requirement for PIP\(_2\) as a cofactor for both endocytosis and exocytosis suggests that its synthesis at localized sites of fusion and fission is tightly regulated, but little is known about the control of PIP\(_2\) synthesis. Recent analyses identified protein kinases and phosphatases that regulate the type I PIP5K (Park et al., 2001). An important advance was the finding that ADP-ribosylation factor (ARF) GTPases directly activate type I PIP5K enzymes (Honda et al., 1999; Jones et al., 2000).

The ARF proteins are GTPases that function as regulators of membrane traffic (Randazzo et al., 2000). ARF6, the sole member of the class III ARF family, resides on endosomes and the plasma membrane and regulates membrane traffic between these compartments (D’Souza-Schorey et al., 1995; Peters et al., 1995). ARF6 functions as a switch that cycles through GTP for GDP exchange and GTP hydrolysis. Correspondingly, studies of its function have used expression of dominant-
negative GTP-binding deficient mutants (e.g., ARF6\(^{T27N}\)) or of constitutively active GTPase-deficient mutants (e.g., ARF6\(^{Q67L}\)) to perturb membrane trafficking. Activation of ARF6 promotes peripheral actin assembly (Schafer et al., 2000), plasma membrane protrusions and invaginations (Peters et al., 1995; D’Souza-Schorey et al., 1998), pinocytosis (Brown et al., 2001), and the internalization of plasma membrane proteins via clathrin-dependent (D’Souza-Schorey et al., 1995; Altschuler et al., 1999; Claing et al., 2001; Palacios et al., 2002) or clathrin-independent (Radhakrishna et al., 1996; Radhakrishna and Donaldson, 1997; Brown et al., 2001; Delaney et al., 2002) endocytosis.

Unlike the results in other cell types, ARF6 was reported to localize to DCVs in adrenal chromaffin cells and to undergo translocation from DCVs to the plasma membrane in response to the stimulation of exocytosis (Galas et al., 1997; Caumont et al., 1998). Whether ARF6 plays a direct role in regulated DCV exocytosis or exerts indirect effects through cytoskeletal rearrangements or plasma membrane-endosomal trafficking remains to be determined. We found that ARF6 localizes to the plasma membrane and recycling endosomes, but not to DCVs in neuroendocrine PC12 cells. Cells expressing the GTPase-deficient ARF6\(^{Q67L}\) mutant exhibited reduced rates of Ca\(^{2+}\)-triggered DCV exocytosis resulting from the diversion of plasma membrane PIP\(_2\) and PIP5K to endosomal membranes. The restoration of DCV exocytosis by addition of PIP5K to permeable cells required Ca\(^{2+}\), which activated PIP5K and increased its association with ARF6. These results indicate that ARF6 plays a role in DCV exocytosis by controlling the activity of PIP5K and the synthesis of a required plasma membrane pool of PIP\(_2\).

**Results**

Expression of ARF6\(^{Q67L}\) inhibits Ca\(^{2+}\)-activated DCV exocytosis in PC12 cells

To determine if ARF6-dependent cellular activities play a role in Ca\(^{2+}\)-activated DCV exocytosis, wild-type and mu-
tant ARF6 proteins (GTPase-deficient ARF\textsuperscript{Q67L} or GTP binding–deficient ARF6\textsuperscript{T27N}) were expressed in PC12 cells (Fig. 1 B). Human growth hormone (hGH), which is stored in DCVs, was coexpressed to enable the detection of regulated secretion in the transfected cells (Zhang et al., 2002). Ca\textsuperscript{2+}-dependent hGH secretion was elicited in brief incubations in high K\textsuperscript{+}-containing buffer to promote Ca\textsuperscript{2+} influx, and was compared with basal secretion in Na\textsuperscript{+}-containing buffer. Expression of wild-type and GTP binding–deficient ARF6\textsuperscript{T27N} did not affect the basal release of hGH, but expression of the GTPase-deficient ARF6\textsuperscript{Q67L} increased basal release approximately twofold (Fig. 1 A, open bars), which may correspond to the Ca\textsuperscript{2+}-independent, GTP-dependent stimulation of DCV exocytosis characterized in permeable PC12 cells (Banerjee et al., 1996). Ca\textsuperscript{2+} influx–dependent hGH secretion stimulated by K\textsuperscript{+} depolarization was unaffected by expression of either wild-type or ARF6\textsuperscript{T27N}, but was consistently inhibited in ARF6\textsuperscript{Q67L}-expressing cells (Fig. 1 A, closed bars). The Ca\textsuperscript{2+}-dependent release of hGH, calculated by subtracting basal release from high K\textsuperscript{+}-induced release measured in the presence of high K\textsuperscript{+}, was inhibited by 43% (24.4 ± 1.1% and 13.8 ± 2.8% hGH release for wild-type and ARF6\textsuperscript{Q67L}, respectively). Because ARF6\textsuperscript{Q67L}, reported to alter peripheral membrane trafficking in other cell types, affected DCV exocytosis in PC12 cells, subsequent analyses were undertaken to determine the basis for the inhibition.

