Synaptic Vesicle Proteins and Early Endosomes in Cultured Hippocampal Neurons: Differential Effects of Brefeldin A in Axon and Dendrites

Olaf Mundigl,* Michela Matteoli,*§ Laurie Daniell,* Annette Thomas-Reetz,* Anne Metcalf,* Reinhard Jahn,*‡ and Pietro De Camilli*

*Howard Hughes Medical Institute and Departments of Cell Biology and ‡Pharmacology, Yale University School of Medicine, New Haven, Connecticut; and §Consiglio Nazionale Per La Ricerca, Center of Cytopharmacology, University of Milan, Italy

Abstract. The pathways of synaptic vesicle (SV) biogenesis and recycling are still poorly understood. We have studied the effects of Brefeldin A (BFA) on the distribution of several SV membrane proteins (synaptophysin, synaptotagmin, synaptobrevin, p29, SV2 and rab3A) and on endosomal markers to investigate the relationship between SVs and the membranes with which they interact in cultured hippocampal neurons developing in isolation. In these neurons, SV proteins are detected as punctate immunoreactivity that is concentrated in axons but is also present in perikarya and dendrites. In the same neurons, the transferrin receptor, a well established marker of early endosomes, is selectively concentrated in perikarya and dendrites. In the perikaryal-dendritic region, BFA induced a dramatic tubulation of transferrin receptors as well as a cotubulation of the bulk of synaptophysin. Synaptotagmin, synaptobrevin, p29 and SV2 immunoreactivities retained a primarily punctate distribution. No tubulation of rab3A was observed. In axons, BFA did not produce any obvious alteration of the distribution of SV proteins, nor of peroxidase- or Lucifer yellow-labeled early endosomes. The selective effect of BFA on dendritic membranes suggests the existence of functional differences between the endocytic systems in dendrites and axons. Cotubulation of transferrin receptors and synaptophysin in the perikaryal-dendritic region is consistent with a functional interconnection between the traffic of SV proteins and early endosomes. The heterogeneous effects of BFA on SV proteins in this cell region indicates that SV proteins are differentially sorted upon exit from the TGN and are coassembled into SVs at the cell periphery.

SYNAPTIC vesicles (SVs)[1] are highly specialized secretory organelles of neurons which store and secrete non-peptide neurotransmitters. In mature neurons, SVs are concentrated in nerve terminals where they undergo exocytosis at specialized regions of the presynaptic plasmalemma, the so-called active zones (see Kelly, 1988; for reviews see De Camilli and Jahn, 1990; Südhof and Jahn, 1991; Trimble et al., 1991). Their exocytosis occurs asynchronously and at very low rates in resting nerve terminals and is greatly stimulated, with minimal temporal delay (fractions of a millisecond), by nerve terminal depolarization (Katz and Miledi, 1968). After exocytosis, SV membranes are rapidly reinternalized and reused for the assembly of new, neurotransmitter-filled SVs (Heuser and Reese, 1973; Ceccarelli et al., 1973; Sulzer and Holtzman, 1989). It remains to be elucidated how SVs are first assembled from newly synthesized components and subsequently regenerated at each cycle of exo-endocytosis. It is still unclear whether SV recycling involves clathrin-coated vesicles and early endosomes (as in the receptor-mediated recycling pathway), or whether endocytosis follows another more specialized pathway (Miller and Heuser, 1984; Pfeffer and Kelly, 1985; Torri-Tarelli et al., 1987; Maycox et al., 1992).

Microvesicles that are closely related to neuronal SVs in biochemical and functional properties (referred to as synaptic-like microvesicles) were identified in a variety of endocrine cells and endocrine cell lines (Navone et al., 1986; Wiedenmann et al., 1988; Baumert et al., 1990; Reetz et al., 1991). These vesicles have been used to study aspects of SV membrane traffic which cannot be easily investigated in neurons (Johnston et al., 1989; Cameron et al., 1991; Linstedt and Kelly, 1991; Régnier-Vigouroux et al., 1991). Most of these studies have focused primarily on synaptophysin as a model SV protein. It was found that a large fraction of synaptophysin in these cells is colocalized with the transferrin receptor (Trf-R), a well established marker of the receptor-mediated recycling pathway (Dautry-Varsat et al., 1983), and it was suggested that synaptic-like microvesicles are regener-

Please address all correspondence to Dr. Pietro De Camilli, Howard Hughes Medical Institute, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, CT 06510.

1. *Abbreviations used in this paper*: BFA, Brefeldin A; SVs, synaptic vesicles; Syt_{lum}-A, lumenal domain of rat Syt I; Trf-R, transferrin receptor.

ated after each cycle of exo-endocytosis by endosomal sorting. By extension, it was suggested that recycling of SVs is closely related to the receptor-mediated pathway. Consistent with this hypothesis, expression of synaptophysin in non-neuronal cells results in its targeting to the same membranes which contain Trf-R, as if the receptor-mediated recycling pathway was the default pathway for SV proteins (Johnston et al., 1989; Cameron et al., 1991; Linstedt and Kelly, 1991).

In neurons, the elucidation of the biogenesis and traffic of SVs is complicated by the presence of distinct cellular domains, e.g., dendrites and axons. SVs are concentrated in axon endings while Trf-R is selectively localized in perikarya and dendrites (Cameron et al., 1991). Given the different characteristics of axons and dendrites, it is possible that the properties of the endocytic systems in the two neuronal domains are different. Membrane proteins recycling via two anatomically distinct populations of early endosomes is well documented for polarized cells of surface epithelia. These two endosomal populations differ in protein composition and may also differ in functional properties (Parton et al., 1989; Rodriguez-Boulan and Powell, 1992). Similarities between the basolateral compartment of epithelial cells and the perikaryal-dendritic region of neurons, as well as between the apical compartment of epithelial cells and axons, have become apparent (Dotti and Simons, 1990; Cameron et al., 1991; Rodriguez-Boulan and Powell, 1992; Pietrini, G., Y. J. Suh, L. Edelmann, G. Rudnick, and M. Caplan. 1992. *Mol. Biol. Cell.* 3:303*a*; Cameron et al., 1993).

The fungal metabolite Brefeldin A (BFA) has been used in a variety of studies to dissect the properties of the endocytic pathway. It causes the formation of long, branching tubular extensions from the Golgi apparatus that lead to a rapid disassembly of the Golgi and to a redistribution of its membrane components to the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Doms et al., 1989). These effects are thought to be caused by interference with the formation of a protein coatomer on Golgi-derived transport vesicles (Donaldson et al., 1990, 1992; Orci et al., 1991; Helms and Rothman, 1992). It also induces an anatomical continuity between elements of the TGN and early endosomes. This is due to the formation of a microtubule-dependent, interconnected reticulum of tubules where proteins of endosomes and the TGN are intermixed (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Hunziker et al., 1991). This tubular reticulum is clearly distinct from the ER-Golgi reticulum. Transport routes between the two BFA-induced hybrid compartments are blocked (Lippincott-Schwartz et al., 1991; Ladinsky and Howell, 1992). Remarkably, vesicular traffic between the TGN-endosomal reticulum and the cell surface continues (Wood and Brown, 1992; Miller et al., 1992; Hunziker et al., 1991; Lippincott-Schwartz et al,. 1991).

The goal of this study was to explore the relationship between the recycling pathway of SV proteins and early endosomes in neurons and to investigate differences between the endocytic systems in axons and dendrites. If SV proteins recycle through sorting compartments which are sensitive to BFA, one may expect the distribution of these proteins to be dramatically affected by this drug. As a model for these studies we have used hippocampal neurons developing in isolation in primary culture (Banker and Cowan, 1977; Goslin and Banker, 1990). These neurons extend axons and dendrites and contain abundant SVs which undergo exo-endocytotic recycling (Fletcher et al., 1991; Matteoli et al., 1992). SV proteins are concentrated in axons but are also present in dendrites (Matteoli et al., 1992). We have investigated the effect of BFA on several SV proteins (synaptophysin, synaptotagmin, synaptobrevin, p29, SV2 and rab3A [for review see Südhof and Jahn, 1991 and Trimble et al., 1991]), and on the Trf-R. We show that BFA induces a massive cotubulation of synaptophysin and Trf-R in the perikaryal-dendritic region. The distribution of other SV proteins in dendrites was affected either to a lower degree or not at all by BFA. Unexpectedly, no obvious effects of BFA were observed in axons on either the distribution of SV proteins or of early endosomes. These results demonstrate that individual SV proteins are differentially sorted in the perikaryal-dendritic region and indicate that important differences exist between the endocytic systems of dendrites and axons.

Materials and Methods

Antibodies and Cytochemical Probes

Rabbit polyclonal antibodies directed against the lumenal domain of rat Syt I (Syt_{lum}-Abs) were generated by using a peptide corresponding to residues 1–19 (NH_2 terminus) of the protein (Perin et al. 1990) with an additional cysteine at the COOH terminus (MVSASHPEALAAPVTTVATC). The peptide was conjugated to KLH via the COOH-terminal cysteine as described by Johnston et al. (1989). Antibodies were affinity purified using the same peptide coupled to an EAH Sepharose column according to the manufacturer's instructions (Pharmacia LKB, Biotechnology, Uppsala, Sweden). Polyclonal and monoclonal antibodies directed against rat synaptophysin (Navone et al., 1986), polyclonal antibodies directed against synaptobrevin (Baumert et al., 1989), and monoclonal antibodies directed against the cytoplasmic domain of synaptotagmin I were previously described (Matteoli et al., 1991; Brose et al., 1992). Monoclonal antibodies directed against SV2 (Buckley and Kelly, 1985) and MAP2 (De Camilli et al., 1984) were kind gifts of Dr. K. Buckley (Harvard University, Boston, MA) and Dr. R. Vallee (Worcester Foundation for Experimental Biology, Worcester, MA), respectively. The mAb recognizing human Trf-R (Cameron et al., 1991) was kindly provided by Dr. I. Trowbridge (Salk Institute, San Diego, CA). A polyclonal antibody against synaptotagmin I and II was a kind gift of Drs. B. Wendland and R. Scheller (Stanford University, Palo Alto, CA). The following reagents were obtained from commercial sources: mAb directed against β-tubulin (Amersham Corp., Arlington Heights, IL), TRITC-conjugated phalloidin, TRITC-conjugated human transferrin and polyclonal antibodies against Lucifer yellow (Molecular Probes, Eugene, OR), FITC-conjugated WGA (Vector Laboratories, Burlingame, CA), HRP type VI (300 U/mg) and Lucifer yellow (Sigma Chem. Co., St. Louis, MO), BFA (Epicentre Technologies, Madison, WI). BFA was stored at 10 mg/ml in ethanol at −20°C.

