ADP Ribosylation Factor 1 Is Required for Synaptic Vesicle Budding in PC12 Cells

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Abstract. Carrier vesicle generation from donor membranes typically progresses through a GTP-dependent recruitment of coats to membranes. Here we explore the role of ADP ribosylation factor (ARF) 1, one of the GTP-binding proteins that recruit coats, in the production of neuroendocrine synaptic vesicles (SVs) from PC12 cell membranes. Brefeldin A (BFA) strongly and reversibly inhibited SV formation in vivo in three different PC12 cell lines expressing vesicle-associated membrane protein–T Antigen derivatives. Other membrane traffic events remained unaffected by the drug, and the BFA effects were not mimicked by drugs known to interfere with formation of other classes of vesicles. The involvement of ARF proteins in the budding of SVs was addressed in a cell-free reconstitution system (Desnos, C., L. Clift-O’Grady, and R.B. Kelly. 1995. J. Cell Biol. 130:1041–1049). A peptide spanning the effector domain of human ARF1 (2–17) and recombinant ARF1 mutated in its GTPase activity, both inhibited the formation of SVs of the correct size. During in vitro incubation in the presence of the mutant ARFs, the labeled precursor membranes acquired different densities, suggesting that the two ARF mutations block at different biosynthetic steps. Cell-free SV formation in the presence of a high molecular weight, ARF-depleted fraction from brain cytosol was significantly enhanced by the addition of recombinant myristoylated native ARF1. Thus, the generation of SVs from PC12 cell membranes requires ARF and uses its GTPase activity, probably to regulate coating phenomena.

1. Abbreviations used in this paper: ARF, ADP ribosylation factor; BFA, brefeldin A; COPI and COPII, coat proteins I-II; HMW, high molecular weight; SV, synaptic vesicle; TAg, T Antigen; VAMP, vesicle-associated membrane protein.

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The GDP-GTP exchange activity that replaces GDP bound to ARF proteins with GTP is inhibited by BFA (Donaldson et al., 1992; Helms and Rothman, 1992). The GDP form of ARF1 is unable to bind membranes and consequently, to recruit coats (Robinson and Kreis, 1992; Donaldson and Klausner, 1994). The selectivity of BFA is such that, if a membrane traffic event is sensitive to BFA, it is predicted to require ARF proteins. Inhibition of intra-Golgi and ER to Golgi traffic by BFA probably involves the COPI coatomers. BFA also interferes with coats other than COPI, especially those involved in budding from TGN. Thus it inhibits the formation of vesicles from the TGN (Simon et al., 1996) and causes the redistribution of assembly protein 1 and clathrin to the cytosol (Robinson and Kreis, 1992). Post-Golgi trafficking of the mannose-6-phosphate receptor (Wood et al., 1991) and the maturation of secretory granules (Dittie et al., 1996) are also sensitive to BFA. In addition to clathrin and COPI, BFA affects the recruitment of other “coating” molecules, such as the p47–βNAP complex (Simpson et al., 1996; Dell’Angelica et al., 1997) and p200 (Narula and Stow, 1995) to TGN membranes.

Some endocytotic pathways are also sensitive to BFA. For example, the delivery of polyimmunoglobulin A (pIgA)
to plasma membrane from the specialized apical endo-
osome in epithelial MDCK cells, or from dendritic endo-
somes in hippocampal neurons, is inhibited by BFA (Hun-
ziker et al., 1991; Barroso and Sztul, 1994). BFA-sensitive
recruitment of COP1-related proteins and ARF proteins
to endosomes has also been reported (Whitney et al., 1995; 
Cavenagh et al., 1996).

The formation of synaptic vesicles at nerve terminals is a
specialized endocytotic pathway that has many similarities
to the formation of carrier vesicles from Golgi mem-
branes. In this case, the donor membrane for synaptic vesicle
formation is the plasma membrane or the endosome
(De Camilli and Takei, 1996). Morphological evidence
strongly suggests that synaptic vesicles are generated in
nerve terminals through a coat-dependent mechanism
(Shupliakov et al., 1997). In lysed nerve terminals, recruit-
ment of dynamin and clathrin coats to membranous or-
ganelles is modulated by nonhydrolyzable GTP analogues
(Takei et al., 1996). Cell-free reconstitution assays of neu-oendocrine synaptic vesicle (SV) formation in PC12 cell
extracts showed that GTPγS blocks the generation of
properly sized SVs (Desnos et al., 1995), but the identity of
the GTP-binding protein or proteins was not determined.

In this paper, we show that reagents that interfere with
the cycling of ARF1 between cytosol and membranes
block SV formation in neuroendocrine PC12 cells. SV for-
mation was reconstituted in vitro using recombinant
ARF1 and a cytosol-derived high molecular weight frac-
tion. Since SV production in vitro is from an endocytotic
pool, these results suggest that coating mechanisms associ-
ated with ER and Golgi biosynthetic pathways are also
associated with at least one endocytotic pathway.

Materials and Methods

125I-Labeled Na and ECL reagents were obtained from Amersham Corp.
(Arlington Heights, IL). Iodogen came from Pierce Chemical Co. (Rock-
ford, IL). ATP, GTPγS, creatine phosphate, creatine kinase, and Sephadex
G25 spin columns were purchased from Boehringer Mannheim Biochem-
icals (Indianapolis, IN). DEAE Sephacel, Superose 6, ProtG-Sepharose 4
Fast Flow, and Bl21 Escherichia coli strain were obtained from Pharma-
cia Biotech AB (Uppsala, Sweden). Ultragel AcA 54 was from Biosepra
(Marlborough, MA). Brefeldin A was purchased from Epicentre Technol-
ogies (Madison, WI). Bafilomycin A1 was obtained from Calbiochem-
Novabiochem Corp. (LaJolla, CA). Iliumaquinone and avarol were kindly
provided by Dr. V. Malhotra (University of California, San Diego, CA).
Cell culture media and reagents were obtained from the University of Cal-
ifornia Cell Culture Facility (San Francisco, CA). Geniticin (G418) and
isopropylthio-β-μ-galactoside were obtained from GIBCO BRL (Gaith-
ersburg, MD). All the other reagent grade chemicals were purchased ei-
ther from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Co.
(Fairlawn, NJ), or Calbiochem-Novabiochem Corp. Female Sprague-
Dawley rats were from Bantin and Kingman (Fremont, CA).

