Identification of SNAREs Involved in Regulated Exocytosis in the Pancreatic Acinar Cell*

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The molecular basis of exocytotic membrane fusion in the pancreatic acinar cell was investigated using an *in vitro* assay that measures both zymogen granule-plasma membrane fusion and granule-granule fusion. These two fusion events were differentially sensitive to Ca^{2+}, suggesting that they are controlled by different Ca^{2+}-sensing mechanisms. Botulinum neurotoxin C (BoNT/C) treatment of the plasma membranes caused cleavage of syntaxin 2, the apical isoform of this Q-SNARE, but did not affect syntaxin 4, the basolateral isoform. BoNT/C also cleaved syntaxin 3, the zymogen granule isoform. BoNT/C treatment of plasma membranes abolished granule-plasma membrane fusion, whereas toxin treatment of the granules reduced granule-plasma membrane fusion and abolished granule-granule fusion. Tetanus toxin cleaved granule-associated synaptobrevin 2 but caused only a small reduction in both granule-plasma membrane fusion and granule-granule fusion. Our results indicate that syntaxin 2 is the isoform that mediates fusion between zymogen granules and the apical plasma membrane of the acinar cell. Syntaxin 3 mediates granule-granule fusion, which might be involved in compound exocytosis. In contrast, the major R-SNARE on the zymogen granule remains to be identified.

The pancreatic acinar cell provides an ideal model to study the molecular basis of exocytosis in non-excitable tissue (1). The action of secretagogues such as acetylcholine and cholecystokinin on receptors at the basolateral pole of these cells causes a release of Ca^{2+} from intracellular stores (2, 3). This in turn leads to fusion of the zymogen granules both with the apical domain of the plasma membrane and with each other (1). The physiological significance of the specific targeting of granules to the apical plasma membrane is illustrated by the fact that a breakdown in the polarity of secretion is associated with a serious clinical condition, acute pancreatitis (4).

It is now widely accepted that the biochemical machinery involved in fusing membranes together is highly conserved (reviewed in Ref. 5). Membrane fusion apparently requires the formation of a complex between SNARE[1] proteins (6) in the two interacting membranes. In neurons, the synaptic vesicle-associated SNARE, synaptobrevin-VAMP, forms a complex with two plasma membrane-associated SNAREs, syntaxin and SNAP-25. These proteins bind together in a parallel manner (7) to form a four-helix bundle, with two helices contributed by SNAP-25 and one each by synaptobrevin and syntaxin (8). Further, preliminary evidence has been presented that the formation of the SNARE complex is by itself sufficient to bring the two lipid bilayers close enough together to cause fusion (9). Originally, SNAREs were classified into v-SNAREs and t-SNAREs (6) based on their location on either the vesicle (v) or the target (t) membrane. However, following the determination of the crystal structure of the neuronal SNARE complex (8), an alternative classification into R- and Q-SNAREs was proposed, based on the contribution of either an arginine (R) or a glutamine (Q) residue to the ionic "0" layer at the heart of the SNARE complex (10). According to this new classification, synaptobrevin would be an R-SNARE and syntaxin and SNAP-25 would both be Q-SNAREs, and the basic requirement for membrane fusion would be the formation of a SNARE complex consisting of four helices (1R and 3Q) of which at least two are anchored in opposing membranes. One advantage of this new convention is that it deals more satisfactorily with homotypic fusion, such as that between pancreatic zymogen granules, where the distinction between "vesicle" and "target" is meaningless.

The most persuasive evidence that the SNAREs are crucial to exocytosis in neurons is the demonstration that each of the SNAREs is a specific target for at least one member of the family of clostridial neurotoxins, which comprise tetanus toxin (TeNT) and the various serotypes of botulinum neurotoxin (BoNTs (11–13)) (reviewed in Ref. 14). These toxins produce their pathophysiological effects by blocking neurotransmitter release from the nerves that become poisoned. All of the toxins are zinc endoproteases, and they are highly homologous with each other, especially in the region of their active sites (reviewed in Ref. 14). As a consequence of their ability to cleave specific SNAREs at particular sites, they are being used widely as molecular scalpels to dissect the mechanism of membrane fusion in various tissues.