Hippocampal Cell Culture

Primary neuronal cultures were prepared from hippocampi of 17-d old fetal rats as described (Banker and Cowan, 1977; Goslin and Banker, 1990). The neurons were cultured at low density (3,000/cm^2) in vitro for 4–5 d before the experiments (stage 3–4 cells according to Dotti et al., 1988). Alternatively, hippocampal neurons were grown without the support of astrocytes but in the presence of a cocktail of biological antioxidants (supplement B27 from GIBCO BRL, Gaithersburg, MD, at the concentration indicated by the manufacturer). It was previously shown that the addition of physiological antioxidants improve the survival and neuritogenesis of hippocampal neurons in primary culture (Brewer and Cotman, 1989). Under these growth conditions neurons with giant axons were more abundant and the typical development of these neurons seemed to be delayed ~1–2 d. The distribution of SV proteins was identical to the two types of cultures regardless of whether BFA was present or not.

Labeling Protocols

Immunofluorescence. BFA (final concentration 10 μg/ml, but concentrations between 5 and 20 μg/ml yielded similar results) was added to culture

media for 20 min before fixation. Syt_{lum}-Abs, TRITC-conjugated human transferrin (50 μg/ml), and FITC-conjugated WGA (5 μg/ml) were added to culture media for 1 h at 37°C. After these incubations, cells were either briefly washed, and then were fixed or incubated for an additional 20 min with BFA (10 μg/ml) in the continued presence of the label. In some experiments Syt_{lum}-Abs and BFA were added together for 20 min before fixation. For fluid phase uptake of Lucifer yellow, neurons were first incubated with BFA for 15 min, and then transferred to 10 mg/ml Lucifer yellow in the continued presence of BFA for 5 min, briefly washed, and fixed. To enhance the signal of Lucifer yellow, the internalized dye was revealed by immunofluorescence using polyclonal antibodies to Lucifer yellow. Fixation was performed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer containing 0.12 M sucrose. Neurons were processed for double immunofluorescence as described (De Camilli et al,. 1983; Cameron et al., 1991). At the end of immunostaining, neurons were mounted in 70% glycerol in PBS containing 1 mg/ml phenylenediamine, examined with a Zeiss axiophot microscope equipped for epifluorescence microscopy, and photographed with T-MAX 100 film (Kodak).

EM Immunoperoxidase

Neurons were incubated with HRP (10 mg/ml) for 30 min at 37°C in absence or presence of 10 μg/ml BFA, which was added 20 min before fixation, briefly rinsed in serum-free medium, and fixed with 1.5% glutaraldehyde, 4% sucrose in 100 mM sodium cacodylate buffer, pH 7.2 for 60 min. Cells were then washed in cacodylate buffer and the HRP was developed in 100 mM ammonium phosphate buffer containing 5 mg/ml diaminobenzidine plus 0.005% hydrogen peroxide. The reaction was monitored by light microscopy and stopped after sufficient color development. Subsequently, cells were washed in cacodylate buffer, postfixed for 60 min with ice cold 2% OsO_4 in the same buffer, block stained in 2% aqueous uranyl acetate, dehydrated in ascending alcohols and propylene oxide, and finally embedded in Epon between a Teflon support and a coverglass. After epon polymerization, the coverglass was removed by dipping the sample alternatively into liquid nitrogen and hot water in a sonicator. Neurons to be sectioned were selected by observing a flat epon layer under the light microscope. Areas containing the selected neurons were cut out and mounted on prepolymerized epon blocks for thin sectioning. 50-nm sections were cut parallel to the coverslip, stained with 2% aqueous uranyl acetate and lead citrate, and examined in a Philips 410 electron microscope.

Results

Polarized Hippocampal Neurons with Giant Axonal Processes as a Model System for the Study of Vesicular Traffic in Axons and Dendrites

Polarized hippocampal neurons developing in isolation in primary culture express high levels of SV proteins including synaptophysin (Jahn et al., 1985; Wiedenmann and Franke, 1985; Navone et al., 1986), synaptotagmin (Matthew et al., 1981; Perin et al., 1990), synaptobrevin (Baumert et al. 1989; Trimble et al., 1988), p29 (Baumert et al., 1990), SV2 (Buckley and Kelly, 1985; Bajjalieh et al., 1992; Feany et al., 1992), and rab3A (Matsui et al., 1988; Fischer von Mollard et al., 1990). These proteins are present both in perikarya and in dendrites, but are primarily concentrated in the axons where they are scattered throughout the axonal arbor (Figs. 1, 7, 8, and 10, see also Fletcher et al., 1991; Matteoli et al., 1991, 1992). Synaptophysin, synaptotagmin, synaptobrevin, p29 and SV2, which are intrinsic membrane proteins, are also concentrated in the perinuclear region that corresponds to the location of the Golgi complex (Figs. 1, 7, 8, 9, and 10). In these neurons, Trf-R is primarily restricted to perikarya and dendrites (Cameron et al., 1991).

Differential sorting of SV proteins and Trf-R can already be seen at the earliest stages of axonal differentiation (beginning of stage 3 according to Dotti et al., 1988). Axons can be recognized morphologically by their distinctive length and/or shape as soon as they start to differentiate. As a rule the axon is the longest process (Goslin and Banker, 1989). The neuron visible in Fig. 1, *a* and *b*, displays a single process that is longer than the others (*arrow*). This process is enriched in synaptophysin (Fig. 1 *a*) but not in Trf-R when compared to the other process (Fig. 1 *b*). The distribution of Trf-R, assessed by steady-state labeling with iron-saturated rhodamine-conjugated transferrin (Fig. 3, *a* and *b*) (Dautry-Varsat et al., 1983; Cameron et al., 1991) or by antibodies directed against the Trf-R (Fig. 1 *b*), yielded identical results. In contrast, synaptophysin and Trf-R had a nearly identical distribution in the perikaryal-dendritic region (Fig. 1). Immunoreactivity for these proteins in dendrites is represented by puncta and elongated elements which may represent short tubules (Parton et al., 1992; Tixier-Vidal et al., 1988).

In some neurons, the process identified by its shape as the axon, had a unique morphology. At variable distances from the perikaryon (in some cases from their very beginning), it became very large and flat (width of 30–40 μm or more), as if rather than growing in length it had expanded in diameter (e.g., Fig. 1 *c*, Fig. 2). The abundance of neurons with giant axons was related to the gestational age of embryos used for the preparation of the culture. They were more abundant in cultures from 17-d old embryos than in those from 18-d old embryos, the age typically used for preparation of cultures. In addition, they were more abundant in cultures where neurons were grown in the absence of astrocytes but in the presence of biological antioxidants (see Materials and Methods). These axonal expansions offer special advantages for the localization of subcellular organelles in axons at the light microscopic level of resolution. Therefore, the axonal properties of these processes were further documented.

The giant processes became heavily labeled when cultures were exposed for 1 h to antibodies directed against the lumenal domain of synaptotagmin I (Syt_{lum}-Abs) (Fig. 2 *a*, see also Fig. 7). This labeling, which is thought to reflect SV recycling, was previously shown to occur predominantly in axons (Matteoli et al., 1992). As in conventional axons (Matteoli et al., 1992), the distribution of internalized antibodies was distinct from that of internalized WGA (Fig. 2 *b*). These processes lacked MAP2 (Fig. 2, *c* and *d*), a microtubule-associated protein selectively concentrated in the perikaryal-dendritic region of differentiated neurons (De Camilli et al., 1984). Like other axons, they accumulate synaptophysin and other SV proteins (Figs. 1 *c* and 2 *c*, and following figures) and exclude Trf-R (Fig. 1 *d*). In a few neurons, Trf-R immunoreactivity partially extends into the stalk (Fig. 3 *d*) and the core (Fig. 4, *b* and *d*) of the giant process. This distribution may reflect an incomplete polarization because we have found that in many neurons a well defined asymmetric distribution of axonal and dendritic markers develops only gradually (unpublished observation). The tips of the giant axonal processes are characterized by a fan-like shape with actin-rich filopodia radiating from an organelle-rich core as demonstrated by immunofluorescence (Fig. 2 *f*) and by electron microscopy (see Fig. 5). However, the structure of these processes is somewhat different from typical growth cones, including the giant growth cones formed by Aplysia bag cells in culture (Forscher and Smith, 1988). Microtubules loop around the tip of these processes (Fig. 2 *e*), rather than terminating abruptly at the interface with the distal actin-rich domain (Forscher and Smith, 1988). Perhaps, a bending of

