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Fusion of Endosomes Involved in Synaptic Vesicle Recycling

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Recycling of vesicles of the regulated secretory pathway presumably involves passage through an early endosomal compartment as an intermediate step. To learn more about the involvement of endosomes in the recycling of synaptic and secretory vesicles we studied in vitro fusion of early endosomes derived from pheochromocytoma (PC12) cells. Fusion was not affected by cleavage of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins synaptobrevin and syntaxin 1 that operate at the exocytotic limb of the pathway. Furthermore, fusion was inhibited by the fast Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid but not by the slow Ca^{2+} chelator EGTA. Endosome fusion was restored by the addition of Ca^{2+} with an optimum at a free Ca^{2+} concentration of 0.3×10^{-6} M. Other divalent cations did not substitute for Ca^{2+}. A membrane-permeant EGTA derivative caused inhibition of fusion, which was reversed by addition of Ca^{2+}. We conclude that the fusion of early endosomes participating in the recycling of synaptic and neurosecretory vesicles is mediated by a set of SNAREs distinct from those involved in exocytosis and requires the local release of Ca^{2+} from the endosomal interior.

INTRODUCTION

Neurotransmitter release in the nerve terminal is mediated by exocytosis of synaptic vesicles. After exocytosis, the vesicle membrane is retrieved by endocytosis and used for the reformation of fusion-competent synaptic vesicles. In recent years, the pathways involved in the vesicle recycling have received considerable attention. Both morphological (Heuser and Reese, 1973; Shupliakov *et al.*, 1997) and genetic evidence (Koenig and Ikeda, 1989; González-Gaitán and Jäckle, 1997) demonstrate that clathrin-coated vesicles are essential intermediates in vesicle recycling, although direct, i.e., non–clathrin-dependent, retrieval mechanisms may coexist in the synapse (Fesce *et al.* 1996).

Currently, it is still debated to which extent endosomal intermediates are involved in synaptic vesicle recycling. Originally, it was assumed that after pinching off the plasma membrane, clathrin-coated vesicles decoat and subsequently fuse with early endosomes. Synaptic vesicles then regenerate by budding from the endosome. This view was mostly based on analogy to clathrin-mediated recycling pathways in non-neuronal cells (Goldstein *et al.*, 1985; Kelly, 1993). Membrane cisternae larger than synaptic vesicles are visible in nerve terminals, at least after intense stimulation, which may correspond to endosomes (Heuser and Reese, 1973). In addition, rab5, a resident of early endosomes (Chavrier *et al.*, 1990), is highly enriched in synapses, suggesting that early endosomes play a prominent role in the synaptic vesicle pathway (Fischer von Mollard *et al.*, 1994). However, recent data suggest that synaptic vesicles may recycle directly without intermediate fusion and budding steps. When vesicles undergoing endocytosis are labeled with the styryl dye FM 1-43, it was found that the amount of dye a vesicle releases upon subsequent exocytosis equals the amount taken up by endocytosis (Murthy and Stevens, 1998), ruling out communication with intermediate endosomal compartments. Additional evidence for direct recycling was obtained when the biogenesis of synaptic vesicles was studied in PC12 cells. Here, vesicles are generated in parallel by two clearly distinguishable pathways. One of them presumably involves direct retrieval from the plasma membrane (Shi *et al.*, 1998). In the second pathway synaptic vesicles bud off from endosomal precursors in a step that is Arf and AP-3 dependent and inhibited by brefeldin A (Faúndez *et al.*, 1997, 1998; Lichtenstein *et al.*, 1998). Thus it appears that endosomes may be bypassed during recycling of synaptic vesicles. Nevertheless, the available data also suggest that

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synaptic vesicles pass through endosomes at least occasionally during repetitive recycling. Clearly, vesicle constituents are subject to endocytic sorting, an event that requires endosomes as functional sorting compartments.

Synaptic vesicles that passage through endosomal intermediates undergo two distinct budding and fusion steps in each cycle. Exocytotic fusion is well characterized. It is highly regulated by intracellular Ca^{2+}-concentrations and is mediated by a set of conserved membrane proteins including synaptobrevin, syntaxin, and SNAP-25, commonly also referred to as SNAREs (soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein receptors). Botulinum and tetanus toxins irreversibly block exocytosis by means of selectively proteolyzing these exocytotic SNAREs (Niemann *et al.*, 1994; Montecucco and Schiavo, 1995). The neuronal SNARE proteins are representatives of a large protein superfamily that appears to be involved in virtually every intracellular fusion reaction. Although still debated, it is currently thought that SNARE assembly directly mediates membrane fusion (Jahn and Hanson, 1998).

The mechanisms of endosome fusion in this pathway are less well understood. However, endosome fusion has been investigated in some detail in non-neuronal cells, mostly because of the availability of convenient cell-free fusion assays (Braell, 1987; Gruenberg and Howell, 1987; Diaz *et al.*, 1989). Both fusion of incoming endocytotic compartments with early endosomes as well as of endosomal vesicles with each other has been described (Mayorga *et al.*, 1988; Diaz *et al.*, 1989; Woodman and Warren, 1991). However, early endosomes cannot fuse with carrier vesicles shuttling between early and late endosomes, or with late endosomes indicating that specific proteins are required for each fusion event (Braell, 1987; Gruenberg and Howell, 1987; Gruenberg *et al.*, 1989). Fusion is sensitive to ionic environment (Diaz *et al.*, 1993) and requires ATP and both soluble and membrane-bound proteins (Diaz *et al.*, 1989). The small GTPase rab5 plays an essential role in endosome fusion (Gorvel *et al.*, 1991). Several putative effector proteins have been described for rab5, including rabaptin-5 (Stenmark *et al.*, 1995), rabex-5 (Horiuchi *et al.*, 1997), and EEA1 (Simonsen *et al.*, 1998), which may operate in conjunction with rab5. Studies on the fusion of vacuole precursors in yeast have suggested that rab proteins and their effectors are required for membrane attachment but do not participate in the fusion reaction itself (Ungermann *et al.*, 1998).