The neuronal SNAREs are known to be prototypical members of SNARE families, which are growing rapidly (15–17). In the pancreatic acinar cell, synaptobrevin 2 is found on the zymogen granule membrane (18, 19), and at least three syn-

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; SNAP-25, synaptosome-associated protein of molecular weight 25,000; SNAP-23, synaptosome-associated protein of molecular weight 23,000; TeNT, tetanus neurotoxin; BoNT/C, botulinum neurotoxin C; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; MES, 2-morpholinoethanesulfonic acid; R18, octadecylrhodamine; PT, 1,10-phenanthroline; VAMP, vesicle-associated membrane protein.

taxin isoforms are also present (syntaxin 2 on the apical plasma membrane, syntaxin 4 on the basolateral membrane, and syntaxin 3 on the granule membrane (20)). SNAP-23, the non-neuronal isoform of SNAP-25 (16), has also been found on the basolateral domain of the plasma membrane (21). To determine how these various SNAREs operate to mediate exocytosis in the acinar cell, a simple functional system is required to which agents such as the clostridial toxins can be applied.

It was shown several years ago (22–24) that acini permeabilized with the bacterial toxin streptolysin O secrete digestive enzymes in response to micromolar Ca^{2+}. More recently, it was shown that TeNT treatment of these cells cleaved synaptobrevin 2 and also caused a reduction in the exocytotic response (18). Unfortunately, permeabilized cell systems are still rather complex and do not permit, for example, manipulation of only one of the interacting membranes. We have developed an *in vitro* assay in which zymogen granules fuse with plasma membranes in a Ca^{2+}-dependent manner (19, 25, 26). This assay provides greater flexibility than the permeabilized cell system and has already been used to demonstrate the effect of TeNT treatment of the zymogen granules on their ability to fuse with plasma membranes (19). Here we show that zymogen granules undergo Ca^{2+}-dependent fusion *in vitro* both with plasma membranes and with each other. In addition, by examining the effects of the BoNT/C and TeNT on membrane fusion, we provide new information about the SNAREs involved in these two membrane fusion events.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used were as follows: HPC-1, a mouse monoclonal antibody against syntaxin 1 (27); a rabbit polyclonal antibody against syntaxin 2 (generously provided by Dr. T. C. Südhof, Dallas, TX); a rabbit polyclonal antibody against syntaxin 3 (28) (kindly provided by Dr. T. Galli, Paris, France); a rabbit polyclonal antibody against syntaxin 4, produced by immunization with the cytoplasmic domain of syntaxin 4 (residues 1–273) tagged with His_6; and 69.1, a mouse monoclonal antibody against synaptobrevin 2 (29).

Constructs and Proteins—cDNAs encoding maltose-binding protein (MBP) syntaxins 1–5 were generously provided by Dr. T. C. Südhof. MBP fusion proteins were isolated from bacterial lysates by binding to amylose-agarose (NEB, Hitchin, United Kingdom). cDNAs encoding His_6-BoNT/C and His_6-TeNT light chains were kindly provided by Dr. H. Niemann (Hannover, Germany). His_6-toxin light chains were generously prepared by M. Margittai (Göttingen, Germany).

SDS-PAGE and Immunoblotting—Proteins were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting. Blots were probed with primary antibodies at dilutions of 1:500–1:1000. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (1:1000) and enhanced chemiluminescence (Pierce and Warriner, Chester, UK).

Preparation of Pancreatic Zymogen Granules and Plasma Membranes—Zymogen granules and plasma membranes were isolated as described previously (30). All buffers contained 1 mM EGTA and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 17 μg/ml benzamidine, and 50 μg/ml bacitracin).

Fluorescence Dequenching Assay for Membrane Fusion—Octadecylrhodamine (R18; Molecular Probes, Eugene, OR) was stored at -20 °C as a 20 mM stock solution in ethanol. Probe (1 μl of the stock solution) was added to 300 μl of zymogen granule suspension in 280 mM sucrose and 5 mM MES buffer, pH 6.0, containing protease inhibitors. Granules were incubated with the probe at 37 °C for 5 min and then collected by centrifugation at 900 $\times$ g for 10 min. Labeled granules were resuspended in the original volume of sucrose/MES buffer, pH 6.0. In a typical fusion assay, 10 μl of a labeled granule suspension was added to 700 μl of sucrose/MES buffer, pH 6.5, at 37 °C. Ca^{2+} was buffered with 1 mM EGTA, and free Ca^{2+} concentrations were calculated as described by Föhr *et al.* (31). Fluorescence was measured continuously in a Hitachi F-2000 fluorescence spectrometer (excitation at 560 nm, emission at 590 nm). After a 1-min equilibration period, unlabeled target membranes (plasma membranes, 10–20 μg of protein; zymogen granules, 200–500 μg of protein) were added in a small volume (10–20 μl)
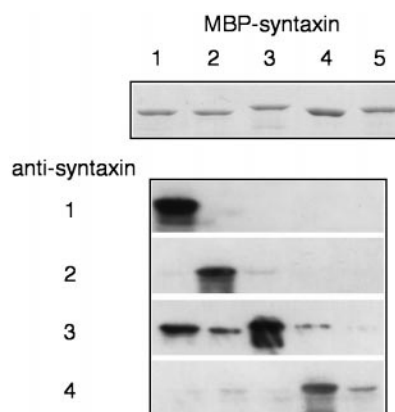