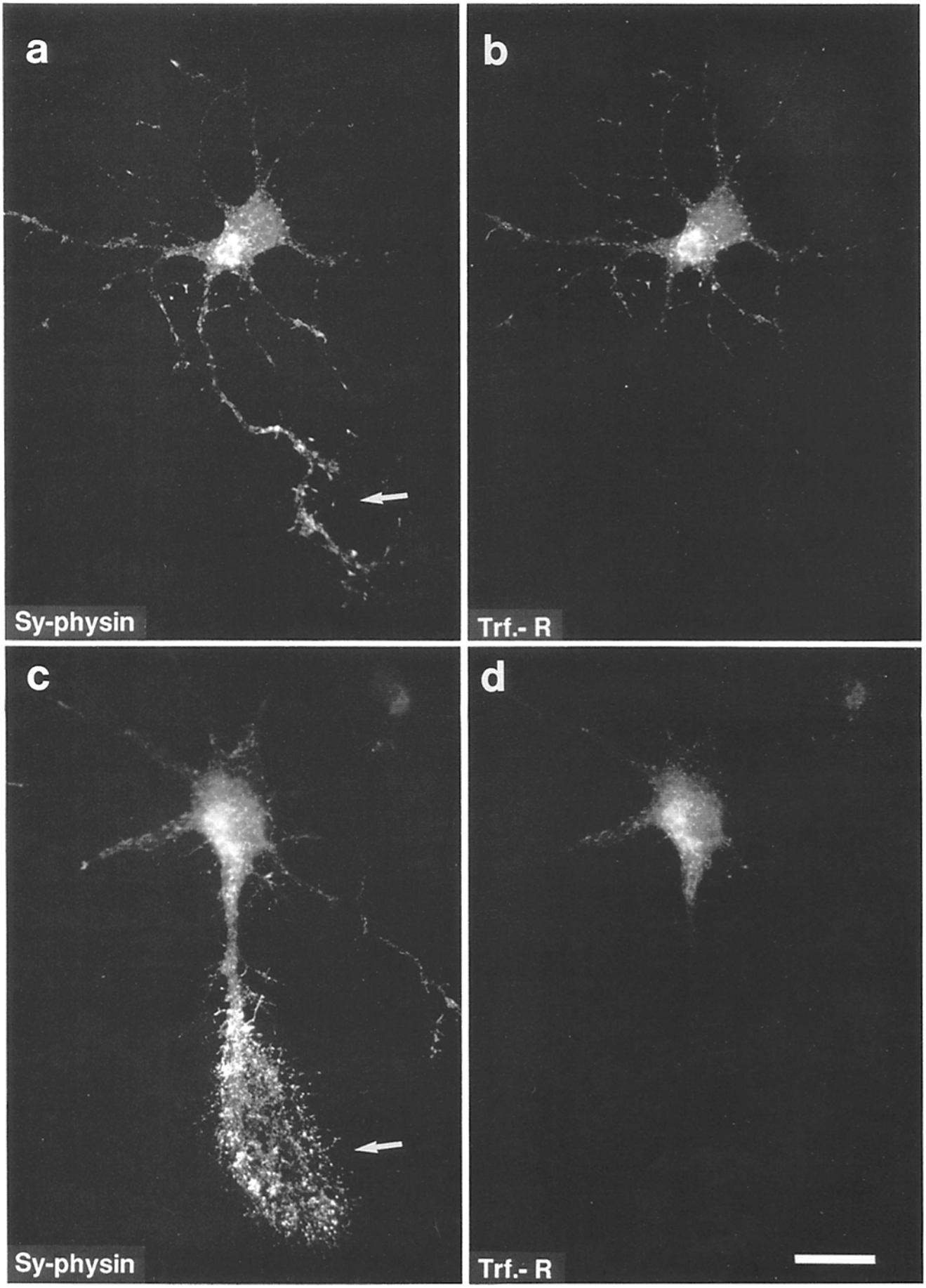


Figure 1. Comparison of the distribution of synaptophysin and Trf-R in isolated hippocampal neurons in primary culture demonstrated by double immunofluorescence. *a* and *b* show a neuron with a typical axon (*arrow*) which has just started to differentiate. *c* and *d* show a cell with a giant axon (*arrow*). The axons of both cells are enriched in synaptophysin but are almost devoid of Trf-R. Both proteins are present in the perikaryal-dendritic region where they are precisely colocalized. The bright areas in the perikarya correspond to the region of the Golgi complex. Bars: (*a* and *b*) 36 μm; (*c* and *d*) 23 μm.

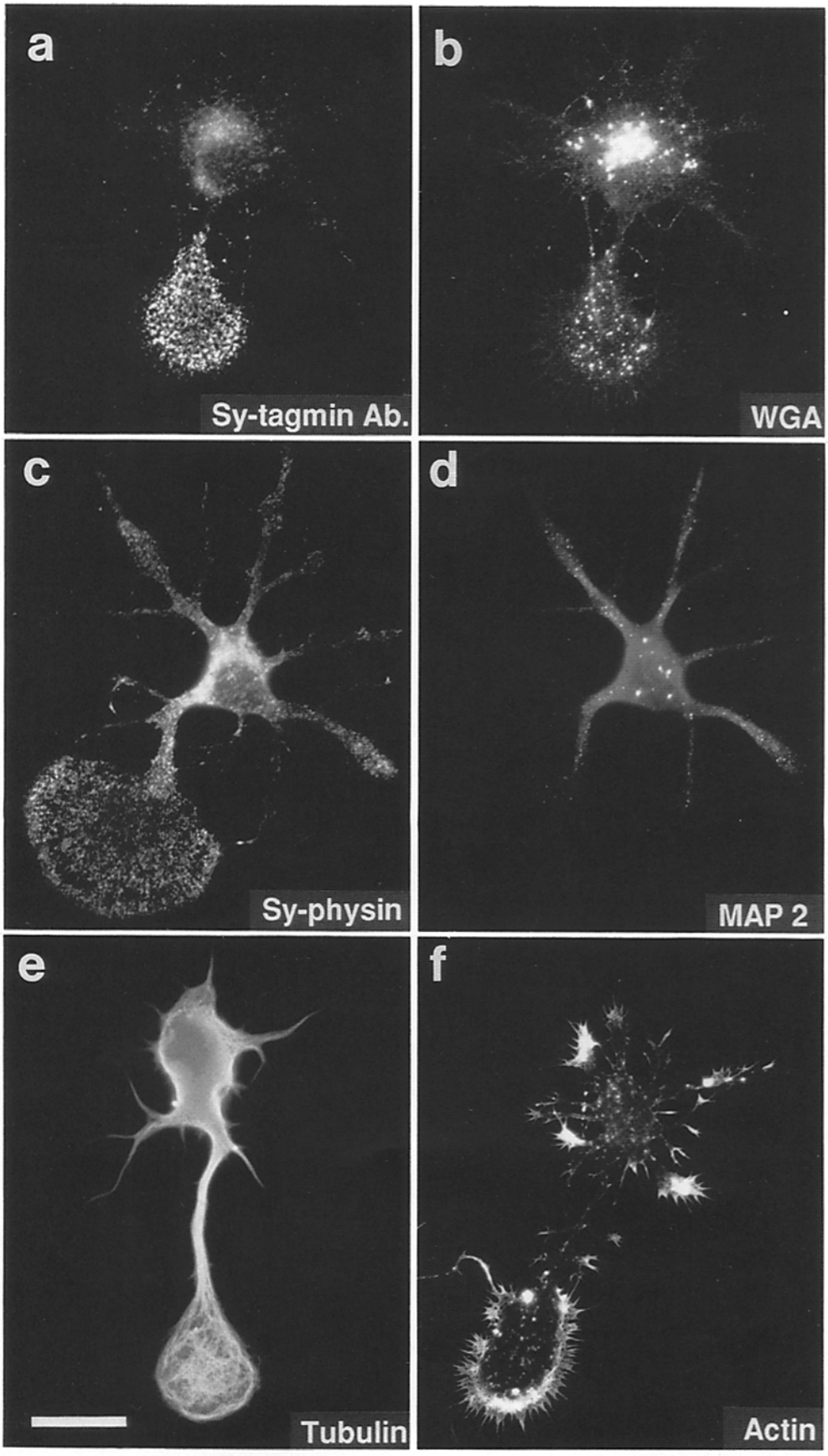


Figure 2. Distribution of vesicular markers and cytoskeletal proteins in isolated hippocampal neurons with giant axons. (*a* and *b*) Comparison of the distribution of internalized antibodies directed against the lumenal domain of synaptotagmin I (Syt_{lum}-*Abs*) (*a*) and of FITC-WGA (*b*) after 1 h simultaneous incubation with both markers. Internalized antibodies were stained with secondary antibodies after fixation. (*c* and *d*) Neuron double-labeled for synaptophysin (*c*) and for the perikaryal-dendritic marker MAP2 (*d*). The giant process is enriched in synaptophysin but is MAP2-negative. (*e*) Comparison of the distribution of β-tubulin (*e*) and actin (*f*) immunoreactivity in two neurons with similar morphology. Note that microtubules loop around the tip of the giant axon (*e*) and that the leading edge is rich in actin-containing filopodia (*f*). Bars: (*a–e*) 23 μm; (*f*) 30 μm.