Peptides

A 16-amino acid peptide (GNIFANLFKGLFGKKE) corresponding to residues 2–17 of the human ARF1 NH2 terminus (ARF 2–17 peptide), and
a scrambled peptide of identical composition (FLKANGIGKNGFE)
(Chen and Shields, 1996) were either a gift of Dr. D. Shields (Albert Ein-
stein College of Medicine, New York) or were purchased from Chiron
Mimotopes (San Diego, CA).

Cell Culture

PC12 cell lines stably transfected with rat vesicle-associated membrane
protein–T Antigen (VAMP-TAg) and with the mutants N49A and del 61–
70 were grown in DMEM H-21 media supplemented with 10% horse serum,
5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and with 250 
μg/ml G418. The cells were treated for 24 h before the experiments by 6 
mM sodium butyrate to induce the expression of the different VAMP con-
 structs as described (Grote et al., 1995).

Cell Labeling and Subcellular Fractionation

PC12 cells containing the different VAMP-TAg constructs were labeled with 125I-KT3 mAb against the TAg epitope tag following the methods of
Desnos et al. (1995). Briefly, confluent dishes of cells were washed at 0°C
with labeling buffer (PBS supplemented with 3% BSA, 0.3 mM CaCl2, 0.3 
M MgCl2, and 1 mg/ml glucose), labeled in the same buffer at 0°C for 15 
min, and then transferred at 15°C for 40 min. After labeling, the cells were
extensively washed in the same buffer, scraped, and sedimented at 800 × 
103 g for 5 min. To examine the effect of reagents on the in vivo generation of
SVs, cells were first labeled at 15°C and then treated in DME H-21, 10 
mm Hepes, pH 7.4, for 15 min at 0°C with different drugs, and then trans-
ferred to 37°C for times indicated. Similar results were obtained by prein-
cubating cells with the drugs for 30 min at 15°C before warming them. La-
abeled cells were chilled at 0°C, and then washed and pelleted as described
above. Cell pellets were gently resuspended in intracellular buffer (38 mM
potassium aspartate, 38 mM potassium glutamate, 38 mM potassium glu-
conate, 20 mM potassium MOPS, pH 7.2, 5 mM reduced glutathione, 5 
mM sodium carbonate, 2.5 mM magnesium sulfate) containing protease
inhibitors. Homogenizations were performed as described using a ball
bearing homogenizer (cell cracker; European Molecular Biology Labora-
tory, Heidelberg) with 12 μm clearance. For in vivo experiments, the dif-
ferent conditions in the same experiment were normalized by seeding
equal amounts of cells and by loading equal amount of homogenate pro-
teins during subcellular fractionation.

A postnuclear supernatant (S1; 1,000 × g for 5 min) was sedimented at
27,000 × g for 35 min, and the supernatant generated (S2) was used to iden-
tify SVs by velocity sedimentation. S2 (250 μl, 3–5 mg/ml) were loaded
onto 5–25% glycerol gradients prepared in intracellular buffer over a 50%
sucrose cushion, and then spun at 218,000 × g for 75 min in a rotor (SW55
 Beckman Instruments, Inc., Palo Alto, CA). Fractions (17 and 18) were
collected from the bottom and counted on a gamma counter. For either in
vivo or cell-free reactions, the amount of labeled synaptic vesicles gener-
ated was determined by integration of the total cpm in the SV peak (frac-
tions 8–13) minus the background cpm determined by integration of the
same fractions in parallel reactions, kept at 4°C.

In Vitro Budding Assay

VAMP-TAg/N49A PC12 cells were labeled at 15°C as described above.
The assay was performed as described by Desnos et al. (1995). Aliquots of
1 mg of homogenate were incubated for 30 min at 37°C or 4°C in the pres-
ence of an ATP regenerating system (1 mM ATP, 8 mM creatine phos-
phate, 5 μg/ml creatine kinase), and 1 or 3 mg/ml of rat brain cytosol pre-
pared as described (Desnos et al., 1995). ARF1 was removed from rat
brain cytosol using a Superose 6 sizing column, preequilibrated in intracel-
llular buffer, as described (Waters et al., 1991; Stamnes and Rothman, 1993).
In reactions containing peptides or recombinant ARF1 proteins, mixtures
were preincubated for 15 min at 0°C before warming, whereas those con-
taining antibodies or glutathione-S-transferase (GST) fusion proteins were
kept at 0°C for 3 h. The reactions were stopped by chilling to 0°C for 10
min before fractionation.