FIG. 1. **Isoform specificities of the four anti-syntaxin antibodies.** Equal amounts of MBP-syntaxins 1–5 (0.5 μg of protein) were analyzed by SDS-PAGE. A Coomassie Blue-stained gel (*top panel*) shows the protein loading. Proteins were electrophoretically transferred to nitrocellulose and probed with antibodies against syntaxins 1–4, followed by horseradish peroxidase-conjugated secondary antibodies (*bottom panel*). Immunoreactive bands were visualized by enhanced chemiluminescence.

and mixed with the labeled granules. Fluorescence was then monitored for 4 min more. Dequenching traces show fluorescence in arbitrary units. Dequenching signals are expressed as a percentage of the Ca^{2+}-stimulated signal given by untreated membranes.

Clostridial Toxin Treatment—Zymogen granules or plasma membranes were incubated with either BoNT/C light chain (5.8 μM) or TeNT light chain (1 μM) for 20 min at 37 °C in sucrose/MES buffer, pH 6.0, containing 1 mM EGTA. Two methods were used to inactivate the toxin for control incubations: boiling the toxin for 5 min and preincubating it for 30 min at 37 °C with 5 mM 1,10-phenanthroline (PT).

Data Analysis—Values given are means $\pm$ S.E. Where individual dequenching traces or immunoblots are shown, they are representative of the results of between 3 and 20 experiments. An immunoblot was carried out for each toxin experiment.

RESULTS

Distribution of SNAREs between Zymogen Granules and Plasma Membranes—The anti-syntaxin antibodies used in this study were first tested for their ability to recognize various syntaxin isoforms. Equal amounts of MBP-syntaxin 1–5 fusions were blotted onto nitrocellulose and probed with the four antibodies. The antibodies against syntaxins 1, 2, and 4 recognized their appropriate targets, with only minor cross-reactivity with the other isoforms (Fig. 1). The antibody against syntaxin 3, however, did show some cross-reactivity with syntaxin 1, as described previously (28).

To determine the distribution of the SNAREs between the zymogen granule membrane and the plasma membrane, membrane samples were analyzed by SDS-PAGE and immunoblotted with the four anti-syntaxin antibodies and with an antibody known to recognize synaptobrevin 2 (29). As shown in Fig. 2, syntaxins 1, 2, and 4 were found only in the plasma membrane. Syntaxin 3 gave a strong signal in the zymogen granule membrane lane and a much weaker signal in the plasma membrane lane. Despite the cross-reactivity of the anti-syntaxin 3 antibody with syntaxin 1, it is safe to conclude from this result that the principal location of syntaxin 3 is on the zymogen granule membrane. Given the fact that syntaxin 1 is present on the plasma membrane, it is possible that the syntaxin 3 signal in this fraction might represent cross-reactivity of the antibody. On the other hand, it is also possible that some syntaxin 3 does reside on the plasma membrane.

Our results are in agreement with those of Gaisano *et al.* (20). Using immunocytochemistry, these authors also showed that the syntaxin isoforms present at the plasma membrane are distributed in a polarized manner, with syntaxin 2 on the
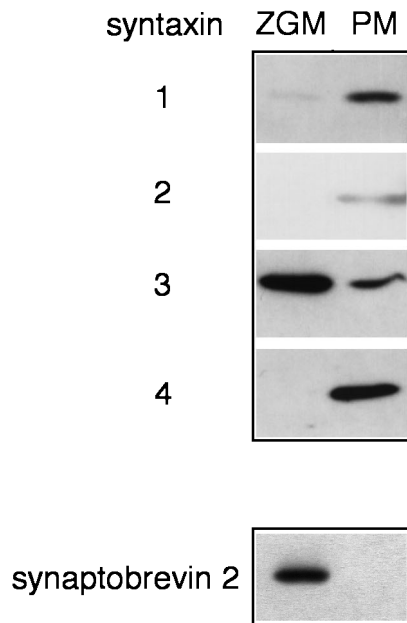
FIG. 2. **Distribution of SNAREs between zymogen granule membranes and plasma membranes.** Equal amounts (10 µg of protein) of zymogen granule membranes (*ZGM*) and plasma membranes (*PM*) were analyzed by SDS-PAGE and electrophoretically transferred to nitrocellulose. Blots were probed with antibodies against syntaxins 1–4 and synaptobrevin 2 and processed as described in the legend to Fig. 1.