Currently, it is unknown which SNARE proteins mediate endosome fusion. During synaptic vesicle recycling, considerable amounts of all three synaptic SNAREs are endocytosed (Walch-Solimena *et al.*, 1995). These proteins are functionally "active" because they form SNARE complexes in the membrane, which can be reversibly disassembled by the ATPase NSF (Otto *et al.*, 1997). Recent evidence has shown that there is little specificity in SNARE pairing, because even only distantly related members of the SNARE family can replace a given SNARE in a SNARE complex (Fasshauer *et al.*, 1999; Yang *et al.*, 1999). In addition, it was reported that fusion of endosomes derived from fibroblasts (BHK-21 cells) is insensitive to pretreatment with clostridial neurotoxins (Link *et al.*, 1993), whereas fusion of aquaporin-containing endosomes derived from kidney papillae is inhibited by tetanus toxin (TeNT) (Jo *et al.*, 1995). Thus it is conceivable that exocytotic SNAREs also function in endosome fusion. Furthermore, calmodulin has been invoked in intracellular fusion events, including endosome fusion (Colombo *et al.*, 1997), raising the possibility that endosome fusion, like exocytosis, is dependent on a rise of intracellular calcium.

In the present study, we have investigated the fusion of early endosomes involved in synaptic vesicle recycling using the neuroendocrine cell line PC12 as a model. PC12 cells possess two types of secretory vesicles: secretory granules (large dense-core vesicles) containing dopamine and protein and small synaptic vesicles containing acetylcholine (Bauerfeind *et al.*, 1993). Although their exocytosis is presumably differentially regulated, they share a common pool of endosomes during recycling (Bauerfeind *et al.*, 1995). Our results show that endosome fusion does not involve exocytotic SNAREs and requires local release of calcium from intraorganellar stores.

MATERIALS AND METHODS

Antibodies

The following antibodies were described previously: synaptobrevin (monoclonal antibody Cl 69.1) (Edelmann *et al.*, 1995), synaptophysin (monoclonal antibody C 7.2) (Jahn *et al.*, 1985), SNAP-25 (Cl 71.1) (Bruns *et al.*, 1997), and cellubrevin (rabbit antiserum) (Annaert *et al.*, 1997). For NSF, mouse monoclonal antibodies were generated using recombinant NSF as antigen. These antibodies, which will be described in detail elsewhere (Rammner, Otto, and Jahn, manuscript in preparation), recognized a single band in immunoblots corresponding to the position of NSF. The following antibodies were kind gifts: syntaxin (monoclonal antibody HPC-1; kindly provided by Dr. C. Barnstable, Yale University, New Haven, CT) and sec61α (rabbit serum; kindly provided by Dr. E. Hartmann, Göttingen University, Göttingen).

Fluid Phase Internalization

When BHK-21 cells were used, the experiments were carried out essentially as described previously (Gruenberg *et al.*, 1989, 1991; Link *et al.*, 1993). For PC12 cells (Greene and Tischler, 1976) the protocol was slightly modified. Briefly, PC12 cells were grown to confluency on collagen-coated culture dishes in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum and 10% heat-inactivated horse serum (Life Technologies, Gaithersburg, MD), 20 mM HEPES, pH 7.4, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 7.5% CO_2. Cells were harvested by pipetting with ice-cold saline PBS and washed repeatedly. Cells were washed once in prewarmed internalization medium (OptiMEM; Life Technologies) supplemented with 10 mM D-glucose). Ten confluent plates of a diameter of 150 mm resulted in a cell pellet of ~1.5 ml vol. The cell pellet was resuspended in 4 vol (vol/vol) of internalization medium that contained either 3.2 mg/ml avidin (Pierce, Rockford, IL) or 1.7 mg/ml biotinylated HRP (prepared according to the method of Gruenberg *et al.*, 1991) for the acceptor and donor compartments, respectively, and incubated for 5 min at 37°C. The cells were rapidly diluted with 3–4 vol of ice-cold PBS containing 5 mg/ml BSA and washed four times at 4°C.

Preparation of Subcellular Fractions

All steps of the preparation were carried out at 4°C or on ice. After fluid phase internalization (see above) the cell pellet was resuspended in 4 vol (vol/vol) of homogenization buffer (250 mM sucrose, 3 mM imidazole-HCl, pH 7.4, and protease inhibitors: 10 μg/ml trypsin inhibitor, 0.7 μg/ml pepstatin, and 0.1 mM PMSF) and homogenized by 20 passages through a stainless steel ball homogenizer with a clearance of 0.0005 inches and eight passages with a clearance of 0.0009 inches for PC12 and BHK-21 cells, respec-

tively. The homogenates were centrifuged for 15 min at 1800 × *g*. The resulting postnuclear supernatants were divided into aliquots, snap frozen in liquid N_2, and stored at −70°C until use. Dilutions of these samples were used as sources for either the acceptor compartment (avidin) or the donor compartment (biotinylated HRP) in the fusion assay. HRP uptake into organelles was determined by measuring the amount of HRP recovered in the membrane fraction that was obtained by centrifugation of the postnuclear supernatant at 45,000 × *g* for 30 min using a TLA-100.3 rotor (Beckman Instruments, Palo Alto, CA). In the membrane fraction an average of 50–200 ng HRP/mg protein of the postnuclear supernatants was internalized.