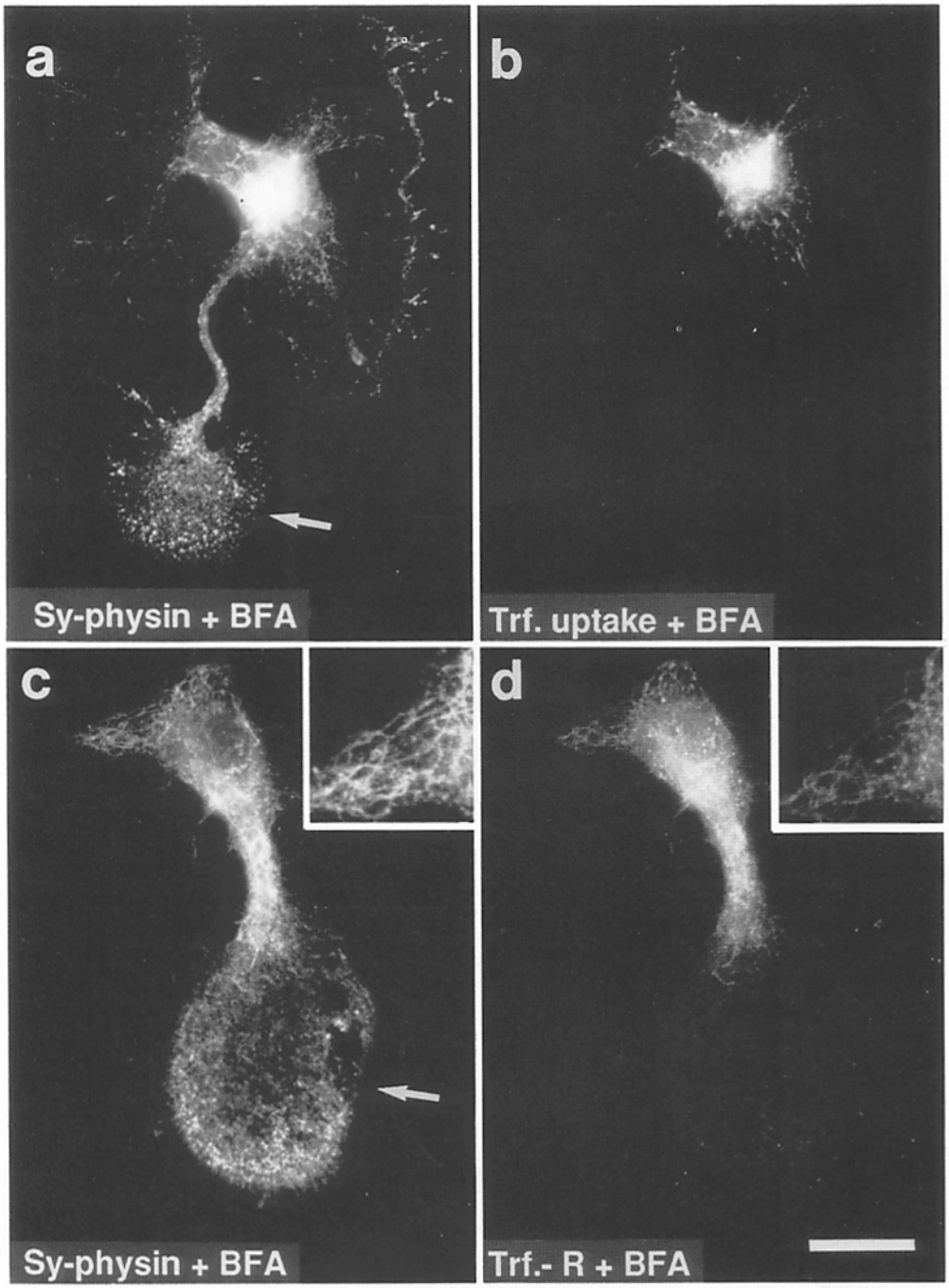


Figure 3. Effect of BFA on the distribution of synaptophysin and Trf-R in isolated hippocampal neurons. (*a* and *b*) Hippocampal neurons were incubated with TRITC-conjugated human transferrin for 1 h, and then treated with BFA (10 μg) for an additional 20 min in the continued presence of TRITC transferrin (*b*), fixed, permeabilized, and double-stained with antibodies directed against synaptophysin (*a*). (*c* and *d*). Neuron double-labeled for synaptophysin and Trf-R after 20 min incubation in the presence of 10 μg/ml BFA. Internalized transferrin (*b*) or Trf-R (*d*) as well as the perikaryal-dendritic pool of synaptophysin (*a* and *c*) are redistributed by BFA into a same reticular network. Insets in *c* and *d* show a detail of the BFA induced reticulum at higher magnification. Note lack of tubulation of synaptophysin in the flat, large axonal processes (*arrows*). Bar, 23 μm, inset, 10 μm.

microtubules may initiate the growth in width at the expense of the growth in length.

Although we focused our observations on neurons with giant axons, in all cases our findings were confirmed on neurons with more conventional axons.

BFA Induces Tubulation of Synaptophysin and Early Endosomes in the Perikarya and Dendrites but Not in Axons

When BFA was added to the cultures for 20 min, a massive redistribution of Trf-R immunoreactivity was observed. The punctate immunoreactivity observed in the perikaryal-dendritic region of control cells became a network of immunoreactive fibers which are known to represent tubules (Lippincott-Schwartz et al., 1991; Wood et al., 1991). Identical results were obtained with anti-Trf-R antibodies (Fig. 3 *d*) and with steady-state labeling with TRITC-conjugated transferrin (Fig. 3 *b*). In addition, BFA induced a massive tubulation of the perikaryal-dendritic pool of synaptophysin (Fig. 3, *a* and *c*). Double immunofluorescence of the same neurons after BFA treatment revealed that the network of Trf-R immunoreactivity precisely coincided with the perikaryal-dendritic network of synaptophysin (compare Fig. 3,

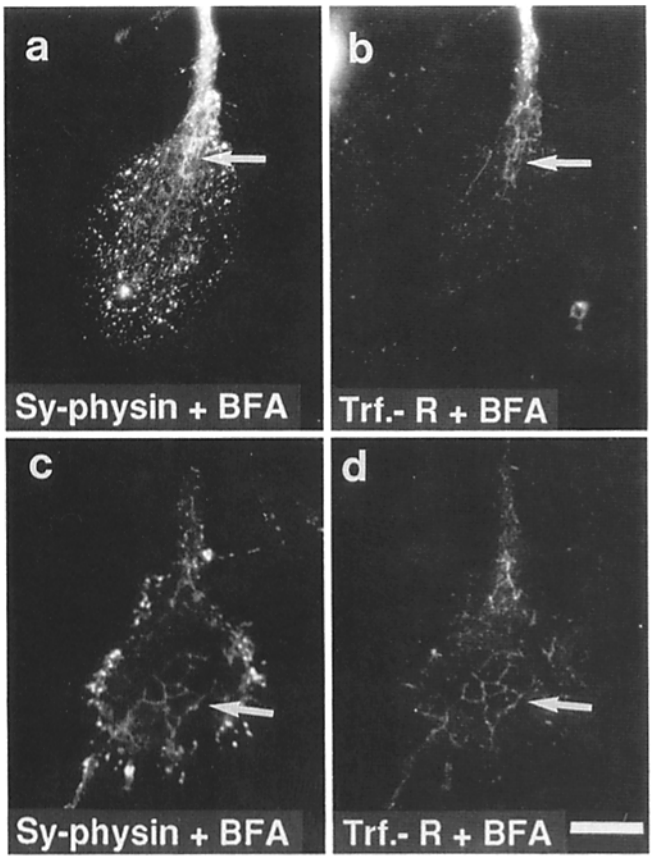


Figure 4. Details of giant axons of hippocampal neurons at an early stage of polarization double-labeled for synaptophysin (*a* and *c*) and Trf-R (*b* and *d*). In these two incompletely polarized neurons a few BFA-induced tubules of synaptophysin immunoreactivity extend into the stalk (*a* and *b*) and center (*c* and *d*) of the giant axonal expansion (*arrows*). These tubules are positive for Trf-R immunoreactivity. Bar, 15 μm.