apical domain and syntaxin 4 on the basolateral domain. They were unable to detect syntaxin 1 using this technique. Taken together, these results indicate that the best candidate for the syntaxin isoform that mediates exocytotic fusion of the zymogen granule with the plasma membrane is syntaxin 2. The location and likely role of syntaxin 1 are currently unknown.

Ca^{2+} Sensitivity of Fusion Events—Fusion between zymogen granules and plasma membranes was measured using an *in vitro* fluorescence dequenching assay based on a method originally described by Hoekstra *et al.* (32). Granules were loaded with the lipid-soluble fluorescent probe R18. When the labeled granules were incubated with unlabeled target membranes, fusion between the two membranes resulted in a dilution-dependent dequenching of the probe's fluorescence. We have previously reported that fusion between zymogen granules and plasma membranes *in vitro* is stimulated by Ca^{2+} (19, 26). In the present study, we examined the Ca^{2+} sensitivity of membrane fusion in detail. As shown in Fig. 3A, virtually no dequenching signal was seen in the presence of EGTA alone. In contrast, in the presence of Ca^{2+} (1 mM) a clear, time-dependent dequenching signal was observed. This stimulation of membrane fusion was very selective for Ca^{2+}, because even at this high concentration (1 mM) three other divalent cations, Mg^{2+}, Sr^{2+}, and Ba^{2+}, supported only a small fusion signal. The sizes of the signals with these three cations, relative to that for Ca^{2+}, were 32 ± 4% for Mg^{2+}, 29 ± 11% for Sr^{2+}, and 28 ± 3% for Ba^{2+} (all *n* = 3).

R18-labeled zymogen granules also gave a dequenching signal when incubated with unlabeled target granules, and this fusion event was again selectively stimulated by Ca^{2+} (Fig. 3B); the sizes of the dequenching signals in the presence of the other three cations (at 1 mM), relative to that for Ca^{2+}, were 20 ± 2% for Mg^{2+}, 27 ± 2% for Sr^{2+}, and 21 ± 1% for Ba^{2+} (all *n* = 3). Interestingly, the sensitivities of the two types of fusion events to Ca^{2+} were different (Fig. 3C), with zymogen granule-plasma membrane fusion being 16-fold more sensitive to Ca^{2+} than granule-granule fusion (EC$_{50}$ values for Ca^{2+}, 5 and 80
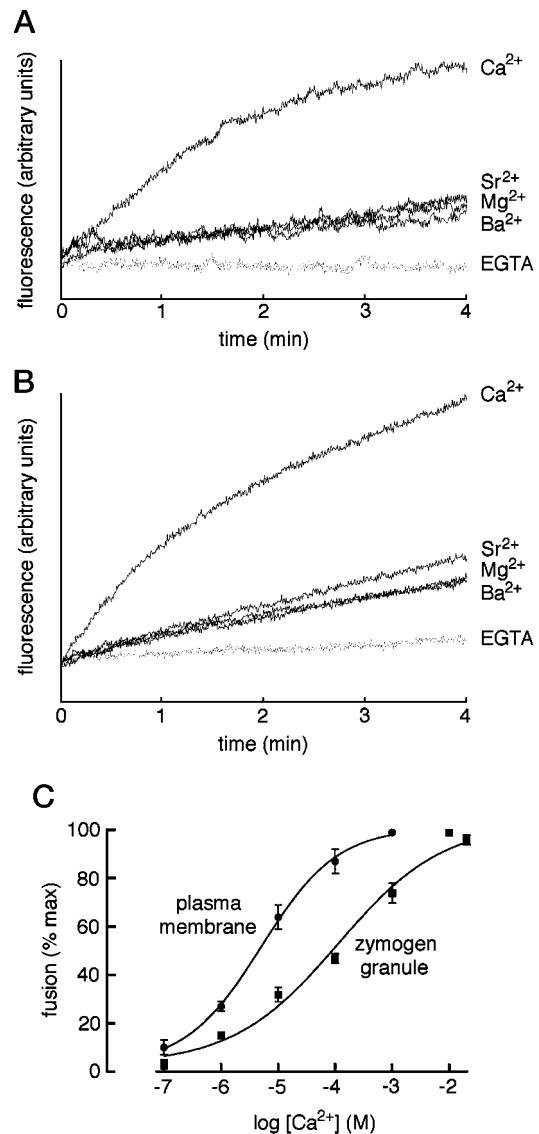