For the preparation of cytosol, BHK-21 or PC12 cells were resuspended in 3 vol of homogenization buffer and homogenized in a stainless steel ball homogenizer with 10 passages (clearance, 0.0009 inches) or 20 passages (clearance, 0.0005 inches), respectively. A postnuclear supernatant obtained as described above was centrifuged at 14,000 × *g* for 25 min. The resulting low-speed supernatant was centrifuged at 185,000 × *g* for 1 h in a TLA-100.3 rotor. The high-speed supernatant was divided into 500-μl aliquots, snap frozen in liquid N_2, and stored at −70°C until use. For the preparation of cytosol from rat brain, brains were homogenized in homogenization buffer (1 ml/g tissue) using a glass-Teflon homogenizer. The homogenate was centrifuged at 3500 × *g* for 20 min. A high-speed supernatant was obtained as described above.

Cell-free Fusion Assay

The assay for in vitro fusion of early endosomes of PC12 and BHK-21 cells and PC12–BHK-21 mixed cells was performed as previously described (Gruenberg *et al.*, 1989; Gorvel *et al.*, 1991; Link *et al.*, 1993) with minor modifications.

Reaction mixtures (200 μl in total) were assembled on ice, containing, as final concentrations, postnuclear supernatants (4 mg protein/ml), cytosol (2.5 mg/ml; if not indicated otherwise, cytosol from rat brain was used), 11.25 mM HEPES, pH 7.0, 1.35 mM magnesium acetate, 0.18 mM dithiothreitol, 45 mM potassium acetate, 0.05 mg/ml biotinylated insulin as quencher, and as ATP-regenerating system 1.6 mM ATP, 13 mM creatine phosphate, and 0.066 mg creatine phosphokinase (800 U/mg; Boehringer Mannheim, Mannheim, Germany). Quantification of the enzymatic activity of biotinylated HRP was performed as described (Al-Kassai and Mostratos, 1983). Fusion efficiency was calculated by relating the amount of biotinylated HRP recovered in the immunoprecipitated complex to the amount of HRP recovered in the membrane fraction of the postnuclear supernatant. ATP-dependent fusion efficiency of PC12 cell endosomes, measured under standard conditions, ranged between 25 and 30%.

For treatment with *N*-ethylmaleimide (NEM), both postnuclear supernatants and cytosol were preincubated separately with 1 mM NEM at 37°C for 30 and 10 min, respectively. For the experiment shown in Figure 1, NEM pretreatment was performed for only 15 min on ice, followed by the addition of 2 mM DTT and another 10-min incubation on ice. For incubation with light chains (L chains) of TeNT or of botulinum neurotoxin C1 (BoNT/C1), the postnuclear supernatants were preincubated separately at 37°C for 30 min in the presence of active or heat-inactivated toxin L chains. An aliquot was removed before the fusion reaction to check for substrate cleavage. Stock solutions for 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; Molecular Probes, Eugene, OR), EGTA, and EGTA-acetoxymethyl ester (AM) (Calbiochem, La Jolla, CA; 200 mM) were carefully adjusted to neutral pH. For treatment with EGTA-AM postnuclear supernatants were preincubated for 30 min on ice and 30 min at room temperature with EGTA-AM (dissolved in DMSO). Controls for the effect of the solvent alone in our endosome fusion assay were also performed.

Immunoisolation

Monoclonal antibodies C 7.2 were covalently coupled to the reactive surface of Eupergit C1Z methacrylate microbeads (1 μm mean diameter; Roehm Pharma, Darmstadt, Germany) as described (Burger *et al.*, 1989). Antibody-containing immunobeads are referred to as synaptophysin beads. Fusion assays were performed as indicated above using PC12 postnuclear supernatants (1 mg protein/ml) and cytosol from rat brain (2.5 mg protein/ml). After completing the fusion reaction, 6.25 μl of synaptophysin beads and control beads, respectively, were added, followed by 30 min of incubation on a rotator at 4°C. The sample was then diluted in 3 vol homogenization buffer, layered on top of a sucrose cushion (0.5 ml, 0.8 M), and centrifuged for 5 min at 4600 × *g* in a microfuge. The supernatants were subjected to a high-speed centrifugation step for 30 min at 200,000 × *g* at 4°C using a Beckman TLA-120.2 rotor to sediment nonbound membranes. The bead pellets were resuspended in 1 ml fusion assay buffer and centrifuged for 5 min at 1800 × *g*. An aliquot of each sample was then solubilized in detergent and the HRP–avidin complex was immunoprecipitated and quantitated as described to determine fusion activity. A second aliquot was analyzed by SDS-PAGE and immunoblotting.

Other Methods

Plasmids encoding His_6-TeNT L chain and His_6-BoNT/C1 L chain were kindly provided by Dr. Heiner Niemann (Medizinische Hochschule, Hannover, Germany). Fusion proteins were purified on Ni^{2+}-Sepharose columns according to the manufacturer's protocol (PROBond; Invitrogen, San Diego, CA). Purity was examined by SDS-PAGE and staining with Coomassie blue. The proteins were dialyzed against HEPES-buffered saline (20 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mM dithiothreitol) and stored at −70°C until use. Recombinant NSF was expressed in bacteria and purified as described (Hanson *et al.*, 1997).

SDS-PAGE and immunoblotting were performed according to standard procedures (Laemmli, 1970). For detection, we used either the enhanced chemiluminescence kit of Amersham (Arlington Heights, IL; HRP-conjugated antibodies) or a colorimetric procedure involving the generation of formazan precipitates (Sambrook *et al.*, 1989).