Clathrin heavy chains were quantitatively removed from cytosol using
the X22 mAb (Brodsky, 1985). Briefly 60 μg of antibody were bound
overnight to 40 μl of packed protein G-Sepharose in 0.5 ml of intracellu-
lar buffer. Unbound Ig was extensively washed in intracellular buffer and
the X22 affinity matrix was incubated with 0.8 mg of cytosol for 2 h at 4°C
with gentle rocking. The gel was spun and the cytosol recovered for in
vitro reactions. The beads were washed from cytosolic proteins and the
clathrin bound to them, and clathrin remaining in the cytosol was deter-
mined by immunoblotting using the TD1 anti-clathrin heavy chain anti-
body (Nathke et al., 1992). Blots were performed using the enhanced
chemiluminescence (Amersham Corp.) system. The amount of clathrin
remaining in the cytosol was determined after exposing films for sev-
eral times. Images were acquired in a digital image system (IS1000; Alpha
Innotec Corp., San Leandro, CA) and quantified using the National Insti-
tutes of Health Image 1.60 program. The clathrin removed was 90–95%
of the total. The cytosol protein concentration before and after the depletion
remained constant.
Expression and Purification of Recombinant Proteins

Wild-type, Q71L, and T31N mutant human ARF1 cDNAs subcloned in the pET11d expression vector and yeast N-myristoyl transferase (pBB131) were kindly provided by Dr. D. Shields. Native and mutant proteins were coexpressed with N-myristoyl transferase in BL21 E. coli strain and purified as described (Randazzo et al., 1992). Myristylated recombinant proteins were extensively dialyzed against intracellular buffer, concentrated to 2–3 mg/ml in a Centriprep 10 (Amicon Corp., Danvers, MA). Aliquots were flash frozen in liquid N\textsubscript{2} and stored at −70°C. Purity, assessed by SDS-PAGE and Coomassie blue staining, was ~80%. The identity of the ARF1 protein was confirmed by immunoblot using the 1D9 mAb (kindly provided by Dr. R. Kahn, Emory University, Atlanta, GA).

It also bound to PC12 membranes in the presence of GTP\textgamma{}S (Walker et al., 1992). WBPI-GST fusion proteins were purified following manufacturer’s instructions. Recombinant proteins were concentrated in a Centriprep 30 (Amicon Corp.) to 1–3 mg/ml and extensively dialyzed against intracellular buffer.

Measurements of VAMP-TAg/N49A Endocytosis and Transfer Recycling

Endocytosis of VAMP-TAg/N49A protein was assessed as described (Grote and Kelly, 1996). Cells were plated 2 d before the assay on poly-D-lysine–coated dishes. Surface labeling was performed with 125\texttextsuperscript{I}-KT3 (3.3 μg/ml) for 2 h at 0°C. N49A/PC12 cells were extensively washed in labeling buffer, and then incubated for 15 min at 0°C either in the absence or presence of BFA (5 μg/ml) in DME H-21, 10 mM Hepes, pH 7.4, before warming to 37°C for different times. Endocytosis was stopped at 0°C for 10 min. Antibody remaining on the cell surface was removed by acid stripping with labeling buffer supplemented with 30 mM glycine adjusted to pH 2.4. Acid-resistant antibody was collected by lysing the cells in 2M NaOH. Calculation and expression of the results were done as described (Grote and Kelly, 1996).

N49A/PC12 cells were incubated in serum-free media for 90 min at 37°C before labeling. 125\texttextsuperscript{I}-rat transferrin (0.2 μg/ml) was bound for 15 min at 0°C and internalized at 15°C for 40 min as described above. Unbound transferrin was washed at 0°C in DME H-21 media, 0.2% BSA, 10 mM Hepes, pH 7.4. Surface-bound ligand was removed at 0°C by three washes of 6 min in mild acidic buffer (0.5 M NaCl, 50 mM MES, pH 5.0) (Martyts et al., 1995), followed by three washes in PBS, 0.3 mM CaCl\textsubscript{2}, 0.3 mM MgCl\textsubscript{2}. These washes removed 70–80% of the surface-associated 125\texttextsuperscript{I}-rat transferrin. N49A cells were then incubated at 0°C in DME H-21 media, 10 mM Hepes, pH 7.4, supplemented with 100 μg/ml of cold iron-loaded rat transferrin in the presence of either methanol (0.001%) or BFA (5 μg/ml) at 15°C for 15 min. Cells were then warmed for different times and the reactions stopped at 0°C. 125\texttextsuperscript{I}-rat transferrin that was released to the supernatant, and that which remained cell associated, were both determined after TCA precipitation.

Confocal Immunofluorescence Microscopy

Immunofluorescence procedures and confocal microscopy have been detailed elsewhere (Bonzellus et al., 1994). N49A/PC12 cells were plated in poly-D-lysine–coated Permanox\textsuperscript{TM} slides (Nunc Inc., Naperville, IL) 2 d before staining. Cells were loaded in vivo with KT3 antibodies (10 μg/ml) in labeling buffer and washed as mentioned above. To observe total VAMP-TAg/N49A, cells were fixed in 4% paraformaldehyde in PBS. For the endocytosis assays, cells (before fixation) were acid stripped in uptake buffer supplemented with 30 mM glycine adjusted at pH 2.4 as previously described (Grote and Kelly, 1996). Fixed cells were permeabilized in 0.2% saponin in PBS, 2% BSA, 1% fish skin gelatin, and incubated with affinity-purified fluorescent-labeled goat anti-mouse IgG (Cappel Laboratories, Malvern, PA). Observation and image acquisition were performed in a Bio Rad MRC 600 confocal laser scanning microscope (Hercules, CA).

Other Procedures

KT3 mAbs and iron-loaded rat transferrin were iodinated in iodogen-coated tubes according to Grote and Kelly (1996). Protein assays were performed using the Bio-Rad Protein Assay Dye Reagent (Hercules, CA) using BSA as standard.