FIG. 3. **Divalent cation sensitivities of zymogen granule-plasma membrane and granule-granule fusion.** Unlabeled target membranes were added to R18-labeled zymogen granules at time zero, and fluorescence was followed for 4 min. *A*, zymogen granule-plasma membrane fusion is shown. *B*, granule-granule fusion is shown. The free concentration of all the divalent cations was 1 mM. *C*, dependence of the extent of granule-plasma membrane fusion (●) and granule-granule fusion (■) on free Ca^{2+} concentrations (*n* = 4) is shown.

µM, respectively). This differential sensitivity to Ca^{2+} suggests that the Ca^{2+}-sensing mechanisms for the two fusion events (and possibly the molecular identity of the Ca^{2+} sensors themselves) are different. Further, the Ca^{2+} sensitivity of zymogen granule-plasma membrane fusion *in vitro* is very close to that of exocytosis in permeabilized pancreatic acini (22–24), indicating that the *in vitro* assay does indeed represent a *bona fide* exocytotic membrane fusion event.

Effects of BoNT/C on Zymogen Granule-Plasma Membrane Fusion—To determine whether either of the syntaxin isoforms present on the plasma membrane of the acinar cell (syntaxins 2 and 4) is involved in fusion between zymogen granule membranes and plasma membranes, we examined the effect of BoNT/C (light chain) on this fusion event *in vitro*. It has been established (33) that BoNT/C cleaves syntaxin 1A at a specific lysine-alanine bond, Lys^{253}-Ala^{254}. As shown in Fig. 4, this bond is also present in syntaxin 2, whereas in syntaxin 3 the lysine is replaced by another basic residue, arginine (15). In
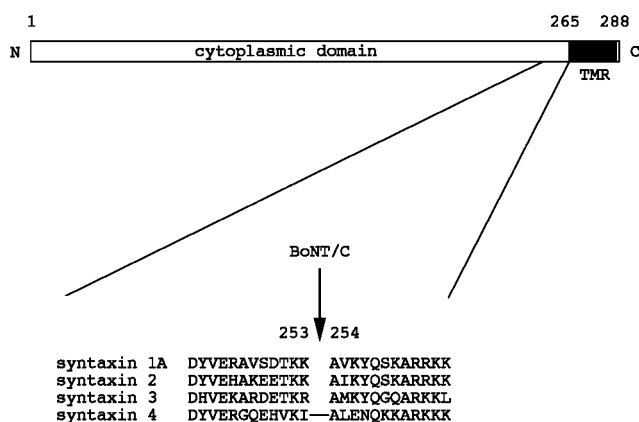
FIG. 4. **Sites of cleavage of syntaxin isoforms by BoNT/C.** Amino acids around the BoNT/C cleavage site are highlighted. Note that syntaxins 1A, 2, and 3 contain either a Lys-Ala bond (Lys^{253}-Ala^{254} in syntaxin 1A) or an Arg-Ala bond, which is cleaved by BoNT/C. In contrast, syntaxin 4 contains an Ile-Ala bond at this position, which is not cleaved by the toxin.

syntaxin 4, on the other hand, the lysine is replaced by the hydrophobic residue, isoleucine, which renders the protein resistant to the toxin (33). BoNT/C should therefore be able to discriminate between syntaxins 2 and 4 and reveal which isoform is principally involved in exocytotic membrane fusion. As shown in Fig. 5*A*, BoNT/C treatment of plasma membranes almost abolished their ability to fuse with zymogen granules (reduction to $11 \pm 5\%$ of control, $n = 4$). Toxin treatment of the zymogen granules also produced a significant, albeit smaller, inhibition of their ability to fuse with plasma membranes (reduction to $63 \pm 7\%$ of control, $n = 4$; Fig. 5*B*). Fig. 5*C* shows that, as expected, BoNT/C treatment of the plasma membranes caused complete cleavage of syntaxin 2 but did not affect syntaxin 4. Syntaxin 1 was also partially cleaved. These results strongly suggest that syntaxin 2, the apical isoform in the intact acinar cell, is the Q-SNARE that mediates exocytotic membrane fusion of zymogen granules and that syntaxin 4, the basolateral isoform, is not involved in this membrane fusion event. The smaller inhibition of fusion observed when granules are treated with BoNT/C might reflect the cleavage of granule-associated syntaxin 3 (see below), which could remove a component of compound fusion occurring *in vitro*.