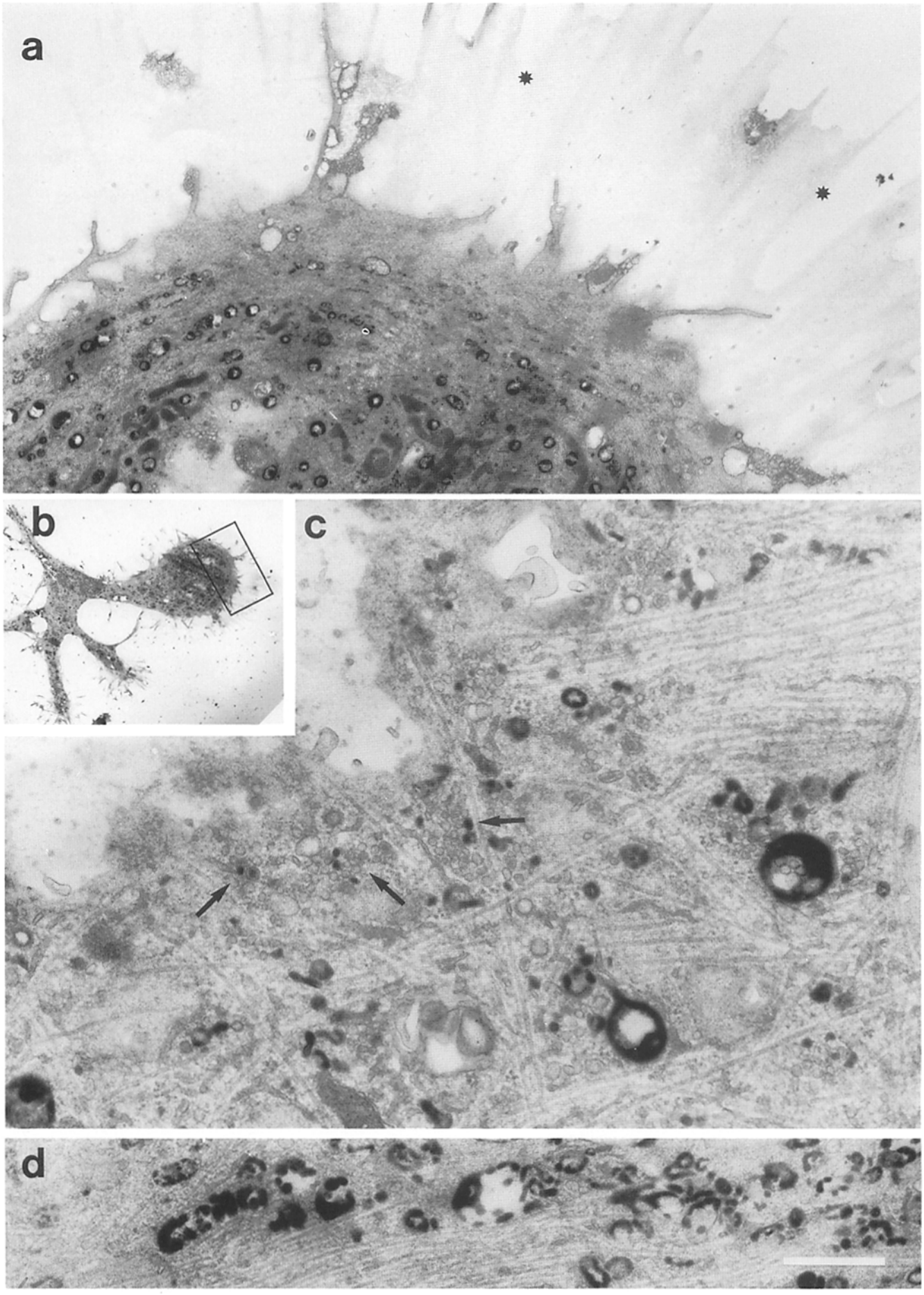
a and *c* with Fig. 3, *b* and *d*, respectively). Notably, BFA did not induce an obvious tubulation of synaptophysin in the axon (Fig. 3, *a* and *c*). Only in the very few incompletely polarized neurons where some Trf-R was detected in the axon (see above), this immunoreactivity became tubular after BFA treatment and these tubules were also positive for synaptophysin (Fig. 4). In principle, lack of synaptophysin tubulation in the giant axonal processes could be explained by absence of typical early endosomes in this cellular compartment, or by the exclusion of synaptophysin from axonal early endosomes.

To determine whether organelles with the typical morphological features of early endosomes (Helenius et al., 1983; Geuze et al., 1984) are present in the giant axonal processes, we investigated the morphology of the endocytic pathway in these processes at the electron microscopic level. Neurons were loaded with HRP, a fluid phase marker, for 30 min, a time sufficient for complete labeling of early endosomes and some late endosomes (Parton et al., 1992). Peroxidase-labeled tubulo-vesicular structures with the typical appearance of early endosomes were observed both in dendrites (not shown) and in the giant axons of control neurons (Fig. 5 and not shown). Many of these structures were represented by highly convoluted tubules forming local networks reminiscent of the tubular endosomes described by Tooze et al. (1991) in cells not treated with BFA (Fig. 5 *d*). In axons, but not in dendrites, these structures were often interspersed with clusters of vesicles with the typical size of SVs (Fig. 5 *c*). These SV clusters were more abundant at the rims of the axonal process. In BFA-treated neurons, longer, more rectilinear, peroxidase-labeled tubules, such as those produced by BFA in nonneuronal cells (Lippincott-Schwartz et al., 1991; Tooze and Hollinshead, 1992), were observed in dendrites but not in axons (not shown). These results were confirmed by experiments in which Lucifer yellow internalized for 5 min was used as a marker of early endosomes (Wood et al. 1991). Lucifer yellow internalization after 15 min of BFA treatment resulted in a tubular distribution of the dye in dendrites but in a punctate distribution in axons (Fig. 6). Unresponsiveness to BFA of axonal early endosomes, as defined by fluid phase markers, ruled out that lack of synaptophysin tubulation in axons upon exposure to the drug might simply be explained by the selective localization of this protein in SVs and its exclusion from early endosomes.

Heterogeneous Localization of SV Proteins in the BFA-induced Tubules

We next compared the distribution of other SV proteins with that of synaptophysin and Trf-R before and after BFA treatment. The distribution of synaptophysin and synaptotagmin was very similar in the perikaryon and the dendrites and was nearly identical in the axon of control neurons (Fig. 7, *a* and *b*). After BFA treatment, a differential distribution of the two proteins could be observed in the perikaryal-dendritic region. The massive tubulation of synaptophysin immunoreactivity induced by the drug was not paralleled by a corresponding redistribution of synaptotagmin immunoreactivity, which remained mostly punctate throughout the cell, although synaptotagmin positive puncta sometimes followed the course of synaptophysin tubules. In axons, the punctate distribution of the two proteins was unchanged after BFA treatment (Fig. 7, *c* and *d*). The few synaptophysin positive tubules which extended from the perikaryal-dendritic region into the stalk of a few axons (which were always Trf-R positive—see above), were negative for synaptotagmin (Fig. 7, *c* and *d*).

The dissociation of synaptotagmin immunoreactivity from the tubules positive for Trf-R and synaptophysin in BFA-treated cells was observed both with mono- and polyclonal antibodies directed against synaptotagmin I or synaptotagmin I and II. This dissociation was observed also when Syt_{lum}-Abs were used to selectively label recently internalized synaptotagmin I (see above, Matteoli et al., 1992). Neurons were first exposed to Syt_{lum}-Abs for 1 h and subsequently incubated in the absence or presence of BFA for 20 min before fixation and immunostaining. Fig. 8, *a–d* shows that the distribution of internalized antibodies in the absence of BFA was precisely identical to that of the total pool of synaptotagmin I immunoreactivity as revealed by counterstaining the neurons with antibodies directed against the cytoplasmic tail of synaptotagmin I. After BFA treatment, the distribution of internalized Syt_{lum}-Abs remained highly punctate not only in axons (not shown) but also in dendrites (Fig. 8, *e* and *f*) in spite of the typical tubulation of the Trf-R.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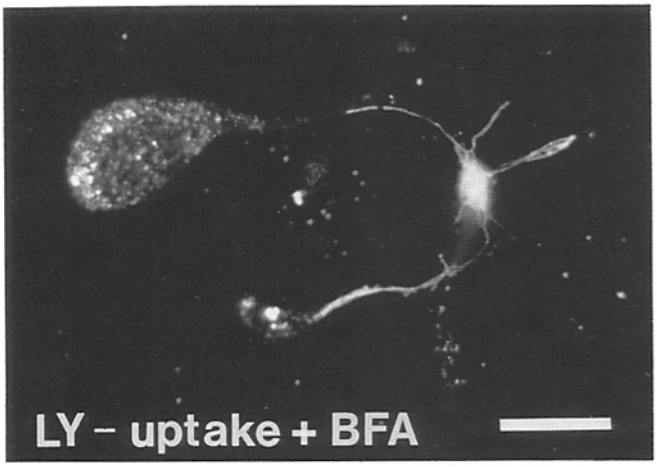

Figure 6. Distribution of internalized Lucifer yellow in a BFA-treated neuron. The neuron was exposed to BFA for 15 min, and then to Lucifer yellow for an additional 5 min in the continued presence of BFA. Tubulation is present in the perikaryal-dendritic region but not in the giant axonal process. Bar, 36 μm.

Similar results were obtained when neurons were incubated simultaneously for 20 min in the presence both of Syt_{lum}-Abs and BFA. Notably, the presence of BFA in the medium did not appear to affect internalization of Syt_{lum}-Abs in either the perikaryal-dendritic region or in axons. This finding is consistent with the observation made in nonneuronal cells that BFA does not block vesicular traffic between endosomes and the plasmalemma (Lippincott-Schwartz et al., 1991; Hunziker et al., 1991; Wood and Brown, 1992; Miller et al., 1992).

Lack of cotubulation with Trf-R and synaptophysin after BFA treatment was also observed in case of three other intrinsic membrane proteins of SVs, synaptobrevin, p29 and SV2. Before BFA treatment, the distribution of these other proteins was the same in the axon and similar, although not identical, in perikarya and dendrites (not shown). After the addition of BFA, the distribution of synaptobrevin (Fig. 9, *a* and *b*), p29 (Fig. 9, *c* and *d*) and SV2 (Fig. 9, *e* and *f*) in perikarya and dendrites remained mostly punctate although a weak tubular immunoreactivity could be observed in some neurons for SV2 (not shown). In axons, synaptophysin, synaptobrevin, p29 and SV2 had a punctate distribution, identical for the four proteins, before and after BFA treatment (not shown).

Finally, we investigated the effect of BFA on the distribution of rab3A, a low molecular weight GTP-binding protein which is thought to participate in the docking and/or fusion of SVs with the plasmalemma (Fischer von Mollard et al., 1990; Mizoguchi et al., 1990). Current models predict that rab3A undergoes cycles of association/dissociation with SV membranes in parallel with their exo-endocytic cycle and associates with SV membranes only at the stages which directly precede exocytosis (Fischer von Mollard et al., 1991; Matteoli et al., 1991). If this model is correct, rab3a should be generally absent from endosomes and consequently be absent from the synaptophysin-positive tubules induced by BFA in the perikaryal-dendritic region. The distribution of rab3A was similar, although not precisely identical, to that of synaptophysin in control neurons (Fig. 9, *a* and *b*). After BFA treatment, rab3A immunoreactivity remained unchanged throughout the cell. Synaptophysin-positive tubules in the perikaryal-dendritic region were rab3A-negative (Fig. 10, *c* and *d*).