Results

In Vivo SV Biogenesis is Reversibly Inhibited by BFA

To determine whether SV biogenesis is an ARF-mediated process, the effect of BFA upon synaptic vesicle biogenesis in PC12 cells was examined in vivo. The PC12 cell line was stably transfected with a luminaly tagged VAMP construct bearing a point mutation in the cytoplasmic tail, N49A (N49A/PC12). This VAMP derivative shows increased targeting to SV compared with wild type (Grote et al., 1995). It shows even more specific targeting to SVs than the del61-70 mutation used in a previous study (Desnos et al., 1995). Incubating intact cells at 15°C with iodinated antibodies (125\texttextsuperscript{I}-KT3) against the luminal epitope labels plasma membrane and intracellular compartments without labeling synaptic vesicles (Desnos et al., 1995). Internalizing the antibody at 15°C, removing free antibody, and then incubating the cells at 37°C caused the appearance of antibody-labeled SVs, monitored by their migration on glycerol velocity gradients (Fig. 1a, ○). Free antibody remains at the top (right) of the velocity gradients. The addition of BFA (5 μg/ml) inhibited the production of labeled vesicles upon warming to 37°C (Fig. 1a, ◯). After washing the drug out, the BFA-mediated block was completely reversed within 15 min (Fig. 1a, ●). The fast reversibility of the block argues against nonspecific toxicity effects.

BFA was effective at concentrations as low as 0.1 μg/ml (Fig. 1b) with a maximal inhibition (80–95%) observed at concentrations between 5–10 μg/ml (n = 25). This dose—

Figure 1. In vivo synaptic vesicle biogenesis is inhibited by BFA in a concentration dependent and reversible way. Stably transfected PC12 cells bearing the N49A VAMP-TAg construct were labeled at 15°C for 40 min in the presence of 125\texttextsuperscript{I}-KT3 antibody, and the unbound antibody washed extensively. (a) After washing out the free antibody, cells were incubated at 0°C for 15 min either in the absence (○) or presence (◇) of BFA (5 μg/ml). Control and BFA-treated cells were warmed for 15 min. To measure reversibility, one of the BFA-containing plates was washed thoroughly at 0°C and then reincubated at 37°C for another 15 min in the absence of BFA (●). The reactions were stopped, cells homogenized, and high speed supernatants processed for velocity sedimentation analysis as described in Methods. The data are one example of two independent experiments. (b) Cells were incubated as described in a with 0.1–10 μg/ml of BFA, and then transferred at 37°C for 15 min and processed as described. BFA inhibited vesicle production in a dose-dependent way (0% inhibition corresponds to 3550 cpm in the SV peak).
response inhibition was similar to that described for the block of transcytosis in MDCK cells (Hunziker et al., 1991) and was slightly higher than that required to disrupt the Golgi complex in fibroblasts (Lippincott-Schwartz et al., 1991b). In the absence of the drug, the generation of 125I-KT3–loaded vesicles from the 15°C compartment reached a plateau in less than 20 min (Fig. 2 a). Inhibition by BFA was a fast phenomenon detected as early as 7.5 min (data not shown) and maintained throughout the incubation period (Fig. 2 a). Similar inhibition was obtained in a PC12 cell line expressing del61–70 VAMP, which also shows enhanced targeting to SVs (Fig. 2 b; Grote et al., 1995). Cells transfected with wild-type VAMP showed similar inhibition (data not shown), but vesicles were labeled less efficiently and so the inhibition appeared to be less dramatic.

The plateau in labeling (Fig. 2) could be due to accumulation of label in a stable, slowly turning over compartment. Alternatively, the plateau could mean that the vesicles recycle and a dynamic equilibrium is reached. To analyze the turnover time of the 125I-KT3–containing synaptic vesicles, N49A/PC12 cells were labeled at 15°C, and then warmed to 37°C for 15 min to allow the formation of 125I-KT3–loaded SVs. The cells were then incubated at 37°C for different times, either in the absence or presence of BFA to block the formation of newly labeled vesicles. In the absence of BFA, the amount of SVs decreased slightly after 60 min (Fig. 3, ○). In contrast, in BFA-treated cells, the amount of 125I-KT3–loaded SVs detected in velocity gradients dropped to 50% in 36 ± 2 min (n = 3) (Fig. 3, ○). About 60% of the labeled SVs disappeared rapidly, indicating that the majority of newly formed SVs are not inert, but are recycling through a pool that is continuously fed from a BFA-sensitive compartment.

**In Vivo Effects of BFA on the Morphology of the KT3-labeled VAMP-containing Endosomes**

To identify the morphological consequences of BFA treatment, VAMP-TAg N49A–containing endosomes were labeled by in vivo uptake of unlabeled KT3 antibody and examined by confocal immunofluorescence microscopy. Intracellular labeling was distinguished from plasma membrane labeling by acid stripping the cell surface. Steady-state KT3-loading at 37°C revealed fluorescent signals in vesicular structures throughout the cytoplasm, with the strongest labeling in the juxtanuclear region (Fig. 4, A and B). The internalized fluorescence signal in these compartments was acid resistant (Fig. 4 B). In contrast, after 15°C KT3 labeling, the signal resistant to acid stripping was in large vesicular structures, some of which were close to the plasma membrane, others were distributed throughout the cytosol (Fig. 4 D). No concentration of fluorescent signal was detected in the juxta- or perinuclear region. However, if cells labeled at 15°C were rewarmed to 37°C for 15 min, the label redistributed to the juxtanuclear region in a pattern similar to the steady-state labeling at 37°C (Fig. 4, E, F, I). These results show that KT3 label moves from the 15°C compartment to the perinuclear region. The addition of BFA before the warming to 37°C did not prevent the accumulation of fluorescent signal in an acid-resistant compartment around the nucleus (Fig. 4, H and J). No BFA-induced tubular endosomal structures were distinguishable. A similar juxta–perinuclear pattern was observed in cells loaded in BFA at 37°C without the 15°C preincubation (Fig. 5 b, C and D). Thus, although both 15°C and BFA inhibit SV formation, transport to the perinuclear region is sensitive only to temperature.