RESULTS

Endosome Fusion Involves Endosomes of the Synaptic Vesicle Recycling Pathway

In the first series of experiments we investigated whether endosome fusion as measured by our assay includes endosomes involved in synaptic or secretory vesicle recycling. Fusion was monitored using an established procedure, which is based on content mixing of two different endosomal populations derived from preloaded cells. PC12 cells were loaded for 5 min either with biotinylated horseradish peroxidase (HRP, donor compartment) or with avidin (acceptor compartment). Endosome-containing cell-free extracts from both cell populations were mixed in the presence of cytosol and ATP. When fusion occurs, a tight complex forms between avidin and biotinylated HRP, which is isolated by immunoprecipitation of avidin and quantitated by measuring HRP activity. To prevent formation of biotinylated HRP–avidin complexes derived from disrupted endosomes, biotinylated insulin was added as quencher. In accordance with endosome fusion reported from other cell lines, fusion was dependent on ATP (also see Figure 6A). Similarly, fusion is prevented by pretreatment of postnuclear supernatants and cytosol with NEM. This inhibition

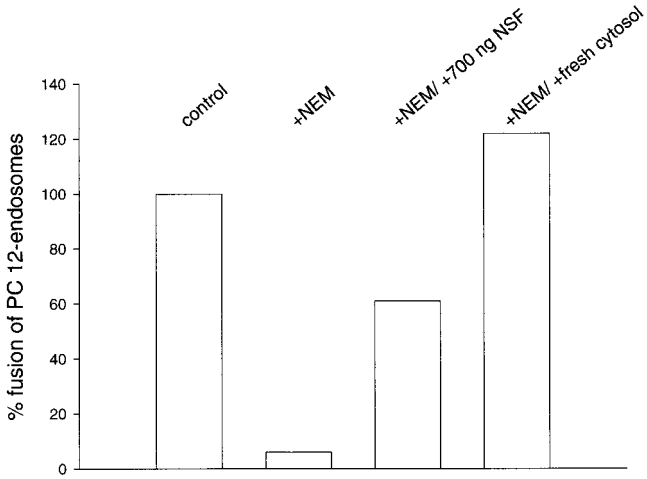

Figure 1. Fusion of PC12 cell-derived endosomes in vitro is inhibited by NEM and restored by subsequent addition of purified NSF or untreated cytosol. The data are derived from a representative experiment.

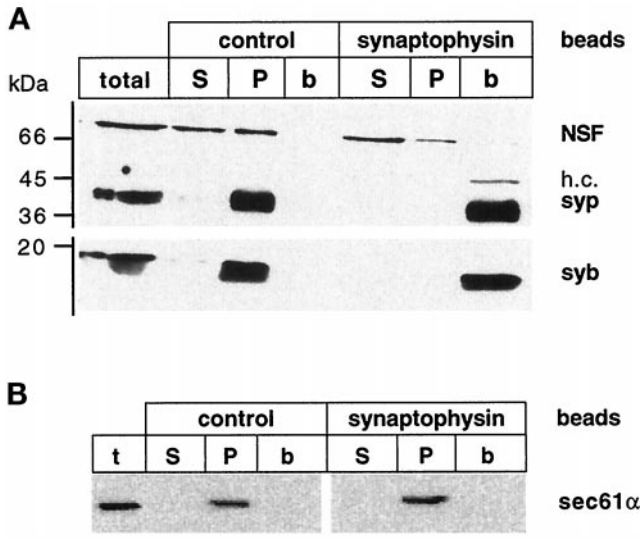

Figure 2. Binding of fused early endosomes to synaptophysin immunobeads. (A) Aliquots of membranes isolated with synaptophysin beads and control beads (b) as well as their corresponding supernatants (S) and high-speed membrane pellets (P) were analyzed by SDS-PAGE and immunoblotting for synaptic vesicle proteins. In the first lane an aliquot of the total fusion reaction before immunisolation was loaded. Note that in addition to endosomes, all synaptophysin-containing membranes are indiscriminately bound to the beads including synaptic-like microvesicles (Cameron *et al.*, 1991). h.c., heavy chain of the antibody C 7.2; syp, synaptophysin; syb, synaptobrevin. (B) Specificity of the immunobeads. The same fractions were analyzed for the distribution of the endoplasmic reticulum-resident sec61α.

is reversed by the subsequent addition of fresh cytosol (Figure 1). Purified NSF partially substitutes for cytosol in this experiment (Figure 1), in agreement with previous reports from other cell lines (e.g., Diaz *et al.*, 1989).

Synaptophysin is specifically localized to synaptic vesicles and, to a lesser extent, to secretory vesicles in PC12 cells and is widely used as a recycling marker in studies addressing vesicle recycling (Linstedt and Kelly, 1991). We therefore used immobilized monoclonal antibodies specific for synaptophysin to immunoisolate all synaptophysin-containing compartments at the end of the fusion reaction and then examined to which extent the fused compartments were bound to the immunobeads. For immunoisolation we used Eupergit C1Z beads, which were shown to yield exceptionally clean fractions with negligible nonspecific binding (Cameron *et al.*, 1991). Immunoisolation of synaptophysin-containing membranes from the fusion assay mix resulted in quantitative binding of all synaptophysin-containing membranes, with virtually no synaptophysin remaining unbound (Figure 2A). No binding was observed when control beads containing no antibody were used. To further control for nonspecific binding of membranes to the immunobeads, we used sec61α, a membrane constituent of the protein import complex from the endoplasmic reticulum, as a marker. As shown in Figure 2B, no sec61α was found in the bead fraction, confirming that the isolation procedure is highly specific.