Discussion

In this study we have used the fungal metabolite BFA as a tool to explore the relationship between the recycling pathway of SV proteins and early endosomes in neurons and to investigate differences between the endocytic systems in axons and dendrites. BFA was found to produce dramatic effects on perikaryal and dendritic endomembranes but did not appear to affect endomembranes in the axonal domain. In addition, BFA unmasked a heterogeneous distribution of SV proteins in the perikaryal-dendritic region. These findings demonstrate that recycling mechanisms are different in axons and dendrites. Furthermore, they indicate a differential sorting of SV proteins in the perikaryal-dendritic region and are consistent with a biogenesis of SVs distal to the region of the TGN.

Membrane trafficking pathways in neurons are still poorly understood. In nerve terminals, SVs undergo local cycles of exo-endocytosis. The precise mechanisms of this recycling remain to be elucidated, but SV proteins are thought to transit through tubulo-vesicular elements that are morphologically similar to early endosomes of other cells (Heuser and Reese, 1973; Broadwell and Cataldo, 1984; Sulzer and Holtzman, 1989). Early endosomes are believed to act as the first sorting compartments that accept internalized plasma membrane components (Helenius et al., 1983). Indeed, endocytic markers label organelles with the morphological characteristics of early endosomes in nerve endings as well as in perikarya and dendrites (this study; Heuser and Reese, 1973; Broadwell and Cataldo, 1984; Sulzer and Holtzman, 1989; Parton et al., 1992). However, it is not known whether the properties of early endosomes in neurons are similar to those of early endosomes in other cells and how SV recycling pathways are related/interconnected with other endocytic pathways that operate in the same cell. These pathways are involved in the recycling of plasma membrane receptors and in the incorporation and/or removal of plasma membrane components during neuronal growth and differentiation.

Figure 5. Ultrastructure of giant axonal processes after 30 min incubation with HRP. (*a*) Leading edge (150-nm thick section) of the giant axonal process shown at lower magnification in *b* (*field enclosed by a rectangle in b*). Note the sharp transition between flat, organelles-free, lamellipodia and filopodia (*asterisks*), and a thicker central region rich in organelles. (*c*) Slightly oblique section of a giant axonal process which grazes the bottom portion of the process. Arrows point to clusters of SVs. Some of these vesicles, as well as tubulo-vesicular structures and larger vacuoles are labeled by HRP reaction product. (*d*) Detail of a large axonal process showing abundance of HRP-labeled tubulo-vesicular structures. Bars: *a*, 2.6 μm; *b*, 28 μm; *c*, 510 nm; *d*, 710 nm.

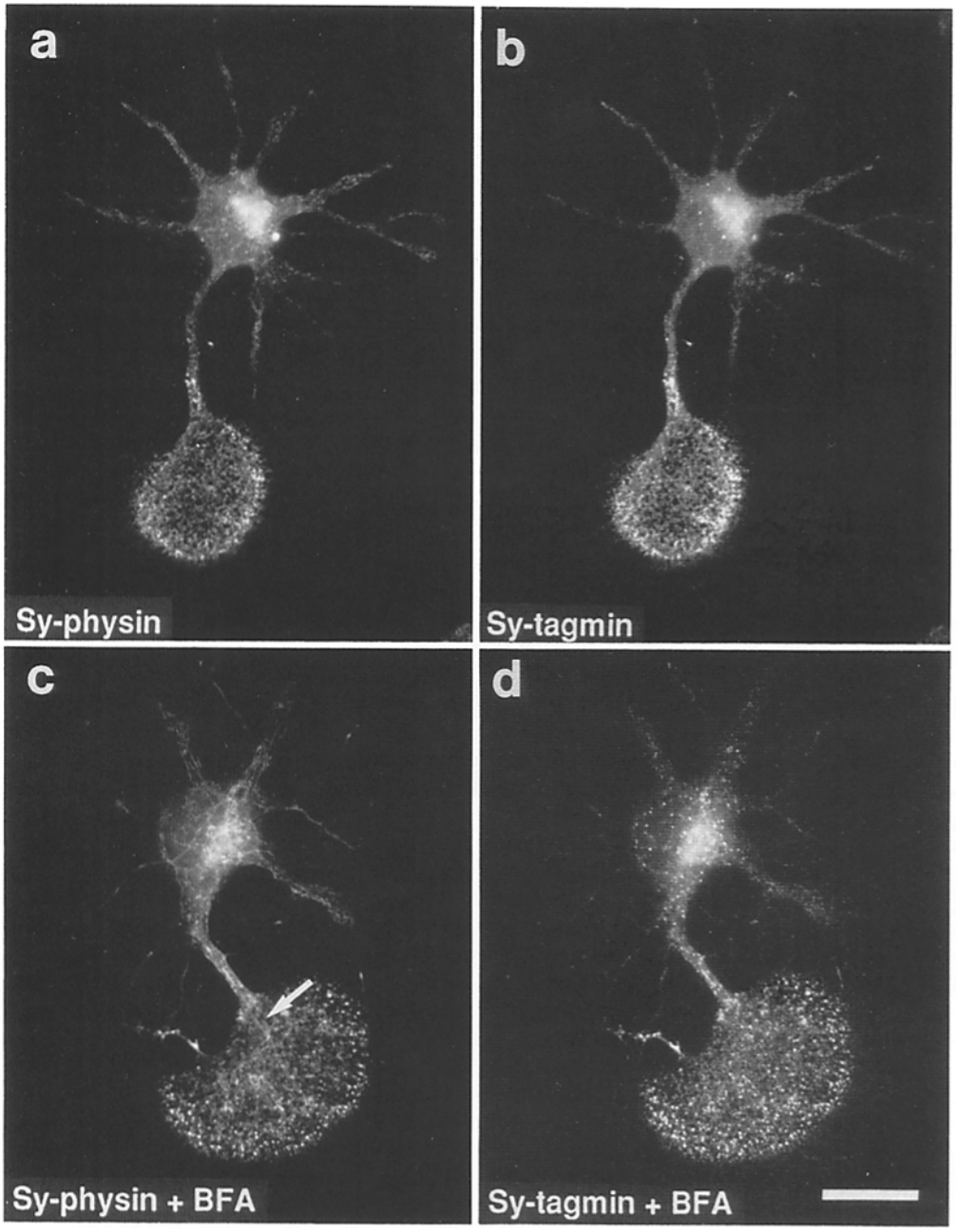

Figure 7. Double immunofluorescence micrographs showing the distribution of synaptophysin and synaptotagmin in control (*a* and *b*) and BFA-treated (*c* and *d*) hippocampal neurons. In the control neuron the distribution of the two proteins is very similar in the perikaryal-dendritic region and identical in the axon. After BFA treatment, the massive redistribution of the perikaryal-dendritic pool of synaptophysin into a reticular network (*c*) is not paralleled by a corresponding tubulation of synaptotagmin (*d*). The few synaptophysin-positive tubules which extend from the perikaryal dendritic region into the stalk of the axon are negative for synaptotagmin (*arrows* in *c*). Bar, 19 μm.

In the present study, we have used BFA to examine whether neuronal early endosomes are perturbed by this drug in a manner similar to early endosomes of other cells (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Hunziker et al., 1991) and to what extent SV proteins are colocalized with markers of the early endocytic pathway after this treatment. We used cultured hippocampal neurons (Banker and Cowan, 1977) at a stage in which they have already established axonal and dendritic polarity but have not yet formed functional synapses. In these cultures some neurons had an extremely large axon or distal region of the axon. This offered the opportunity to study the distribution of SV protein-containing membranes in the axonal compartment at a high level of resolution using immunofluorescence. It remains to be established whether the neurons with these axons represent a specific subpopulation of hippocampal cells. However, with the exception of the size, the properties of these axons appeared to be very similar to the properties of more conventional axons.

The Trf-R, a well established marker of early endosomes in nonneuronal cells (Dautry-Varsat et al., 1983; Klausner et al., 1983; Jing et al., 1990) is generally restricted to the perikaryal-dendritic region of these neurons (Cameron et al., 1991 and this study). Already at an early stage of neuronal polarization, when one of the processes becomes dominant in size and starts accumulating SV proteins, Trf-R is present in this process at a much lower concentration than in the other processes. As the neuron further differentiates, Trf-R is excluded from the prospective axon in spite of the presence in both axons and dendrites of organelles with the