Expression of wild-type or either of the ARF6 mutants did affect the cellular distribution of hGH (Fig. 1 C, right panels) in the Golgi or in DCVs, indicating that the synthesis and transport of hGH through the secretory pathway were unaffected by ARF6 expression. The wild-type and ARF6\textsuperscript{Q67L} expressed proteins localized to the plasma membrane and to cytoplasmic vesicular membranes, whereas the ARF6\textsuperscript{T27N} protein was predominantly localized to cytoplasmic vesicular membranes (Fig. 1 C, left panels), as reported for other cell types (D’Souza-Schorey et al., 1995; Peters et al., 1995). ARF6-containing cytoplasmic vesicles (Fig. 1 C, arrows) were readily distinguished from hGH-immunoreactive DCVs. Neither docked DCVs containing hGH (Fig. 1 C, arrowheads) nor cytoplasmic DCVs (Fig. 1 C, insets) colocalized with ARF6-containing cytoplasmic vesicles. Endogenous ARF6 was weakly detected in cytoplasmic vesicular membranes and on the plasma membrane (Fig. 1 C), and this localization was clearly distinct from that of chromogranin B, an endogenous DCV constituent (Fig. 1 C). Overall, these results indicated that ARF6 proteins are not present on DCVs, and that the inhibition of Ca\textsuperscript{2+}-dependent DCV exocytosis by ARF6\textsuperscript{Q67L} was unlikely the result of either an altered DCV localization or of a direct effect of ARF6 on DCVs.

ARF6 colocalizes with transferrin- and Rab11-positive endosomes in PC12 cells

In other cell types, ARF6 is present in endosomal compartments and on the plasma membrane (D’Souza-Schorey et al., 1995; Peters et al., 1995; Radhakrishna and Donaldson, 1997). Although ARF6 was reported to be present on DCVs in chromaffin cells (Galas et al., 1997; Caumont et al., 1998), this was not the case for neuroendocrine PC12 cells (Fig. 1 C). To identify the cytoplasmic vesicles containing ARF6, colocalization studies were conducted for proteins characteristic of secretory and endocytic compartments. Cytoplasmic ARF6 (Fig. 2 A, arrows) did not colocalize with either TGN38 or with mannosidase II. ARF6-containing vesicles were more diffusely localized around the perinuclear region after treatment with brefeldin A (BFA; Fig. 2 A), which causes tubulation of endosomes, the TGN, and lysosomes (Lippincott-Schwartz et al., 1991). BFA
The treatment also affected mannosidase II–containing and TGN38-containing Golgi elements (Fig. 2 A), as well as transferrin-positive endosomal compartments (unpublished data). The distributions of wild-type and ARF6\textsuperscript{Q67L} (unpublished data) and endocytosed transferrin, a marker for recycling endosomes, overlapped extensively, but not precisely (Fig. 2 B, inset), indicating that cytoplasmic ARF6 proteins were localized to a subdomain of recycling endosomes. Consistent with this, ARF6-containing membranes colocalized extensively with Rab11-positive late endosomes (Fig. 2 B, arrows). The localization of ARF6\textsuperscript{Q67L} at the plasma membrane and in recycling endosomes suggested that the ARF6\textsuperscript{Q67L} inhibition of DCV exocytosis was indirect and caused by alterations in endosomal plasma membrane trafficking.

Recruitment of PIP5K by ARF6\textsuperscript{Q67L} alters the cellular distribution of PIP\textsubscript{2}.