We then examined the distribution of the fused endosomes in the various fractions. As shown in Figure 3, the vast majority of reaction product was recovered in the immunobead fraction. When inactivated beads were used, most of the reaction product remained in the supernatant, again demonstrating the specificity of the immunoisolation procedure. When membranes present in this supernatant were sedimented by centrifugation, most of the reaction product was recovered in the pellet. This proves that endosomal lysis was negligible in the experiment, because lysis would release the reaction product into the soluble fraction. We conclude that early endosome fusion as monitored by our assay predominantly involves endosomes participating in the trafficking pathway of secretory and synaptic vesicles.

SNAREs Mediating Exocytosis of Synaptic Vesicles Are Not Involved in Endosome Fusion

We next studied whether the SNARE proteins involved in calcium-dependent exocytosis of synaptic vesicles and secretory granules play a role in endosome fusion in PC12 cells. As outlined in INTRODUCTION, the SNARE proteins synaptobrevin 2, syntaxin 1, and SNAP-25 are essential for exocytotic membrane fusion and are present on endocytic compartments. To inactivate exocytotic SNAREs, cell-free extracts were incubated with the L chains of TeNT (specific for synaptobrevin), BoNT C1 (specific for syntaxin 1), and BoNT A (specific for SNAP-25; our unpublished results) before the fusion assay. Incubation with TeNT L chain resulted in virtually complete cleavage of synaptobrevin and its close relative cellubrevin (Figure 4A). Cleavage of syntaxin by BoNT C1 L chain was less effective (in agreement with earlier results; Blasi *et al.*, 1993), but cleavage products were clearly detectable (Figure 4A). When endosome fusion was monitored, no significant change in fusion activity was observed under any of the conditions examined (Figure 4B). The fusion reaction was still ongoing at the time the incubation was terminated (our unpublished results; also see

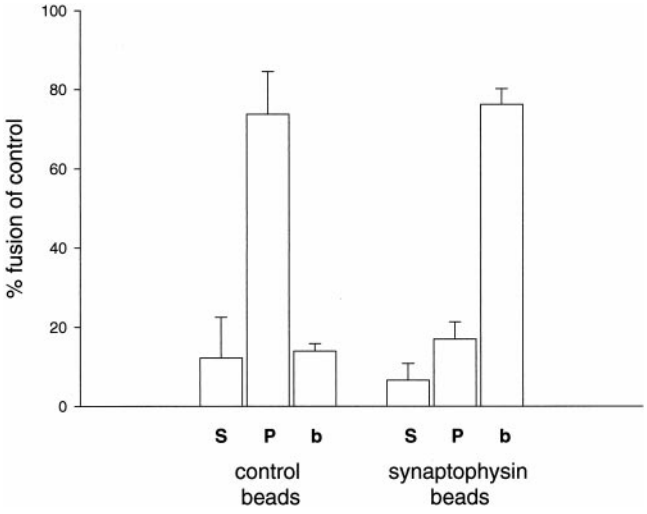

Figure 3. Fused early endosomes were bound to synaptophysin immunobeads. The amount of fusion product in the different fractions, supernatant (S), membrane pellet (P), and bead pellet (b), was monitored. Fusion activity under standard conditions is defined as 100%. The data are represented as means with SD from four independent experiments.

Link *et al.*, 1993), suggesting that not only the extent but also the rate of fusion was unaffected by toxin treatment. We conclude that fusion of early endosomes in PC12 cells involves SNAREs different from syntaxin 1 and synaptobrevin/cellubrevin.

Ca^{2+} Dependence of Early Endosome Fusion in PC12 Cells

Next we analyzed whether the fusion of early endosomes is dependent on the presence of calcium ions for two reasons. First, we wanted to determine whether endosome fusion involved in the recycling of membranes exocytosing in a Ca^{2+}-dependent manner is also dependent on Ca^{2+}, thus resembling exocytotic fusion. Second, evidence has recently accumulated showing that Ca^{2+}- and/or calcium-binding proteins are generally required for intracellular fusion events. For instance, activation of calmodulin appears to be involved in the fusion of endosomes and yeast vacuole precursors (Colombo *et al.*, 1997; Peters and Mayer, 1998). In the latter case it has been found that the calmodulin-dependent step operates after SNARE assembly. It was suggested, that this step is an essential component of the overall fusion reaction. Furthermore, in vitro fusion of nuclear envelope membranes depends on the local release of Ca^{2+} ions from an unknown vesicular store that is probably mediated by inositol 1,4,5-triphosphate (IP_3) receptors (Sullivan *et al.*, 1993). The Ca^{2+}-binding protein that is acted on by Ca^{2+} is not known in this case. The experiments described here should therefore clarify whether 1) fusion of early endosomes from PC12 cells is dependent on calcium; 2) calcium required for fusion is derived from a local pool; and 3) Ca^{2+} dependence is a general feature of endosome fusion irrespective of the cell type and recycling pathway.