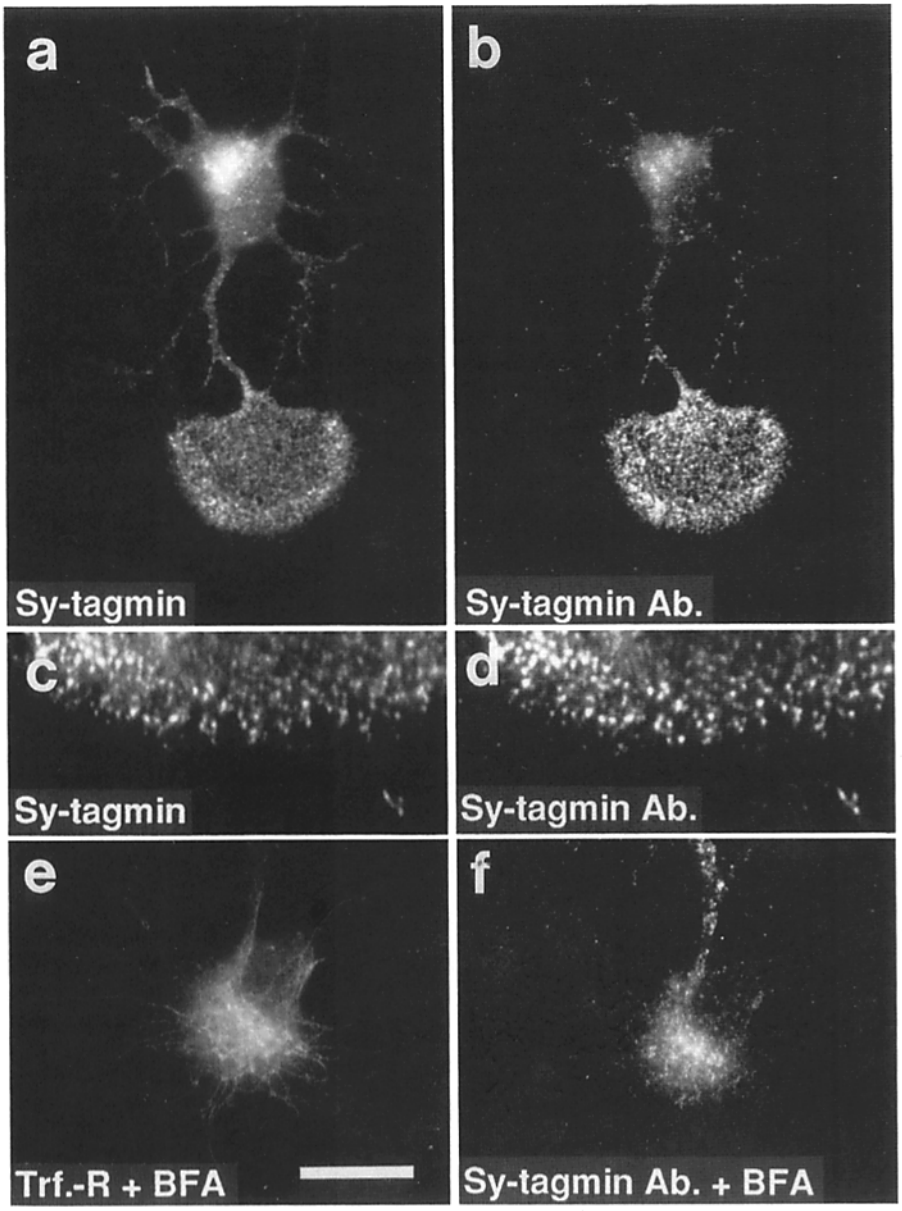

Figure 8. Distribution of internalized antibodies directed against the lumenal domain of synaptotagmin I before and after BFA treatment. (*a–d*) Neurons were incubated for 1 h at 37°C in the presence of Syt_{lum}-Abs, fixed, permeabilized, reacted with TRITC-conjugated goat anti-rabbit IgGs (*b* and *d*) and double-stained with a mAb directed against the cytoplasmic domain of synaptotagmin I (*a* and *c*). *c* and *d* show the identity of the two fluorescent patterns in the leading edge of a giant axon at high magnification. (*e* and *f*) Neurons previously exposed for 1 h to Syt_{lum}-Abs were subsequently incubated in the presence of BFA for 20 min in the continued presence of the antibodies and finally fixed and double-stained for Trf-R (*e*) and for the internalized antibodies (*f*). The distribution of internalized antibodies remained highly punctate in the neuronal perikaryon and dendrites (*f*) in spite of the typical tubulation of the Trf-R network (*e*). Bars: (*a* and *b*) 22 μm; (*c* and *d*) 7.6 μm; (*e* and *f*) 16 μm.

typical characteristics of early endosomes: tubulovesicular structures which become labeled by HRP or Lucifer yellow under conditions which produce a massive labeling of the early endocytic system. All SV proteins investigated, synaptophysin, synaptotagmin, synaptobrevin, p29, SV2 and rab3A, are enriched in the axonal compartment but are also present at significant concentration in dendrites. Thus, at least in neurons developing in isolation, the exclusion of Trf-R from axons is not paralleled by a concomitant exclusion of SV proteins from dendrites. Perhaps, the accumulation of SV proteins in axons is the result of their selective retention in axons and not, or not exclusively, the result of a preferential targeting (Cameron et al., 1993).

The polarized distribution of Trf-R and SV proteins in axons and in dendrites correlates with different functional properties of the endocytic system in the two compartments, as demonstrated by the BFA experiments. After BFA treatment, the punctate Trf-R immunoreactivity observed in control cells was converted into a network of interconnected tubules coursing throughout the perikaryal-dendritic region. This was in agreement with the well documented property of BFA to tubulate early endosomes in a variety of other cell types including endocrine cells (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Hunziker et al., 1991). After BFA treatment, the largest fraction of the perikaryal-dendritic pool of synaptophysin underwent cotubulation with Trf-R, while the distribution of synaptophysin in axons remained primarily punctate. It was of interest, however, that the few synaptophysin-positive tubules visible in axons of incompletely polarized cells were, without any exception, also positive for Trf-R. It appears that the synaptophysin-containing membranes which respond to BFA consistently contain Trf-R, defining most likely the perikaryal-dendritic early endosome. In some studies, long endosomal tubules

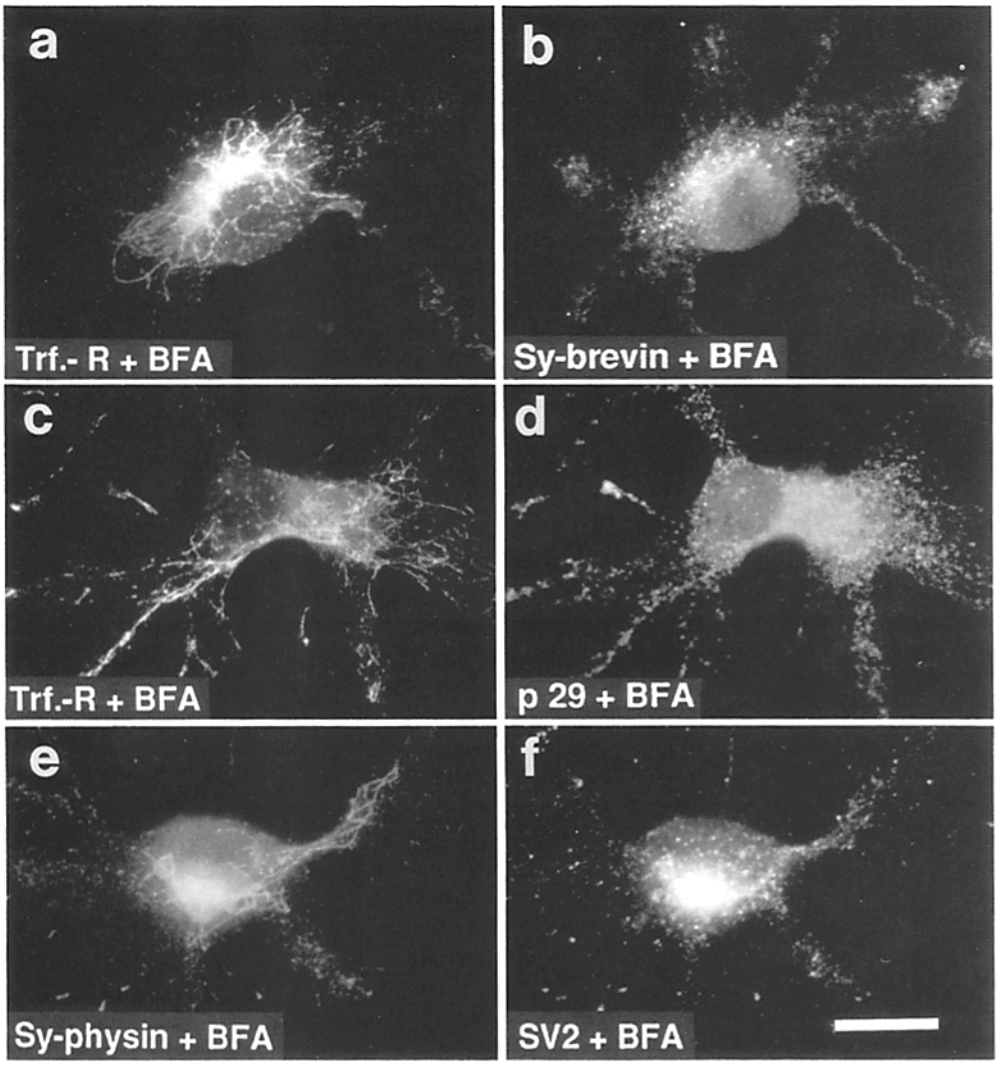

Figure 9. Effect of BFA treatment on the distribution of synaptobrevin, p29 and SV2. Double-immunofluorescence micrographs of the perikaryal-dendritic region of BFA-treated neurons. (*a* and *b*) comparison of Trf-R and synaptobrevin; (*c* and *d*) comparison of Trf-R and p29; (*e* and *f*) comparison of synaptophysin and SV2. Bar: (*a–f*) 13 μm.

were observed in neurons even in the absence of BFA. Even in this case, they were observed in dendrites but never in axons (Tixier-Vidal et al., 1988; Parton et al., 1992).

Unexpectedly, in perikarya and dendrites, synaptotagmin, synaptobrevin, p29 and SV2, four intrinsic SV membrane proteins were not redistributed in parallel with synaptophysin into the BFA-induced reticulum. The reticulum was virtually negative for synaptobrevin, p29, SV2, and synaptotagmin even when Syt_{lum}-Abs (Matteoli et al., 1992) were used to track synaptotagmin recently internalized by antigen-mediated endocytosis. Lack of rab3A from the tubules is consistent with current models predicting that rab3A is associated with SV membranes only at stages preceding exocytosis and is not present on endosomes (Fischer von Mollard et al., 1991; Matteoli et al., 1992).