In other cell types, the expression of the GTPase-deficient ARF6\textsuperscript{Q67L} induces an accumulation of endosomal membranes leading to the intracellular trapping of plasma membrane constituents that normally recycle (Brown et al., 2001; Naslavsky et al., 2003). We considered the possibility that a plasma membrane constituent essential for regulated DCV exocytosis was depleted from the plasma membrane in cells expressing ARF6\textsuperscript{Q67L}. Indeed, SNAP-25, a plasma membrane SNARE required for DCV exocytosis (Banerjee et al., 1996), was found to substantially relocalize to an endosomal compartment in ARF6\textsuperscript{Q67L}-expressing cells (unpublished data); however, we were unable to rescue regulated hGH release by overexpressing SNAP-25. Another
plasma membrane constituent required for DCV exocytosis is PIP$_2$ (Hay et al., 1995), and we found that plasma
membrane pools of PIP$_2$ were dramatically altered by ARF6$^{Q67L}$ expression. A PLC$\delta$1 pleckstrin homology (PH)
domain–GFP fusion protein was expressed to detect membrane-
associated PIP$_2$ as described previously (Varnai and Balla,
1998). PC12 cells expressing high levels of wild-type ARF6
exhibited a uniform distribution of the PH-GFP at the
plasma membrane (Fig. 3 A), which colocalized with
plasma membrane ARF6. In contrast, cells expressing
ARF6$^{Q67L}$ exhibited a very different distribution of the PH-
GFP protein. At low expression levels, the PH-GFP local-
ized to the plasma membrane as well as to intracellular en-
dosomal membranes that contained ARF6$^{Q67L}$ (Fig. 3 A).
Cells expressing higher levels of ARF6$^{Q67L}$, representing
$\sim$25% of the cell population, accumulated intracellular in-
clusions enriched in the PH-GFP protein as well as
ARF6$^{Q67L}$ (Fig. 3 A, arrow). Plasma membrane–associated
PH-GFP was markedly decreased in these cells (Fig. 3 A,
insets), and the cells resembled ARF6$^{Q67L}$-expressing fibro-
blasts in which PIP$_2$-positive vacuoles accumulated (Brown
et al., 2001). This feature was unique for ARF6$^{Q67L}$
expression and was not observed for ARF6$^{WT}$ or ARF6$^{T27N}$ (Fig. 3 A).
Quantitation of digital images documented that the
PH-domain of PLC$\delta$1 was depleted from the plasma mem-
brane (Fig. 3 B, left) and preferentially accumulated on en-
dosomal membranes (Fig. 3 B, right) in cells expressing
ARF6$^{Q67L}$ and, to a much lesser extent, wild-type ARF6,
but not ARF6$^{T27N}$ or ARF6$^{N12I}$, another GTP-binding-
defective mutant (Fig. 3 B, P < 0.005).

A shift of the PH-GFP protein from plasma membrane to
endosomes in ARF6$^{Q67L}$-expressing cells implied that the
distribution of PIP$_2$ was markedly altered. To confirm this
directly, we determined the plasma membrane content of
PIP$_2$ in permeable cells using a PIP$_2$ antibody that revealed a
punctate staining pattern (Fig. 3 C, red channel). The PIP$_2$
content of the plasma membrane was not altered in cells ex-
pressing ARF6$^{WT}$ or ARF6$^{T27N}$ (Fig. 3 C), but was strongly
decreased in cells expressing ARF6$^{Q67L}$ (Fig. 3 C, arrows).
Quantitation of digital images documented a highly signifi-
cant decrease for plasma membrane PIP$_2$ in cells expressing
ARF6$^{Q67L}$ (Fig. 3 D). Overall, the results indicated that
ARF6$^{Q67L}$ expression promotes a redistribution of PIP$_2$ in
PC12 cells from the plasma membrane to an intracellular
endosomal compartment.

The preferential accumulation of PIP$_2$ in an endosomal
compartment might result from either the translocation of
plasma membrane containing PIP$_2$ or the redirected synthe-
thesis of PIP$_2$ on endosomes. ARF proteins directly regulate
PIP5K activity (Honda et al., 1999; Jones et al., 2000), and
the constitutively active ARF6$^{Q67L}$ localized to endosomes
may persistently activate PIP5K on endosomal membranes.
To examine this possibility directly, we coexpressed HA-
tagged PIP5K$\gamma$ with ARF6-GFP and determined their dis-
tribution (Fig. 4). PIP5K$\gamma$ preferentially localized to the
plasma membrane in cells expressing wild-type ARF6 or low
levels of ARF6Q67L where there was extensive colocalization of PIP5KIγ with the ARF6 proteins (Fig. 4). Cells expressing higher levels of ARF6Q67L exhibited a striking redistribution of PIP5KIγ to intracellular membranes where the enzyme colocalized with the ARF6 protein (Fig. 4, arrow). Plasma membrane PIP5KIγ was substantially decreased in these cells (Fig. 4, insets), indicating that ARF6Q67L diverts PIP5K to endosomal membranes. This redistribution of PIP5K would account for the accumulation of PIP2 in an endosomal compartment (Fig. 3 A, arrow). The results indicated that the GTPase-deficient ARF6Q67L induces a shift in the distribution of PIP5K and PIP2 from the plasma membrane to intracellular membranes.

Restoration of Ca2+-dependent exocytosis by PIP5K by a Ca2+-dependent mechanism
To further probe the mechanism by which ARF6Q67L inhibited Ca2+-dependent DCV exocytosis, we determined whether inhibition was preserved in permeable PC12 cells where DCV exocytosis can be directly triggered by Ca2+ addition (Hay and Martin, 1992). Expression of ARF6Q67L (but not wild-type ARF6 or ARF6T27N) resulted in the inhibition of Ca2+-triggered hGH release by ~48% in permeable cells (Fig. 5 A, middle bars), which corresponded closely to the intact cell assay results (Fig. 1 A). However, when MgATP and rat brain cytosol were included in the incubations to fully reconstitute Ca2+-dependent exocytosis, the inhibitory effect of ARF6Q67L was not observed (Fig. 5 A, right bars). This reversal of inhibition in the permeable cells was dependent on MgATP concentration (Fig. 5 C) and the presence of cytosol (compare Fig. 5 D with Fig. 5 A).