**The BFA-dependent SV Block Is Selective**

Although formation of synaptic vesicles from the 15°C compartment was sensitive to BFA, other parts of the endocytotic pathway were not. Internalization of VAMP-TAg from the cell surface was assessed as acid-resistant

![Figure 2](Image)

**Figure 2.** Time course of the BFA inhibition in two VAMP-transfected PC12 cell lines. Stably transfected PC12 cells bearing the N49A (a) or del61–70 VAMP-TAg (b) constructs were labeled at 15°C for 40 min in the presence of 125I-KT3 antibody and incubated in methanol (0.001%) (○) or BFA (5 μg/ml) (●) as described. Cells were chased at 37°C for different times. The reactions were stopped at 0°C and the vesicle production was determined by velocity sedimentation in glycerol gradients as described. BFA blocked synaptic vesicle biogenesis in both cell lines with no detectable delay. Similar results were obtained in two independent experiments. The amount of SV production at 15 min was designated as 100%. The radioactivity corresponding to 100% was 2,000 cpm in a and 10,000 cpm in b.

![Figure 3](Image)

**Figure 3.** Newly formed synaptic vesicles turnover. To evaluate the life span of newly synthesized synaptic vesicles, PC12/N49A cells were labeled with 125I-KT3 antibody at 15°C as described. The free antibody was washed at 4°C and the cells were then incubated for a further 15 min at 37°C to allow SVs to form. Vesicle biogenesis was stopped by placing cells on ice followed by treatment in the absence (○) or presence (●) of BFA (5 μg/ml). After rewarmed the cells back to 37°C for different times; the remaining vesicles were assessed by velocity sedimentation. The data are one example of three independent experiments.
bound 125I-KT3 antibody and found to be similar in the control and BFA-treated cells (Fig. 5a). Insensitivity of internalization to BFA was also shown by immunofluorescence (Fig. 5b). Recycling of transferrin from endosomes to the cell surface was also relatively unaffected by BFA, as shown by the kinetics of 125I-labeled rat transferrin recycling from endosomes to the plasma membrane after BFA treatment (Fig. 6). To measure recycling, 125I-labeled rat transferrin was internalized at 15°C, cells chilled to 4°C, and plasma membrane–associated ligand removed by washing. Cells were then incubated in the absence or presence of BFA for different times at 37°C. As previously reported in K562 and NRK cells (Lippincott-Schwartz et al., 1991a; Schonhorn and Westling-Resnick, 1994), the change in the rate of transferrin externalization was not very dramatic after BFA treatment in N49A/PC12 cells, either evaluated as cell-associated radioactivity or released radioactivity.

The drug illimaquinone also inhibits binding of β-COP and ARF to Golgi membranes, vesicle production, and protein secretion; but it has no observable effect on the endocytic pathway (Takizawa et al., 1993). Illimaquinone also had no effect on SV formation from 15°C-labeled cells (Fig. 7, 16.3 ± 1.7% inhibition, n = 3) at a concentration that stops protein traffic through the Golgi complex.

Figure 4. BFA does not prevent the accumulation of KT3 antibody in perinuclear endosomes. PC12 cells expressing N49A VAMP-TAg were incubated in the presence of KT3 mAb (10 μg/ml) under different conditions to label endocytic organelles. After labeling, the cells were chilled, washed extensively, fixed and processed for immunofluorescence using a labeled secondary antibody either before (A, C, E, and G) or after (B, D, F, and H–J) stripping KT3 from the cell surface by an acid wash. A and B show the total and internal label when antibody uptake was permitted for 40 min at 37°C; significantly less perinuclear staining was seen if the uptake was at 15°C for 40 min (C and D). BFA did not prevent movement to perinuclear endosomes (G, H, and J). If cells were labeled at 15°C for 40 min, and then warmed to 37°C for 15 min, the distribution of immunofluorescence was identical whether or not BFA was present (G, H, and J) or absent (E, F, and I). I and J represent higher magnifications of F and H, respectively. Bars: 10 μm.
Higher illimaquinone concentrations or avarol, a structurally similar inhibitor, also did not modify SV production (data not shown).

Bafilomycin A1, a specific inhibitor of vacuolar type ATPases, perturbs endosomal pH and membrane trafficking, in both early endosomes and from early to late endosomes (van Weert et al., 1995; Aniento et al., 1996). It inhibited SV production by 44 ± 6% (Fig. 7, n = 3). The inhibition reached a plateau at 250 nM (data not shown). A similar dose-response has been described in the inhibition of endocytosis and trafficking of transferrin receptor back to the cell surface. The vacuolar ATPase inhibitor, bafilomycin A1 and illimaquinone, a Golgi-specific inhibitor of β-COP accumulation, are much less effective inhibitors and have not been studied further.

Cell-free SV Biogenesis Requires ARF1 Protein

Involvement of ARF1 protein in SV biogenesis was investigated directly using a cell-free reconstitution assay. ARF1 is believed to interact with effectors through its NH2-terminal domain, since peptides corresponding to its NH2-terminus can block ARF1 function (Balch et al., 1992; Kahn et al., 1992; Randazzo et al., 1994; Boman and Kahn, 1995). The effects on SV production of an ARF1-derived, (2–17) NH2-terminus peptide were therefore examined.

In a standard cell-free assay (Desnos et al., 1995), labeled SVs are generated at 37°C, whereas no production of SV appears in a reaction at 4°C (Fig. 8, a and c). Under this condition the addition of a peptide spanning the NH2-terminus effector domain of ARF1 (2–17) at a concentration of 100 μM, inhibited the SV production by 42 ± 4%, (n =

Thus, BFA affects SV formation selectively having no effect on internalization or recycling of transferrin receptor back to the cell surface. The vacuolar ATPase inhibitor, bafilomycin A1 and illimaquinone, a Golgi-specific inhibitor of β-COP accumulation, are much less effective inhibitors and have not been studied further.