The heterogeneous distribution of intrinsic membrane proteins of SVs in perikarya and dendrites indicates a differential sorting of SV proteins in these cell regions and demonstrates that developing neurons contain a substantial fraction of SV proteins which are not yet coassembled in SVs or SV precursor membranes. This assembly is likely to require a well defined hierarchy of protein–protein interactions (Bennet et al., 1992) because SVs within a given neuron are highly homogeneous morphologically and biochemically. Our results suggest that not all these interactions are established as newly synthesized SV proteins exit the TGN, and are consistent with a biogenesis of mature SVs distal to the TGN. Recently, Régnier-Vigouroux et al. (1991) have reported that in a neuroendocrine cell line, newly synthesized synaptophysin enters a compartment with the sedimentation characteristics of SVs only after several cycles of exo-endocytosis. The pool of synaptophysin which is tubulated by BFA may reflect the fraction of this protein which has not yet been assembled into mature SVs.

With regard to the heterogeneous distribution of intrinsic SV membrane proteins in perikarya and dendrites, several explanations may be considered. The five proteins may be in the same membrane compartment but have a different distribution throughout the compartment. Synaptophysin may be homogeneously distributed in the BFA-induced tubules, while synaptotagmin, synaptobrevin, p29 and SV2 may have a discrete localization. Alternatively, all SV proteins may transit through these tubules, but be sorted away from these tubules into SVs or SV precursor membranes with variable degrees of efficiency. The intensity of the tubular staining may be indirectly related to the efficiency of the sorting. An-

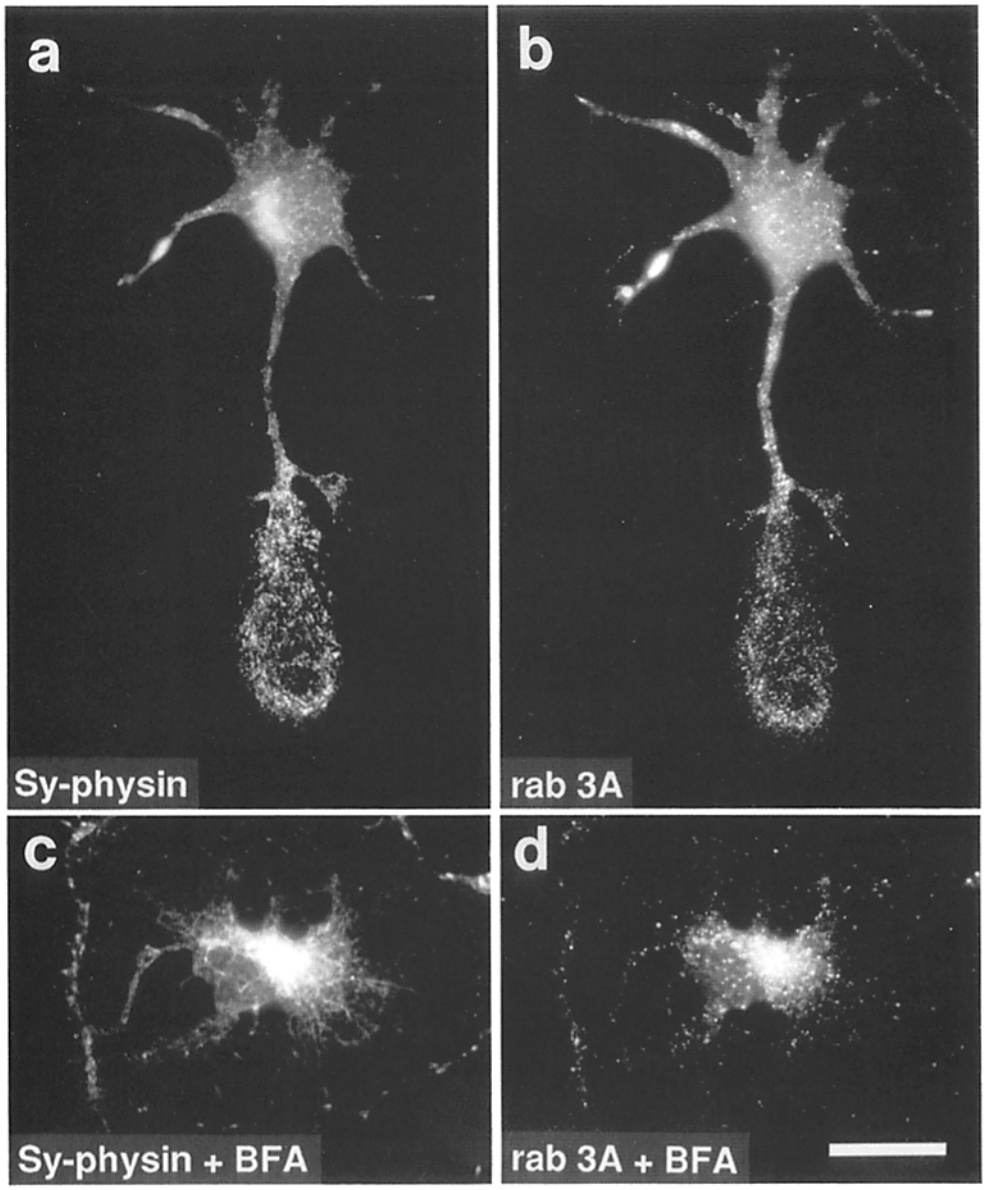

Figure 10. Distribution of synaptophysin and rab 3A before (*a* and *b*) and after (*c* and *d*) BFA treatment. Double immunofluorescence micrographs. The two proteins have a similar distribution in control neurons although the distribution of rab3A is more finely punctate than that of synaptophysin. After BFA treatment rab3A immunoreactivity remained punctate and did not codistribute with the tubular network of synaptophysin immunoreactivity visible in dendrites. Bars: (*a* and *b*) 23 μm; (*c* and *d*) 16 μm.

other possibility is that in dendrites two endosomal populations may coexist: one sensitive to BFA and rich in synaptophysin and the other enriched with all SV proteins, resistant to BFA and corresponding to axonal endosomes. Additionally, a fraction of synaptotagmin, and possibly a small pool of other synaptic vesicle proteins, may be localized on large dense-core vesicles, the peptide-containing secretory organelles (Schmidle et al., 1991; Walch-Solimena et al., 1993). However, the few LDCVs present in dendrites of hippocampal neurons cannot account for the bulk of dendritic synaptotagmin or other SV proteins. Clearly, the dramatic tubulation exhibited by synaptophysin is not shared by at least four of the SV proteins, raising the possibility that synaptophysin might have an important role in the function of classical early endosomes as well as in SV function (Lah and Burry, 1993).

BFA did not produce any obvious morphological change in axons. In this neuronal compartment lack of synaptophysin tubulation, as well as the close colocalization of all SV proteins investigated, may be partially explained by an abundance of mature SVs and by their predominance over endosomes. This may mask any tubulation of SV protein-containing endosomal membranes. On the other hand, our electron microscopic observations of axons after HRP uptake demonstrated a lack of the typical tubulation pattern induced by BFA (very long interconnected tubules following the course of microtubules [Tooze and Hollinshead, 1992]). Additionally, no tubulation of recently internalized (5 min) Lucifer yellow was observed by immunofluorescence. Thus, it seems likely that axonal endosomes may be resistant to the action of BFA. It is of interest that BFA did not appear to have any effect on recycling of SVs as demonstrated by Syt_{lum}-Ab uptake (Matteoli et al., 1992 and this study). It also did not impair neurotransmitter release at the frog neuromuscular junction even after sustained and prolonged stimulation, i.e., after conditions expected to involve at least a complete recycling of the whole SV population (Fesce, D., M. Matteoli and P. De Camilli, unpublished observations). Although

BFA is known not to block recycling between endosomes and the plasmalemma (Lippincott-Schwartz et al., 1991; Wood et al., 1991), in principle, the fidelity of SV budding from axonal endosomes could have been affected by BFA.

Our results demonstrate some fundamental differences between the endocytic system in axons and in dendrites. A segregation of the endosomal system in two distinct compartments is found also in polarized epithelial cells (Parton et al., 1989). In these cells the Trf-R is restricted to basolateral endosomes (Fuller and Simons, 1986), which undergo massive tubulation in response to BFA (Hunziker et al., 1991; Wagner, M., D. Hanzel, I. R. Nabi, and E. Rodriguez-Boulan. 1993. *J. Cell. Biochem.* 17B:278*a*. The property of BFA to tubulate perikaryal-dendritic endosomes, which are positive for Trf-R, supports the concept of a close similarity between basolateral sorting in MDCK cells and perikaryal-dendritic sorting in neurons (Dotti and Simons, 1990; Rodriguez-Boulan and Powell, 1992; Cameron et al., 1993). BFA also perturbs polarized protein sorting in MDCK cells, including transcytosis from the basolateral to the apical region (Hunziker et al., 1991; Low et al., 1992; Wagner, M., D. Hanzel, I. R. Nabi, and E. Rodriguez-Boulan. 1993. *J. Cell. Biochem.* 17B:278*a*). Whether BFA blocks transcytosis from dendrites to axons, remains to be established. This transcytotic route is well documented in mature neurons (Kuypers and Ugolini, 1990). It is of interest that BFA was found to block axonal growth and to induce the retraction of axons already formed in isolated hippocampal neurons at early stages of differentiation (Jareb, M. I., and G. A. Banker. 1991. *Soc. Neurosci.* 17/1:296.10*a*).

In conclusion, our present study suggests that SV biogenesis takes place at the cell periphery and that their exo-endocytotic recycling in nerve terminals involves an endosomal system with some unique properties. Altogether, our findings are consistent with the existence of functional differences between the endocytic systems in dendrites and axons.

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