To determine whether MgATP and cytosol were sufficient for reversing the inhibitory effects of ARF6Q67L, a two-stage assay was used. MgATP- and cytosol-dependent priming reactions were conducted before Ca2+-triggering incubations, which contained Ca2+ and cytosol, but not MgATP (Hay and Martin, 1992). We found that the inhibition by ARF6Q67L was preserved in the two-stage assay over the entire time course of Ca2+-evoked hGH release (Fig. 5 B). Together with the other findings, this result indicates that all three constituents, MgATP, cytosol, and Ca2+, but no two of these alone, were essential for restoring regulated DCV exocytosis in ARF6Q67L-expressing cells.

Because the reversal of ARF6Q67L inhibition was dependent on MgATP and cytosol in permeable cells in which plasma membrane PIP2 and PIP5K had been depleted, it was very likely that the soluble PIP5K enzymes present in cytosol were responsible for the restoration of DCV exocytosis. To test the role of PIP5K directly, we attempted to rescue the inhibitory effect of ARF6Q67L by expressing the type Iγ PIP5K. Expression of PIP5KIγ successfully counteracted the inhibitory effect of ARF6Q67L on Ca2+-activated hGH release in permeable cells (Fig. 6 A). As anticipated, rescue by PIP5KIγ was only evident in incubations that included MgATP and failed to occur with expression of a kinase-dead truncation mutant of PIP5KIγ Δ345 (Fig. 6 A), which did not localize to the plasma membrane (unpublished data). In addition, the reversal of ARF6Q67L inhibition by expression of the wild-type PIP5KIγ enzyme did not require addition

**Figure 4.** PIP5KIγ is recruited to an endosomal compartment in ARF6Q67L-expressing PC12 cells. PC12 cells were cotransfected for 48 h with plasmids encoding EGFP-ARF6 proteins (green) and HA-PIP5KIγ (detected by HA antibody, red). Insets show magnified regions of the plasma membrane. Cells expressing relatively high levels of ARF6Q67L exhibited an endosomal accumulation of PIP5KIγ (arrow) and its depletion from the plasma membrane (inset). Bar, 10 μm.
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of cytosol (Fig. 6 A). These results indicate that reversal of ARF6Q67L inhibition can be achieved by PIP5KIγ overexpression, and they imply that the factor in cytosol required for the reversal is one of the abundant cytosolic PIP5K enzymes (Hay et al., 1995; Wenk et al., 2001). Indeed, direct addition of a pure recombinant PIP5KIγ protein to the permeable cell assay was effective in counteracting the inhibitory effects of ARF6Q67L (Fig. 6 B).

Multiple isoforms of PIP5K have been characterized consisting of types Iα, Iβ, and Iγ (Ishihara et al., 1998; Anderson et al., 1999). We found that expression of the type Iα (human) PIP5K also reversed the ARF6Q67L inhibition, whereas the kinase-dead (D309N/R427Q) PIP5KIα failed to provide rescue (Fig. 6 C). Both active and inactive enzymes were equally well expressed and associated with the plasma membrane (Fig. 6 D). Expression of the type Iβ (human) PIP5K provided only weak rescue, but was poorly expressed (unpublished data). Overall, the results strongly support the conclusion that the depletion of PIP2 from the plasma membrane induced by long-term expression of ARF6Q67L was responsible for the inhibition of regulated DCV exocytosis.

PIP5KIγ is regulated by phosphorylation- and ARF6-dependent mechanisms

These results on PIP5K provided an explanation for why MgATP and cytosol counteracted the inhibition of exocytosis mediated by ARF6Q67L expression, but they did not account for the additional requirement for Ca2+ in the rescue. Thus, we determined whether a requirement for Ca2+ in addition to MgATP and cytosol for counteracting ARF6Q67L inhibition might be mediated by the Ca2+-dependent activation of PKC, which may enhance PIP5K activity. Consistent with this, we found that the specific pseudosubstrate inhibitor of PKC fully blocked the reversal of ARF6Q67L inhibition observed with Ca2+ or MgATP in the absence of cytosol (RBC). Asterisks indicate significant (P < 0.01) differences with mock-transfected cells.
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Figure 6. **Expression or addition of PIP5KIy to permeable cells replaces cytosol in restoring DCV exocytosis in ARF6Q67L-expressing cells.** PC12 cells were transfected for 48 h with plasmids encoding hGH and either ARF6Q67L or PIP5KIy as indicated, and permeable cell hGH secretion assays were conducted. (A) Expression of PIP5KIy restored Ca2+-dependent hGH secretion in ARF6Q67L-expressing cells in the presence (but not in the absence) of MgATP. Expression of the A345 PIP5KIy mutant failed to restore Ca2+-dependent secretion. (B) Purified PIP5KIy replaced cytosol and restored Ca2+-dependent hGH secretion in ARF6Q67L-expressing cells. Incubations similar to those of A were conducted with Ca2+, MgATP, and 0.6 μg/ml (~7 nM) PIP5KIy where indicated. (C) Expression of PIP5KIy, but not a kinase-dead (D309N/R427Q) mutant, replaced cytosol in restoring Ca2+-dependent hGH secretion in ARF6Q67L-expressing cells in the presence (but not in the absence) of MgATP. Results are representative of at least two experiments with mean hGH values ± SEM (n = 4). (D) Wild-type and kinase-dead FLAG-tagged PIP5KIy proteins were expressed at similar levels and were plasma membrane localized. Immunocytochemistry with FLAG antibodies was conducted. Bar, 10 μm.