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In a standard cell-free assay (Desnos et al., 1995), labeled SVs are generated at 37°C, whereas no production of SV appears in a reaction at 4°C (Fig. 8, a and c). Under this condition the addition of a peptide spanning the NH2-terminus effector domain of ARF1 (2–17) at a concentration of 100 μM, inhibited the SV production by 42 ± 4%, (n =

Higher illimaquinone concentrations or avarol, a structurally similar inhibitor, also did not modify SV production (data not shown).

Bafilomycin A1, a specific inhibitor of vacuolar type ATPases, perturbs endosomal pH and membrane trafficking, in both early endosomes and from early to late endosomes (van Weert et al., 1995; Aniento et al., 1996). It inhibited SV production by 44 ± 6% (Fig. 7, n = 3). The inhibition reached a plateau at 250 nM (data not shown). A similar dose-response has been described in the inhibition of endocytosis and trafficking of transferrin receptor back to plasma membrane and in the transfer of markers from early endosomes to late endosomes (van Weert et al., 1995; Aniento et al., 1996). These results show that perturbing the pH gradients along the endocytic pathway affects SV production but does not mimic the severity of the BFA effect.

Thus, BFA affects SV formation selectively having no effect on internalization or recycling of transferrin receptor back to the cell surface. The vacuolar ATPase inhibitor, bafilomycin A1 and illimaquinone, a Golgi-specific inhibitor of β-COP accumulation, are much less effective inhibitors and have not been studied further.

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Figure 5. BFA does not block the 125I-KT3 internalization. (a) PC12/N49A cells were surface labeled with 125I-KT3 antibody (3.3 μg/ml) for 2 h at 0°C. The unbound label was washed away and the cells were incubated in media containing either methanol (0.001%) (C) or BFA (5 μg/ml) (D) for 15 min on ice. The cells were warmed up to 37°C for different times and endocytosis was stopped by putting the cells back on ice. The internalized ligand was determined by surface-acid stripping, and expressed as fraction of the total cell-associated counts. Error bars represent standard deviation of triplicates in one of three independent experiments. (b) Internalization was assessed by immunofluorescent detection of the KT3 antibody uptake. PC12/N49A cells were incubated with unlabeled antibody for 2 h at 4°C and equilibrated in the absence (A and B) or presence (C and D) of BFA as described in a. Cells were chased at 37°C for 15 min. Reactions were stopped at 4°C and the total (A and C) and internalized VAMP-TAg (B and D) determined by acid stripping of the cell surface. Cells were fixed and processed for immunofluorescence as described. Bar, 10 μm.
Figure 8. The ARF1 NH$_2$-terminus peptide (2–17) reversibly inhibits synaptic vesicle biogenesis in a cell-free system. Standard reaction mixtures containing homogenates from PC12/N49A cells labeled with 125I-KT3 antibody at 15°C, ATP regenerating system, and rat brain cytosol (1 mg/ml or 0.35 mg/assay) were prepared and kept on ice for 15 min, either in the absence or presence of the (2–17) NH$_2$-terminus peptide, or the control scrambled peptide. Budding reactions were initiated by warming for 30 min at 37°C. Vesicle production was assessed by velocity sedimentation as described. (a) The (2–17) NH$_2$-terminus peptide (100 µM) inhibited the generation of vesicles by 42 ± 4% of control. a Shows a representative experiment out of seven experiments. The random peptide had no effect on the budding reaction at the highest concentration tested (200 µM). In b, the inhibitory effect of the (2–17) NH$_2$-terminus peptide (100 µM) was reverted by adding additional cytosol. Reaction mixtures similar to those described in a were incubated at 0°C either with (2–17) NH$_2$ terminus or the scrambled peptide (100 µM) for 15 min. The reactions were warmed at 37°C for 15 min, and then supplemented or not with more cytosol. The reactions were allowed to proceed for an additional 15 min before being stopped and analyzed. Dilution of peptide by the addition of cytosol did not exceed 20%. In c, the inhibition mediated by the (2–17) NH$_2$-terminus peptide was reversed by the addition of purified myristoylated recombinant wild-type ARF1 (50 µM).

An effect of this magnitude was not detected using a scrambled peptide of identical composition (105 ± 7%, n = 4) (Fig. 8 a). Since the (2–17) peptide has been reported to inhibit Golgi membrane traffic irreversibly by damaging membranes (Weidman and Winter, 1994), the reversibility of its effect upon SV formation was tested. Identical in vitro reactions containing low levels of brain cytosol (1 mg/ml) were run in parallel for 15 min at 37°C, in the presence of the scrambled or (2–17) peptide (100 µM). To test reversibility, additional brain cytosol was added, and the incubation continued at 37°C for another 15 min. Inhibition by (2–17) peptide was reversed by adding more cytosol (Fig. 8 b), indicating that if the peptide concentration was 100 µM or less, the inhibitory effect was not solely due to membrane damage. Since this peptide is able to inhibit ARF-independent processes (Fensome et al., 1994), we tested if the addition of wild-type ARF1 could induce a recovery of the (2–17) peptide inhibitory effect on SV formation. In standard reactions containing 100 µM of (2–17) peptide, the addition of 50 µM myristoylated wild-type ARF1 together with the peptide (Fig. 8 c) decreased the inhibition to 24 ± 10% (n = 3). These results show a competitive interaction between ARF1 and the peptide, and imply that an ARF is involved in the budding reaction.