Effects of BoNT/C on Granule-Granule Fusion—Syntaxin 3 is specifically localized on the zymogen granule and is the only syntaxin isoform known to be present on this organelle. Because this isoform should be sensitive to BoNT/C (33), it was possible to use this toxin to test the role of syntaxin 3 in membrane fusion. It was found (see Fig. 6*A*) that treatment of either R18-labeled or unlabeled zymogen granules with BoNT/C reduced their ability to fuse with untreated granules by about half ($49 \pm 9\%$ for treatment of labeled granules and $52 \pm 6\%$ for treatment of unlabeled granules, respectively, $n = 4$). The implication of this result is that granule-granule fusion is mediated by interactions between a Q-SNARE (syntaxin 3) on one membrane and one or more cognate binding partner(s) on the other membrane. As expected from this model, botulinum neurotoxin treatment of both labeled and unlabeled granules almost abolished granule fusion ($7 \pm 7\%$ of control, $n = 4$). In parallel with these effects of BoNT/C on the functional assay, syntaxin 3 on the zymogen granules was completely cleaved (Fig. 6*C*). The effects of the toxin on fusion could be abolished either by boiling the toxin ($92 \pm 5\%$ of control fusion, $n = 6$) or by preincubating it with the zinc chelator PT (5 mM; $93 \pm 1\%$ of control fusion, $n = 3$; Fig. 6*B*). Boiling and PT treatment also abolished the ability of the toxin to cleave syn-
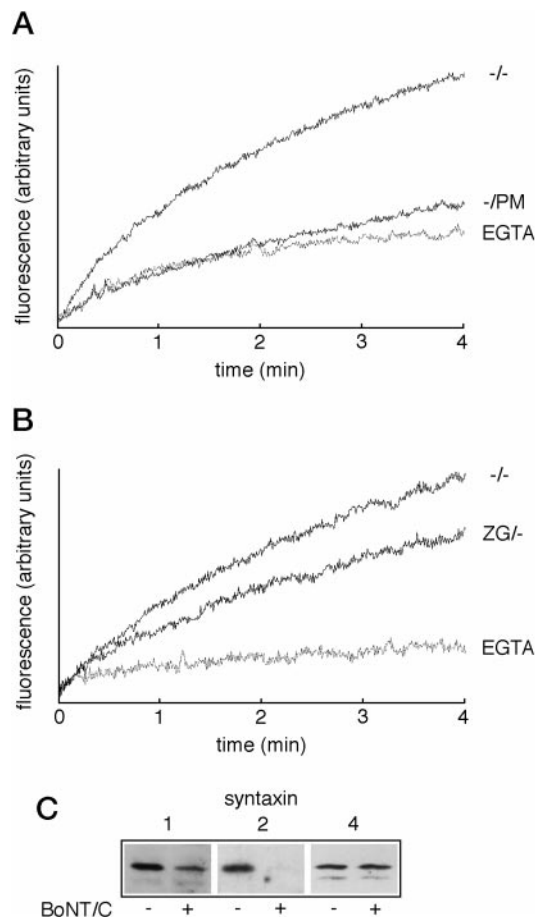

FIG. 5. **Sensitivities of zymogen granule-plasma membrane fusion and of syntaxins 1, 2, and 4 to BoNT/C.** Unlabeled plasma membranes were added to R18-labeled zymogen granules at time zero, and fluorescence was followed for 4 min. The free Ca^{2+} concentration was 100 μM. *A*, the effect of treatment of unlabeled plasma membranes with BoNT/C (5.8 μM for 20 min at 37 °C; $-/PM$) on their ability to fuse with labeled granules is shown. *B*, the effect of treatment of labeled granules with BoNT/C ($ZG/-$) on their ability to fuse with unlabeled plasma membranes is shown. *C*, the effect of BoNT/C treatment of the plasma membranes on the integrity of the plasma membrane-associated syntaxin isoforms is shown.

taxin 3 (Fig. 6*C*). These results indicate that the effects of the toxin were a consequence of specific proteolytic activity.