Asterisks indicate significant (P < 0.01) differences with mock-transfected cells.

Phosphorylatable S264A mutant of PIP5KIy, which was anticipated to be active relative to the wild-type enzyme. Both wild-type and S264A PIP5KIy were expressed at similar levels in transfected PC12 cells, and each localized to the plasma membrane (Fig. 7 D, left panels). Expression of the S264A PIP5KIy mutant enhanced Ca2+-dependent hGH secretion by ~30% (Fig. 7 B), whereas expression of wild-type PIP5KIy had little effect, consistent with the possible constitutive activity of the S264A PIP5KIy enzyme. Indeed, cells expressing the S264A mutant PIP5KIy exhibited substantially greater increases in plasma membrane PIP2 than those expressing the wild-type enzyme (Fig. 7 C and Fig. 7 Da, right). Quantitation of plasma membrane immunoreactive PIP2 (Fig. 7 Db) indicated that >32% of S264A PIP5KIy-expressing cells exceeded threshold values of immunoreactive PIP2, compared with <10% of wild-type PIP5KIy-expressing cells. Overall, the results indicate that phosphorylation at Ser264 may control the activity of PIP5KIy, and that plasma membrane PIP2 levels regulate Ca2+-dependent exocytosis.

ARF proteins were reported to directly activate purified type Iα (mouse) PIP5K (Honda et al., 1999; Jones et al., 2000) and ARF6 was shown to be recruited with PIP5K Iα to plasma membrane sites in fibroblasts (Honda et al., 1999), but a direct interaction of ARF6 with PIP5K has not been demonstrated. To determine whether ARF6 associates with PIP5K, immunoprecipitation experiments were conducted (Fig. 8 A). ARF6 was found to co-immunoprecipitate with PIP5K in resting cells, and this was enhanced by Ca2+ influx stimulated by K+ depolarization (Fig. 8 A, a and b). ARF6Q67L and ARF6N122I proteins exhibited similar associations with PIP5KIy that were enhanced by Ca2+ influx (Fig. 8 Ba). The results indicate that the association of ARF6 with PIP5K is regulated by a Ca2+-dependent mechanism.

To determine if this Ca2+-dependent mechanism involves PIP5K dephosphorylation, immunoprecipitations were conducted with cells expressing the nonphosphorylatable S264A PIP5KIy mutant. ARF6 exhibited substantial association with the S264A enzyme in resting cells, and this was not further enhanced by K+ depolarization and Ca2+ influx (Fig. 8 A, a and b). ARF6Q67L and ARF6N122I proteins exhibited a similar association with the S264A PIP5KIy that was not enhanced by Ca2+ influx (Fig. 8 Bb). The results indicate that, in addition to enhancing PIP5K activity, Ca2+ increases the association of PIP5K with ARF6. Interactions between ARF6 and PIP5K appear to be governed principally by the phosphorylation state of PIP5K rather than the guanine nucleotide–bound state of ARF6. The association of PIP5K with membrane-bound ARF6 may be important for targeting the
enzyme to its membrane PI(4)P substrate. Overall, the results suggest a pathway by which Ca\(^{2+}\) enhances PIP\(_2\) synthesis for DCV exocytosis via an ARF6-dependent PIP5K (Fig. 9).

**Discussion**

**Basis of the inhibition of DCV exocytosis by ARF6\(^{Q67L}\) expression**

In this work, we sought to characterize the role of ARF6 in the mechanisms that regulate Ca\(^{2+}\)-dependent DCV exocytosis in neuroendocrine PC12 cells. Expression of the constitutively active GTPase-deficient ARF6\(^{Q67L}\) mutant exerted a strong inhibitory effect on regulated DCV exocytosis. Subsequent analyses clarified the nature of the inhibitory effect as consisting of a diversion of plasma membrane constituents that are required for DCV exocytosis, PIP5K, and PIP\(_2\), to endosomal membranes with their corresponding depletion from the plasma membrane.