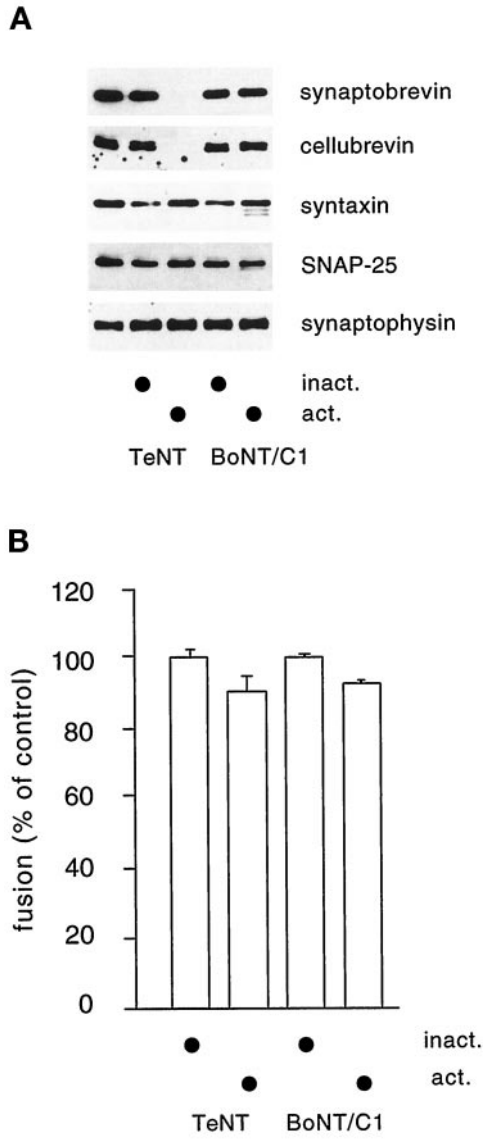

Figure 4. Fusion of early endosomes from PC12 cells is not inhibited by preincubation of the cell-free extract with TeNT-L chain and BoNT/C1-L chain. (A) Aliquots of the toxin-treated extracts were analyzed by SDS-PAGE and immunoblotting for substrate breakdown. In the first lane untreated cell-free extract was loaded. Note that the syntaxin signal is variable; however, partial breakdown is evident by the appearance of bands of increased mobility reflecting cleavage products. No higher degree of breakdown was observed when the BoNT/C1 L chain concentration was increased 50-fold (our unpublished results). (B) Cell-free extracts were incubated for 30 min before the fusion assay with 10 nM TeNT and BoNT/C1, respectively, using either active (act.) or heat-inactivated (inact.) recombinant toxin L chains. The data were derived from five independent experiments.

First, we used the chelator BAPTA to reduce the Ca^{2+} concentration during the fusion reaction. When the assay was performed in the presence of 10 mM BAPTA, fusion was largely inhibited (Figure 5). In contrast, no inhibition was observed when EGTA was added at equal concentra-

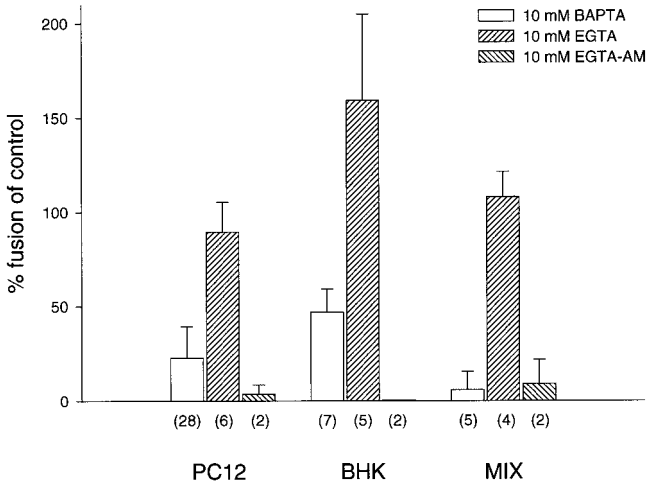

Figure 5. In vitro fusion of early endosomes is inhibited by BAPTA and EGTA-AM but not by EGTA. Fusion was performed under standard conditions (see MATERIALS AND METHODS for details). The numbers in parentheses give the numbers of independent experiments. Error bars represent SD.

tions (Figure 5). To test whether the sensitivity to BAPTA is common to all endosome fusion reactions, we compared fusion of PC12 cell-derived endosomes with that of fibroblast-derived endosomes (BHK-21 cells). As shown in Figure 5, fusion of BHK-21 cell-derived endosomes was also inhibited, but to a lesser degree. The result was independent from the source of cytosol used to support the fusion reaction, including rat brain, PC12 cells, and BHK-21 cells (our unpublished results), and excluding that the differential sensitivity to chelators is due to a tissue-specific cytosolic factor. Similar results were obtained in mixed fusion reactions in which donor and acceptor populations of endosomes were derived from PC12 and BHK-21 cells, respectively (Figure 5, right).

Because both BAPTA and EGTA have very similar affinities for various divalent cations at neutral pH, the strikingly different effects of these chelators on endosome fusion cannot be explained by cation preference. However, BAPTA has >100-fold higher ion association and dissociation rates than EGTA (Tsien, 1980; Fabiato, 1985; Adler *et al.*, 1991). This difference allows for the discrimination of processes that are dependent on fast and local changes of Ca^{2+} concentrations. For example, exocytosis in neurons is sensitive to intracellularly applied BAPTA but largely resistant to EGTA (Adler *et al.*, 1991). Thus, our data suggest that fusion of early endosomes is mediated by a rapid and local release of Ca^{2+} from an intracellular store that is close to, probably identical to, the endosomes participating in the fusion reaction. To test this hypothesis, we examined the effects of a membrane-permeable derivative of EGTA on endosome fusion. Preincubation with such an analogue is expected to chelate Ca^{2+} in vesicular stores that are inaccessible to BAPTA. As shown in Figure 5, preincubation of the endosome-containing extracts with EGTA-AM inhibited fusion by >80% irrespective of whether PC12-derived or BHK-21-derived endosomes were used. We conclude that Ca^{2+} released locally from an intracellular store plays an important regulatory role in triggering endosome fusion.