Effects of TeNT on Fusion Events—The only R-SNARE so far identified on the membrane of the zymogen granule is synaptobrevin 2 (19, 20). We have shown previously (19) that TeNT treatment has only a small effect on the ability of zymogen granules to fuse with plasma membranes despite complete cleavage of synaptobrevin 2. In the present study, we examined the effect of TeNT on the ability of the zymogen granules to fuse with both plasma membranes and zymogen granules. As shown in Fig. 7*A*, TeNT treatment of the zymogen granules had only a small effect on their ability to fuse with plasma membranes, in agreement with our previous finding that fusion was reduced to $84 \pm 1\%$ of control, $n = 20$ (19). As expected, treatment of plasma membranes with the toxin had no effect on their ability to fuse with zymogen granules. TeNT treatment of one population of granules had no effect on their ability to fuse with untreated granules, although there was a small inhibition of fusion when both populations of granules were treated with toxin (reduction to $82 \pm 6\%$ of control, $n = 13$; Fig. 7*B*). Despite its unimpressive effects on the functional assays, however, TeNT did cause complete cleavage of synaptobrevin 2 in a manner that was once again sensitive to boiling and to PT pretreatment (Fig. 7*C*).
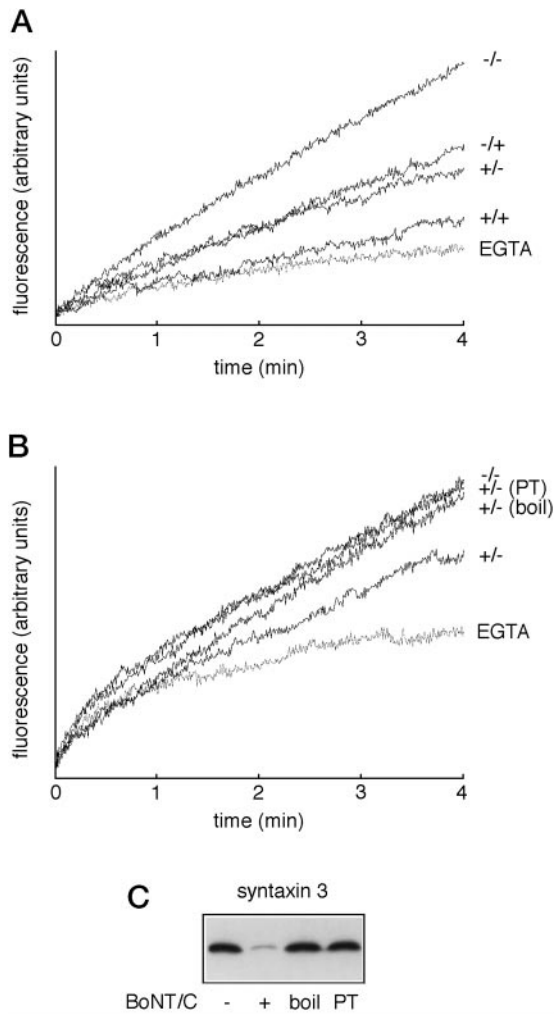
FIG. 6. **Sensitivities of granule-granule fusion and of syntaxin 3 to BoNT/C.** Unlabeled zymogen granules were added to R18-labeled granules at time zero, and fluorescence was followed for 4 min. The free Ca^{2+} concentration was 1 mM. *A*, the effect of treatment of labeled granules ($+/-$), unlabeled granules ($-/+$), or both ($+/+$) with BoNT/C (5.8 μM for 20 min at 37 °C) on their ability to fuse is shown. *B*, the effect of inactivation of BoNT/C either by boiling (5 min) or PT treatment (5 mM for 30 min at 37 °C) on the ability of the toxin to inhibit fusion is shown. Only the labeled granules were treated with toxin ($+/-$). *C*, the effect of treatment of granules with BoNT/C (either active or inactivated by boiling or PT) on the integrity of granule membrane-associated syntaxin 3 is shown.