As reported for other cell types (D’Souza-Schorey et al., 1995; Peters et al., 1995), the distribution of ARF6 proteins in PC12 cells was restricted to the plasma membrane and endosomal membranes. The restricted localization of ARF6 to these compartments in PC12 cells was anticipated in view of the reported biochemical localization of ARF6 to the DCVs of chromaffin cells (Galas et al., 1997; Caumont et al., 1998). However, the degree of purity of DCVs was not assessed in those experiments, and immunocytochemical localization analyses were not reported. Vitale et al. (2002) reported immunocytotoxicomical experiments in PC12 cells indicating that ARF6 proteins colocalize with DCV markers.
However, there was a broad cytoplasmic distribution of both ARF6 and DCV markers by fluorescence microscopy that would preclude resolution of the clearly distinct distributions that we observed (Fig. 1 C). Moreover, an immunogold electron microscopic analysis (Vitale et al., 2002) was consistent with the localization of ARF6 proteins to cytoplasmic membranes other than DCVs.

In other cell types, ARF6 traffics between endosomal and plasma membrane compartments and regulates endosome-plasma membrane trafficking. Perturbations in endosome-plasma membrane trafficking that result from expression of the constitutively active ARF6 Q67L mutant vary with cell type, internalized protein, time of expression, and distribution of the actin cytoskeleton (Radhakrishna et al., 1996; Radhakrishna and Donaldson, 1997; Altschuler et al., 1999; Brown et al., 2001; Delaney et al., 2002; Palacios et al., 2002). Long-term expression of the constitutively active ARF6 Q67L protein in PC12 cells resulted in the accumulation of a subset of plasma membrane constituents in an endosomal compartment. This likely resulted from both accelerated internalization as well as inhibited recycling of constituents back to the plasma membrane. Activated ARF6 likely enhances both clathrin-dependent and -independent pathways of inter-

Figure 8. ARF6 associates with PIP5K1y. PC12 cells were cotransfected with plasmids encoding EGFP-ARF6WT, EGFP-ARF6Q67L, or EGFP-ARF6N122I and HA-tagged wild-type or S264A PIP5K1y as indicated. (A, a) Ca2+ influx and dephosphorylation promote the association of ARF6 with PIP5K1y. Cells cotransfected for 48 h with indicated plasmids were incubated in Na+-containing (control, CNT) or K+-containing (stimulated, S) buffers for 5 min. HA antibody immunoprecipitates were prepared from detergent lysates and analyzed by Western blotting for EGFP-ARF6 and HA-PIP5K1y. Co-immunoprecipitation of EGFP-ARF6 with HA antibody was enhanced by K+ depolarization in PIP5K1y-expressing cells. In S264A PIP5K1y-expressing cells, coimmunoprecipitation was increased in resting cells and not enhanced by K+ depolarization. (A, b) Quantitation of ARF6 coimmunoprecipitation with PIP5K1y from 3 experiments (± SEM) similar to that in panel a. (B) Co-immunoprecipitation studies similar to those in panel A were conducted for cells cotransfected with plasmids encoding ARF6 WT, ARF6Q67L, and ARF6N122I with (a) wild-type HA-PIP5K1y or (b) HA-S264A PIP5K1y. Values under each lane pair indicate fold stimulation by K+ depolarization.

Figure 9. ARF6 regulation of Ca2+-dependent DCV exocytosis in PC12 cells. (1) Ca2+ influx stimulates exocytosis of docked DCVs, but only if plasma membrane PI(2)P3 is available. (2) Ca2+ influx promotes the dephosphorylation of PIP5K1y. (3) Dephospho-PIP5K1y associates with ARF6 and is activated to synthesize PI(2)P3 for DCV exocytosis. (4) Increased PI(2)P3 synthesis drives endocytosis and retrieval of the DCV membrane after exocytosis. (5) Constitutive endocytosis may be enhanced by ARF6Q67L stimulation of PIP5K1y. (6 and 7) Lack of GTP hydrolysis on ARF6Q67L and constitutive PI(2)P3 production on endosomes prevent recycling to the plasma membrane. Entrapment of plasma membrane constituents and diversion of PIP5K1y and PI(2)P3 to endosomes in ARF6Q67L-expressing cells results in the inhibition of DCV exocytosis.
nalization in PC12 cells. Plasma membrane SNAP-25 is co-
internalized with PIP_{2}-containing, ARF6^{Q67L}-enriched mem-
branes by a dynamin-independent pathway (unpublished data),
but ARF6^{Q67L}-expressing cells also exhibit increased ini-
tial rates of transferrin uptake (unpublished data) presumed
to be via clathrin- and dynamin-mediated endocytosis. ARF6^{Q67L}
expression also accelerates the formation of synaptic vesicle-like endosomes in PC12 cells (Powelka and Buckley,
2001) that are formed by a clathrin-dependent pathway
(Strasser et al., 1999). These results indicate that multiple en-
docytic pathways are enhanced by expression of the constitu-
tively active ARF6^{Q67L} in PC12 cells.