To further address ARF1 involvement, we tested the effects of purified ARF1 mutants Q71L, a GTPase null, and T31N (a GTP-binding defective mutant) on the formation of properly sized SVs, assessed by velocity gradient sedimentation. The amount of labeled SVs was unchanged by supplementing the standard reaction mixtures with 50 µM wild-type ARF1 (Fig. 9 a). In contrast, in standard reactions supplemented with 50 µM of either dominant-positive (Q71L) or dominant-negative (T31N) mutants, there was a drastic reduction in the amount of labeled vesicles detected in velocity gradients. Both recombinant mutant proteins were effective at concentrations as low as 1 µM (Fig. 9 b). This concentration is similar to the amount of ARF1 estimated to be present in the rat brain cytosol used in the standard reaction mixtures (data not shown; Kahn et al., 1988). Consistently Q71L was more potent when compared with T31N. Both mutants were also able to induce a reduction in the production of labeled SVs in the suboptimal conditions when only PC12 cell cytosol is present without added brain cytosol (Desnos et al., 1995). Even under these conditions, however, additional wild-type ARF1 had no stimulatory effect (data not shown), indicating that the endogenous ARF1 protein present in the PC12 extracts was sufficient for the vesicle budding reaction.

To demonstrate ARF dependence directly, the cell-free assay was changed by removing the ARF1 present in rat brain cytosol. This was done by passing the cytosol through a Superose 6 sizing column and taking high molecular weight (HMW) fractions reported to be enriched in coat complexes (Stamnes and Rothman, 1993; data not shown). These HMW fractions were pooled and added to the cell-free reaction in the absence of added rat brain cytosol. The addition of HMW fractions alone stimulated the
formation of vesicles by 1.7 ± 0.15 times \((n = 5)\). However, the combined addition of HMW fractions plus myristoylated wild-type ARF1 increased the vesicle production 2.6 ± 0.15 times. Further, both mutant ARFs inhibited the appearance of vesicles in the presence of HMW (Fig. 10). These results show that ARF1 is required for SV biogenesis. A likely possibility is that it recruits coating molecules present in the HMW fraction.

ARF molecules have been implicated in recruiting coats made up either of coatomers or of adaptor/clathrin complexes. To test if clathrin was involved in the budding process, it was quantitatively removed from rat brain cytosol using mAb to clathrin (Fig. 11a). No significant reduction in budding efficiency was observed (Fig. 11b). To examine the involvement of COPI coatomers in vesicle formation, we used conditions that are known to inhibit COPI function during endosomal sorting (Whitney et al., 1995) or binding of COPI to membranes (Lowe and Kreis, 1995). Addition of antibodies to β-COP, a fusion protein corresponding to the KXXX signal motif of WBPI (GST-KK) (Cosson and Letourneur, 1994), or a combination of the antibody and the inhibitory peptide had no effect on vesicle formation.
Reactions containing normal rat brain cytosol (supplemented with either 11–22 μg/ml of affinity-purified anti-β-COP antipeptide antibody EAGE. 1 μM of either GST-WBP1-SS or GST-WBP1-KK, or a combination of EAGE antibody plus GST-WBP1-KK) were kept for at least 3 h at 4°C before warming to 37°C. None of the treatments substantially modified the budding of SV.

Discussion

By using BFA, wild-type, and mutant ARF1 proteins, we have demonstrated that ARF1 is required for the formation of SVs in PC12 cells from the 15°C compartment. The physiological relevance of ARF1 involvement in PC12 SV formation is supported by several arguments. In vivo SV formation is reversibly sensitive to BFA at concentrations known to affect budding from Golgi membranes. The inhibition occurs in three PC12 clones expressing different forms of VAMP. The number of SVs is reduced in PC12 cells incubated in the presence of BFA. ARF1 and a cytosol-derived HMW fraction are sufficient to promote SV production from a PC12 homogenate. Micromolar concentrations of mutant recombinant ARF1s block SV formation; and cell-free incubation of homogenates in the presence of a GTP-binding defective mutant of ARF1 generates antibody-loaded compartments of density different to those formed in the presence of a GTPase-defective mutant. Since most of the ARF1 isoforms are functionally equivalent (Kahn et al., 1991; Boman and Kahn, 1995), however, we cannot be certain that only ARF1 is involved in SV formation.

Specialized Endosomal Compartments as a Source of SV?

BFA has been useful in characterizing functionally specialized endosomes in polarized cells. In MDCK cells and in primary hippocampal neuron cultures, transfer of pIgA from apical/perikaryal endosomes to the apical/axonal membrane exhibits a BFA sensitivity similar to that obtained in SV production (Hunziker et al., 1991; Barroso and Sztul, 1994; de Hoop et al., 1995). However, BFA effects on nonspecialized endocytic routes are subtle. Although BFA induces morphological alterations in endosomes, it does not block the transit of dyes to lysosomes or the recycling of transferrin back to the cell surface (Lippincott-Schwartz et al., 1991a; Tooze and Holinshead, 1992; Wood and Wood, 1992; Schonhorn and Wessling-Resnick, 1994). The transferrin externalization rate is slowed but not blocked by BFA (Schonhorn and Wessling-Resnick, 1994; Fig. 6). In addition to being BFA sensitive, the transit of proteins through apical/perikaryal endosomes and through synaptic vesicle-generating endosomes are both unusually sensitive to low temperature blocks (15–17°C) (Barroso and Sztul, 1994; Desnos et al., 1995). This suggests that the specialized pathways in epithelia and neuronal cells may use a temperature-sensitive “coating” function, absent from nonspecialized or “housekeeping” pathways.