Both EGTA and BAPTA preferentially chelate Ca^{2+} ions but they are not entirely cation selective. Using PC12 cell endosomes, we therefore tested whether fusion was restored by Ca^{2+} and/or other divalent cations. When increasing concentrations of $CaCl_2$ were added in the presence of 10 mM BAPTA, fusion returned to almost normal levels (85% of control; Figure 6A). Further increase of $CaCl_2$ reduced fusion. The free Ca^{2+} concentration yielding maximal fusion was calculated to be 0.3 μM (Figure 6B). Under all conditions, fusion was strictly dependent on the presence of ATP and cytosol and was sensitive to inhibition by NEM (Figure 6A). None of the other divalent cations tested was able to substitute for Ca^{2+} (Figure 6C). Similarly, the block caused by EGTA-AM was reversed by increasing the free Ca^{2+} concentration (our unpublished results). Together, these results demonstrate that fusion of early endosomes is dependent on the local release of Ca^{2+} and requires Ca^{2+} concentrations that are severalfold higher than the resting levels normally found in cells.

DISCUSSION

In this study we have demonstrated that in the neuroendocrine cell line PC12 fusion of early endosomes predominantly involves recycling membranes of the regulated secretory pathway. Furthermore, the data document that the SNAREs mediating endosome fusion are most probably different from the SNAREs involved in exocytosis. PC12–endosome fusion is dependent on the local release of Ca^{2+} from intraorganellar stores, a feature that appears to be shared by non-neuronal cells.

In the original version of the SNARE hypothesis, it was postulated that each intracellular fusion step is mediated by a unique set of SNAREs. Recently, however, it has become clear that some SNAREs operate in multiple trafficking steps, for example, the yeast Q-SNAREs Sed5p and Vti1p (Götte and Fischer von Mollard, 1998). In particular, there is currently no direct evidence supporting the idea that in a cyclic trafficking pathway (e.g., vesicular traffic between the endoplasmic reticulum and the Golgi apparatus) the anterograde and retrograde fusion steps, respectively, are mediated by different sets of SNAREs. Furthermore, synaptobrevin, like Sed5p, forms complexes with multiple syntaxins in vitro, and a similar promiscuity was observed for syntaxin isoforms. These findings suggest that these proteins may participate in multiple SNARE complexes. However, our data clearly show that the fusion of endocytosed membranes of secretory vesicles involves SNAREs different from those operating during exocytosis of the vesicles. The SNAREs mediating fusion are not yet known, although some SNAREs (endobrevin/VAMP8, syntaxin 7, and syntaxin 12/13) have recently been localized to early endosomes (Advani *et al.*, 1998; Prekeris *et al.*, 1998; Tang *et al.*, 1998; Wong *et al.*, 1998a,b). Intriguingly, we have previously reported that clathrin-coated vesicles involved in synaptic vesicle recycling not only contain the R-SNARE synaptobrevin but also the Q-SNAREs syntaxin and SNAP-25 (i.e., a full set of exocytotic SNAREs), and furthermore, that these proteins form NSF-sensitive ternary complexes (*cis*-complexes) in these membranes (Otto *et al.*, 1997). Thus, early endosomes may contain two or