ARF proteins directly activate PIP5K enzymes and regulate
the synthesis of PIP_{2} (Honda et al., 1999; Jones et al.,
2000). Plasma membrane PIP_{2} is an essential cofactor for the
 trafficking of recycling endosomes back to the cell surface (Franco et al., 1999; Naslavsky et al.,
2003). Because this fails to occur in the GTPase-deficient ARF6^{Q67L} mutant, plasma membrane constituents are
entrapped in an endosomal pool (Brown et al., 2001; Delaney et al., 2002; Palacios et al., 2002; Naslavsky et al., 2003). In PC12 cells expressing ARF6^{Q67L}, an endosomal compart-
ment containing ARF6^{Q67L}, PIP_{2}, and PIP5K formed,
which resulted in the depletion of these constituents from
the plasma membrane.

Plasma membrane PIP_{2} is essential for Ca^{2+}-dependent
DCV exocytosis at the plasma membrane (Hay et al., 1995;
Holz et al., 2000). That the depletion of PIP_{2} from the
plasma membrane in ARF6^{Q67L}-expressing PC12 cells was
responsible for the inhibition of DCV exocytosis was estab-
lished by demonstrating that expression or addition of
PIP5K was capable of restoring regulated exocytosis in per-
meable cells incubated with MgATP. These results ac-
counted for the absence of inhibition in permeable cell re-
constitutions in which cytosol and MgATP were included,
and strongly support the conclusion that plasma membrane
PIP_{2} is rate-limiting for DCV exocytosis in ARF6^{Q67L}--
expressing cells. These results reinforce the conclusion that
PIP_{2} is essential for DCV exocytosis and indicate that ARF6
regulation of PIP5K plays a role in maintaining plasma
membrane pools of PIP_{2} required for exocytosis.

Regulation of PIP5K by ARF6 for Ca^{2+}-dependent
DCV exocytosis
The restoration of Ca^{2+}-dependent DCV exocytosis in
ARF6^{Q67L}-expressing permeable cells by PIP5K expression
or addition explained the roles of cytosol and MgATP in the
restoration, but not that of Ca^{2+}. The role of Ca^{2+} in
the restoration may be mediated by PKC based on the effective-
ness of a specific PKC pseudosubstrate inhibitor in blocking
it. Although the involvement of PKC in Ca^{2+}-dependent
DCV exocytosis is well established (Nishizaki et al., 1992;
Chen et al., 1999), its precise role and the nature of PKC
substrates involved in DCV exocytosis remain unknown.
Park et al. (2001) reported that PKC contributes to the
maintenance of cellular PIP_{2} levels by regulating protein
phosphatase-1, which catalyzes the dephosphorylation and
activation of PIP5KI. The dephospho-PIP5KI\textalpha enzyme ex-
hibits increased lipid kinase activity in vitro (Park et al.,
2001), and we found that the cognate nonphosphorylatable
S264A PIP5KI\textgamma enzyme was activated to synthesize plasma
membrane PIP_{2} and enhance Ca^{2+}-dependent DCV exocy-
tosis in PC12 cells. This result indicates that a nonphosphor-
ylatable, activated PIP5KI\textgamma enzyme is a positive effector for
regulated exocytosis, which is particularly significant because
Wenk et al. (2001) found that PIP5KI\textgamma undergoes dephos-
phorylation in response to Ca^{2+} influx into synaptosomes.
Eberhard and Holz (1991) also reported that Ca^{2+} influx in-
creases PIP_{2} synthesis in chromaffin cells. Together, these
results indicate that PIP5K enzymes are Ca^{2+}-dependent ef-
fectors that are activated to promote PIP_{2} synthesis at the
plasma membrane.

ARF6 at the plasma membrane likely mediates the mem-
brane targeting and localization of the activated PIP5K en-
zymes (Honda et al., 1999), and we found that wild-type
PIP5K exhibited an increased association with ARF6 in re-
response to Ca^{2+} influx into PC12 cells. The Ca^{2+}-dependent
increase in ARF6 association may result from PIP5K dephos-
phorylation because the S264A mutant exhibited a strongly
enhanced association with ARF6 that was not further in-
creased by Ca^{2+} influx. These results are consistent with a
model (Fig. 9) in which Ca^{2+} influx triggers the dephosphor-
ylation of PIP5K, its increased association with ARF6, and
its activation for plasma membrane PIP_{2} synthesis.

Although the ATP-dependent priming of DCVs for Ca^{2+}--
dependent exocytosis is known to require PIP_{2} synthesis
(Hay et al., 1995), our results provide the first indication that
PIP_{2} synthesis is regulated through an ARF6-dependent
Ca^{2+}-triggered mechanism. The reported Ca^{2+} regulation
of DCV priming (Bittner and Holz, 1992; Rettig and Neher,
2002) may in part be mediated by this ARF6-dependent
pathway. The Ca^{2+}-triggered stimulation of PIP5K activity,
particularly of the type \textalpha isoform that is enriched at syn-
apses (Wenk et al., 2001), would play an important role in
enhancing Ca^{2+}-triggered exocytosis and endocytosis. In
nerve terminals, plasma membrane PIP_{2} levels decrease im-
mediately upon Ca^{2+} influx (Michaela et al., 2001), and the
Ca^{2+}-dependent recruitment and stimulation of PIP5K may
be essential for the synthesis of additional PIP_{2} needed to
maintain rates of vesicle exocytosis and the endocytic re-
trieval of the vesicle membrane.