The endosomes of nerve terminals in primary hippocampal neurons (Mundigl et al., 1993) and of processes in differentiated PC12 cells (Bonzelius et al., 1994) are specialized compared with those in the cell body, particularly in their lack of transferrin receptor. Although it would be tempting to conjecture that these axonal endosomes might be more BFA sensitive, morphological data show that BFA causes tubulation of cell body but not axonal endosomes (Mundigl et al., 1993). One interpretation of such data is that the BFA-sensitive pathway of SV biogenesis in PC12 cells represents a more elementary form of biogenesis that is replaced in nerve terminal maturation with a more efficient and uniquely neuronal process. Alternative explanations are that the two pathways exist side by side in neurons, or the biogenesis of neuroendocrine SVs might be mechanistically unrelated to the biogenesis of neuronal SVs. Further experiments are required to distinguish between these alternatives.

PC12 SVs Are Dynamic Organelles

In immature hippocampal nerve cells, SVs go through a spontaneous rate of fusion with the plasma membrane in the absence of stimulation (Matteoli et al., 1992). Our results provide evidence that newly formed SVs in PC12 cells also cycle in the absence of stimulation. After loading the cells at 15°C, the production of vesicles at 37°C reaches a maximum in about 15 min. This equilibrium represents a balance between the kinetics of formation and disappearance. When the SV pool of PC12 cells is labeled at 37°C, and BFA added to suppress further SV formation, about half the vesicles disappear in 30 min. Although this reduction of labeled vesicles is probably due to exocytosis, it could also be due to fusion back to the donor or to other intracellular membrane compartments. BFA inhibition in vivo (Figs. 1 and 2) could be attributed to an enhancement of exocytosis rather than fusion. This is unlikely however, since BFA (data not shown) and ARF mutants block SV formation in cell-free assays, where fusion with the plasma membrane is not probable.
A Possible Mechanism of SV Biogenesis Revealed by ARF1 Mutants

It has been proposed that ARF-GTP recruits coats to the nascent vesicle that is budding from precursor Golgi membranes (Serafini et al., 1991; Rothman and Wieland, 1996). In Golgi- and ER-derived membranes, the ARF-GTPase activity is required for the uncoating of already formed vesicles (Tanigawa et al., 1993; Bednarek et al., 1995; Rothman and Wieland, 1996). Preventing GTP hydrolysis results in the formation of carrier vesicles that contain mature cargo and fusion proteins but retain their coats (Ostermann et al., 1993; Oka and Nakano, 1994; Bednarek et al., 1995; Rothman and Wieland, 1996). Since GTPyS effects are pleiotropic and can induce mistargeting of the adaptor (Seaman et al., 1993), the Q71L mutant is more informative for in vitro studies. For example, Q71L ARF can discriminate COPI- and II-dependent budding events simultaneously occurring from yeast ER (Bednarek et al., 1995).

Formation of SVs in a cell-free system resembles the production of Golgi-derived vesicles in its sensitivity to GTPyS (Desnos et al., 1995), its sensitivity to BFA and the need for ARF and a HMW protein fraction. If the inhibition of SV formation by the ARF mutants is interpreted in light of the Golgi results, then the mutant T31N ARF is predicted to prevent the binding of coat and ARF to membranes, and the Q71L ARF mutant would allow coated vesicles to form, but prevent their uncoating. Consistent with those predictions, the donor membranes became less dense when incubated in vitro with T31N ARF, and denser when incubated with Q71L. Unfortunately, the quantities of membrane currently available and their contamination with nondonor membranes preclude biochemical analyses of the two intermediate forms.

If ARFs were recruiting a coat to the SV precursor membranes, the coat would be expected to be clathrin (Heuser and Reese, 1973; Shupliakov et al., 1997). We could, however, find no evidence that the factor or factors contributed by rat brain cytosol were either clathrin or COPI. The data do not eliminate the involvement of either coat in budding, however, since sufficient coat molecules may remain attached to the donor membranes. Peripheral proteins removed from PC12 donor membranes by Tris-stripping are able to support the in vitro budding reaction (Horng, J.-T., unpublished observations). Unfortunately the quantities of proteins extractable from PC12 membranes using Tris are too small for detailed analysis. The active components in the rat brain cytosol are unlikely to include dynamin because extensive depletion using a grb2 affinity column also has no effect on SV formation in vitro (Horng, J.-T., unpublished observations). One possible coat that remains to be tested is the AP-3 coat, implicated in post-Golgi, and probably endosomal sorting events (Spinson et al., 1996; Dell’Angelica et al., 1997). It is also possible that the donor compartments contain partially coated vesicles. The addition of ARF and the high molecular weight fraction could complete the coat, allowing uncoating to proceed. Alternatively, the synaptic vesicle precursor could be a complete coated vesicle that accumulated at 15°C. For this model to be plausible, the T31N dominant negative ARF1 mutant and BFA must inhibit uncoating as well as coating, a property that has not heretofore been attributed to ARF and to BFA.

It has been noted that Golgi budding in some cell types, such as MDCK cells, is BFA-resistant and does not require the addition of exogenous ARF (Kitstakis et al., 1995, 1996). The formation of SVs more closely resembles budding from CHO Golgi membranes in its BFA and exogenous ARF sensitivity. Examination of Golgi budding in the BFA-resistant cell types generated evidence that the role of ARF is catalytic, not stoichiometric and involves the activation of phospholipase D activity by ARF (Brown et al., 1993). The role, if any, of phospholipase D in SV formation needs to be examined further.

In summary, our data show that SV biogenesis in PC12 cells is a process that requires ARF and that the GTP/GDP status of the ARF regulates the intermediates with which it is associated. Because the ARF family is so strongly linked to coating events, our data strongly suggest the participation of coat proteins in the budding of synaptic vesicles from PC12 membranes. The next step is to identify the proteins to which ARF1 binds, and the coating molecules that are recruited.

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