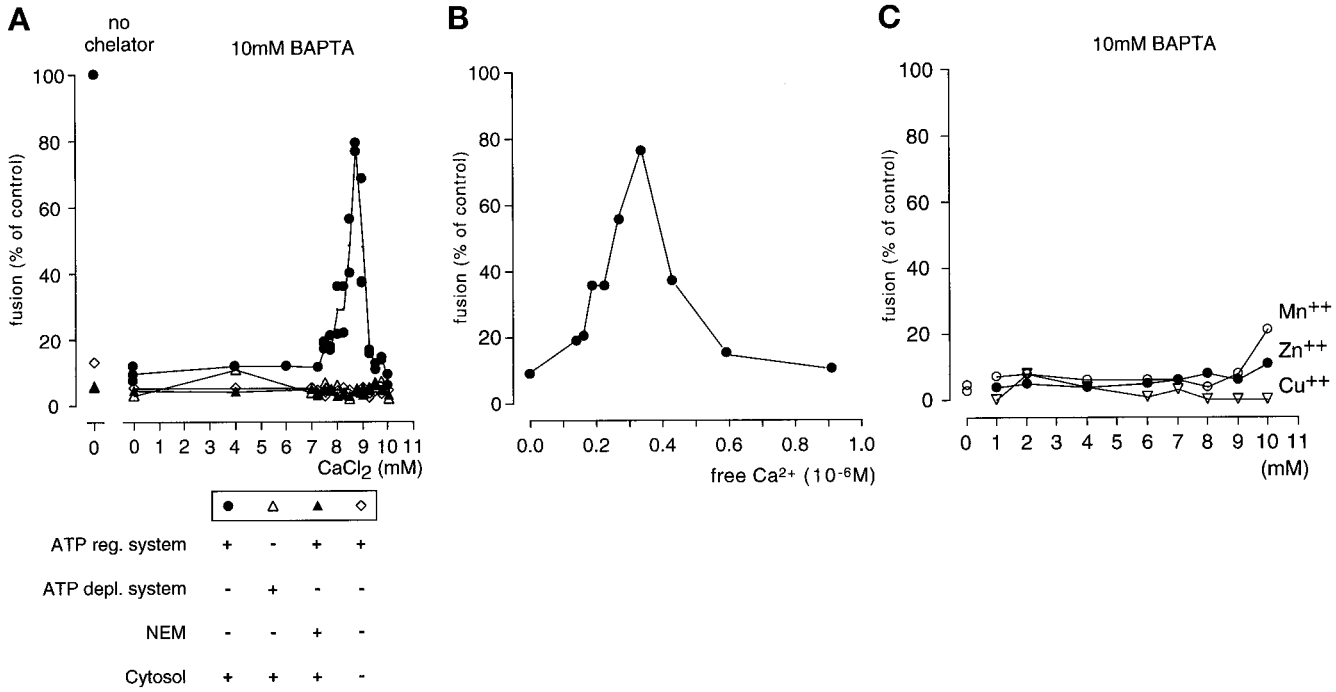

Figure 6. Fusion of early endosomes from PC12 cells is dependent on Ca^{2+}. (A) Endosome fusion inhibited by BAPTA is restored by the addition of $CaCl_2$. No fusion is observed at any Ca^{2+} concentration when cytosol is omitted, ATP is depleted, or the extracts are pretreated with NEM. (B) Calculation of the free Ca^{2+} concentration, based on the data shown in the left panel. Fusion was optimal at a free Ca^{2+} concentration of 0.3 μM. For calculations the program of Föhr *et al.* (1993) was used. (C) Mn^{2+}, Zn^{2+}, and Cu^{2+} ions are unable to substitute for Ca^{2+} in promoting fusion of early endosomes. The results are representative of three (A and B) and two (C) independent experiments.

more sets of SNAREs, which recycle together with the other vesicle constituents. Consequently, mechanisms need to exist that define which of the resident SNARE proteins in the membrane is used for a given fusion step.

One of the most striking observations is that endosome fusion was strongly inhibited by the fast Ca^{2+} chelator BAPTA but was not affected by the slower Ca^{2+} chelator EGTA despite identical affinities of both chelators for Ca^{2+}. Insensitivity to EGTA is in agreement with an earlier study (Mayorga *et al.*, 1994). In contrast, the membrane-permeant analogue of EGTA, EGTA-AM, inhibited the reaction. These results allow us to conclude 1) that the Ca^{2+} pool promoting fusion is derived from a local pool, probably only a few nanometers away from the Ca^{2+} receptor; and 2) that this local pool resides in the interior of a vesicular compartment, most likely the endosomes themselves. Similar observations were reported previously from the fusion of nuclear membranes (Sullivan *et al.*, 1993) and, more recently, from the fusion of vacuolar precursor membranes derived from yeast (Peters and Mayer, 1998). In these cases, local release of Ca^{2+} was needed for fusion, raising the possibility that Ca^{2+} release from intraorganellar stores is a general requirement for intracellular fusion reactions.

Which is the protein Ca^{2+} is acting on in these fusion reactions? In the fusion of yeast vacuoles, calmodulin has emerged as a strong candidate, suggesting that a calmodulin-binding protein operates in the control of membrane fusion. Using stage-specific inhibitors of the fusion reaction, it was demonstrated that calmodulin exerts its effect at a late step in the fusion reaction, probably after assembly of the SNARE complex. Furthermore, calmodulin antagonists were previously shown to block endosome fusion in a macrophage cell line, and this inhibition was reversed by the addition of purified calmodulin (Colombo *et al.*, 1997). A partial inhibition of endosome fusion by calmodulin antagonists was also observed in our experiments (our unpublished observations). Together, these observations suggest that calmodulin may be involved in mediating the Ca^{2+} effects described here. The targets of calmodulin remain to be established. The protein EEA1, which possesses a calmodulin binding domain, has recently been implicated as an effector of Rab5. Thus it is possible that at least parts of the results obtained here may be attributed to a regulation of EEA1. Another candidate Ca^{2+} target is the Ca^{2+}-binding protein annexin II, which was identified as a major component of early endosomes (Emans *et al.*, 1993) and which appears to play an as yet undefined role in endosome fusion (Mayorga *et al.*, 1994).

How is Ca^{2+} getting into the early endosome? Clearly, one of the major Ca^{2+} sources for endocytotic organelles is contributed by the extracellular fluid, which contains Ca^{2+} concentrations in the millimolar range. It remains to be established whether in addition an active refilling mechanism operates, e.g., by means of a Ca^{2+}-ATPase. Because

early endosomes are transient compartments, active uptake may not be required to maintain a high intraendosomal Ca^{2+} concentration. This view is supported by the fact that thapsigargin, an inhibitor of the members of the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases, did not affect Ca^{2+}-dependent fusion of early endosomes even when added at supramaximal concentrations (our unpublished results). Alternatively, Ca^{2+} sequestration may occur through a thapsigargin-insensitive intracellular calcium pump distinct from the classical sarco(endo)plasmic reticulum Ca^{2+}-ATPases (Waldron *et al*., 1995). Recently, an intracellular Ca^{2+} pool that is insensitive to thapsigargin and IP_3 has been observed in mammalian cell lines, including PC12 cells (Pizzo *et al*., 1997), which may be related to the pool described here.

In summary, the endosome fusion reaction in PC12 cells has some intriguing similarities with neuronal exocytosis but also important differences. In both cases, local release of Ca^{2+} drives membrane fusion. During exocytosis, Ca^{2+} release is triggered by depolarization, which leads to the opening of voltage-gated Ca^{2+} channels clustering at the release sites. In endosome fusion, the events triggering Ca^{2+} release are unknown. It is possible that recognition and/or docking of the fusion partners generates a signal that activates endosomal Ca^{2+} channels, for instance, IP_3 or polyphosphoinositides. Alternatively, it is possible that putative endosomal Ca^{2+} channels fire spontaneously, creating local Ca^{2+} gradients that are only effective when the participating membranes are close to each other. The molecular mechanisms involved in Ca^{2+}-mediated control of endosome fusion remain to be established.

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