In conclusion, we present evidence that ARF6, through
its regulation of PIP5K activity and PIP_{2} synthesis, controls
at least two types of peripheral membrane trafficking events
in PC12 cells (Fig. 9). Ca^{2+}-dependent DCV exocytosis re-
quires a plasma membrane pool of PIP_{2} whose synthesis
may be acutely regulated by Ca^{2+}-dependent activation of
ARF6-dependent PIP5K enzymes. ARF6-dependent
PIP5K enzymes also regulate endosome-plasma membrane
trafficking. An imbalance in the ARF6-regulated endosome
recycling pathway can result in the depletion of plasma membrane constituents such as PIP2 that are essential for DCV exocytosis.

Materials and methods

 Constructs

The plasmid pXS-SRAs containing COOH-terminal HA-tagged human ARF6 wild type, Q67L, and T27N were provided by J. Donaldson of the National Institutes of Health (Bethesda, MD). Constructs encoding COOH-terminal fusions of wild-type, Q67L, and T27N ARF6 proteins with EGFP were generated by PCR using the primers 5’-CGATAACCCTATATCGACCC-3’ and 5’-CGATATCTGACGATTTGC-3’. The PCR product was cleaved with HindIII and PstI and inserted into HindIII-PstI-digested pEGFP-N1 (CLONTECH Laboratories, Inc.). All constructs were confirmed by sequencing. Coding sequences from a pBS plasmid containing HA-tagged murine full-length PIPSy provided by H. Ishihara (University of Tokyo, Tokyo, Japan) were excised with HindIII and VspI and subcloned into pcDNA 3.1 (Invitrogen). HA-tagged murine Δ 344–635 PIP5KSy was excised with HindIII and Alw441 and subcloned into pcDNA 3.1. The QuikChange® Site-Directed Mutagenesis method (Stratagene) was used to generate ARF6 PIP5K (S264A) mutant using the sense primer 3’-CACGTA-

Preparation of permeable PC12 cells and hGH secretion assays

PC12 cells were cultured as described previously (Hay and Martin, 1992). Cells were transfected with 50 μg DNA in cytotransfert buffer (120 mM KCl, 0.15 mM CaCl2, 10 mM KH2PO4, 2 mM EGTA, 5 mM MgCl2, 2 mM MgATP, 5 mM glutathione, and 25 mM Hepes, pH 7.6) using an electroporation apparatus (Electroporator II; Invitrogen) set at 1,000 μF and 330 V. 48 h after transfection, cells were washed with PBS, fixed with 4% formaldehyde (wt/vol), permeabilized with PBS containing 0.3% Triton X-100, and blocked in 10% FBS in PBS before antibody incubations. For PIP2 localization in intact cells using anti-PIP2 antibody, we used methods described previously (Laux et al., 2000). For PIP, localization in permeable PC12 cells, the permeable cells were tethered to glutaraldehyde–activated, polylysine–coated glass coverslips (Wiedman et al., 1993). Antibodies were used polyclonal ARF6 (J. Donaldson and V. Klenchin; University of Wisconsin, Madison, WI); monoclonal and polyclonal HA (AbCam); monoclonal FLAG M2 (Sigma-Aldrich); monoclonal SNAP-25 (Stemberger); monoclonal TGN38 (K. Howell, University of Colorado, Denver, Co); polyclonal mannosidase II (K. Moremen, University of Georgia, Athens, GA); monoclonal Rab11 (Zymed Laboratories); monoclonal PIP2 (K. Fukami, University of Tokyo); and monoclonal chromogranin B (W. Hunter, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). For transferrin uptake, transfected cells were incubated in medium containing 50 μg/ml Texas red–conjugated transferrin (Molecular Probes, Inc.) for 30 min at 37°C, washed in PBS, and fixed. Images were obtained on an imaging microscope (MRC-600; Bio-Rad Laboratories) with a 63× oil immersion objective. Quantitation of fluorescence was conducted with MetaMorph® software (Universal Imaging Corp.) or SCION Image software (SCION Corp.).

Immunoprecipitation assays

After transfection, cells were washed and incubated for 5 min with Locke’s or elevated K+ buffer before harvesting in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 or NP-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and Boehringer complete protease inhibitor cocktail). Lysates were sedimented and supernatants were assayed with protein G-Sepharose beads before conducting 3–16-h incubations with HA-tagged human ARF6 or HA-Sepharose beads. Beads were washed five times with lysis buffer and eluted in sample buffer containing 0.2 M DTT for analysis by immunoblotting with ARF6 or HA antibodies.

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