FIG. 7. **Sensitivities of zymogen granule-plasma membrane and granule-granule fusion and of synaptobrevin 2 to TeNT.** Unlabeled zymogen granules were added to R18-labeled granules at time zero, and fluorescence was followed for 4 min. *A*, the effect of treatment of labeled granules (*ZG*/$-$) or unlabeled plasma membranes ($-$/*PM*) with TeNT (1 μM for 20 min at 37 °C) on their ability to fuse is shown. The free Ca^{2+} concentration was 100 μM. *B*, the effect of treatment of labeled granules ($+/-$), unlabeled granules ($-/+$), or both ($+/+$) with TeNT (1 μM for 20 min at 37 °C) on their ability to fuse is shown. The free Ca^{2+} concentration was 1 mM. *C*, the effect of treatment of granules with TeNT (either active or inactivated by boiling or PT) on the integrity of granule membrane-associated synaptobrevin 2 is shown.

DISCUSSION

When the dequenching assay for exocytotic membrane fusion was first introduced (25) there was no evidence for an effect of Ca^{2+} on the extent of fusion. In the course of the present study, we found that the key to observing Ca^{2+}-dependent fusion *in vitro* is the preparation of the membranes in buffers from which Ca^{2+} is effectively absent (*i.e.* in the presence of EGTA). If the membranes are prepared in buffers containing Ca^{2+}, then fusion occurs in a Ca^{2+}-independent manner. This suggests that during membrane preparation, an inhibitor of fusion is being inactivated in a Ca^{2+}-dependent manner. The identification of the mechanism involved in this process represents a major future goal of this work.

In the acinar cell, zymogen granules normally fuse with the apical domain of the plasma membrane, where syntaxin 2 is the predominant Q-SNARE (20). The observation that cleavage of syntaxin 2 by BoNT/C treatment of the plasma membranes results in a parallel inhibition of zymogen granule-plasma membrane fusion represents the first direct evidence that this
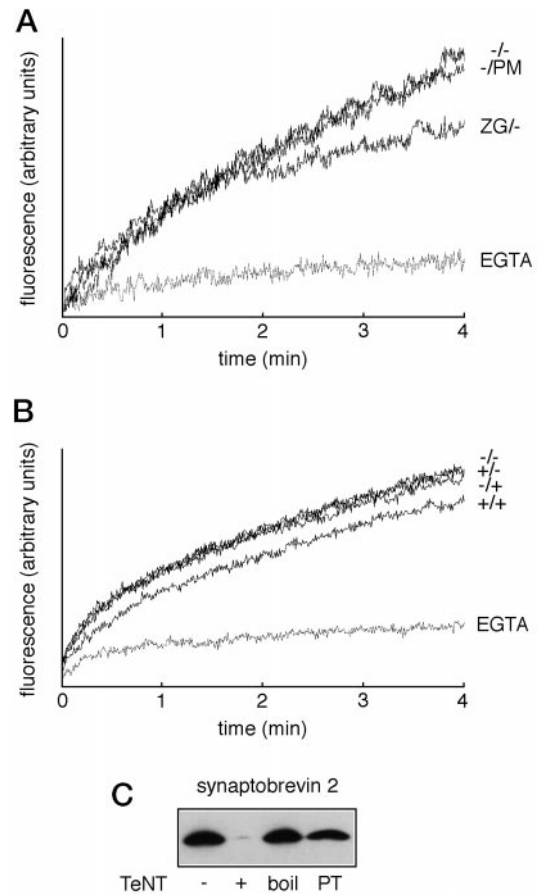
Q-SNARE is, as expected, a major participant in this membrane fusion event. The fact that syntaxin 4, the predominant basolateral Q-SNARE, remains intact in the same experiment indicates that it plays no role in this fusion event, which again would be expected on the basis of its cellular location and the known polarity of secretion in the acinar cell. It has recently been suggested (21), in fact, that syntaxin 4 and SNAP-23, another basolateral Q-SNARE, might interact with synaptobrevin 2 to mediate the inappropriate fusion of zymogen granules with the basolateral domain of the plasma membrane that is a characteristic feature of experimental pancreatitis induced, for example, by hyperstimulation of the pancreas with the secretagogue cerulein (4). The ability of BoNT/C to cleave all of the syntaxin 2 must also mean, incidentally, that all of the apical membrane fragments present in the plasma membrane are in the form of either sheets or "inside-out" vesicles, which should be capable of fusion with zymogen granules.

During exocytosis in the acinar cell, the fusion of zymogen granules with the apical plasma membrane is accompanied by fusion between the granules themselves, in a process known as compound exocytosis (1). Here we have shown that these heterotypic and homotypic fusion events can both be reconstituted *in vitro*. Indeed, when R18-labeled granules are incubated with

unlabeled plasma membranes, the dequenching observed is probably a result of both types of fusion occurring together (*i.e.* R18-labeled granules fusing with granules that have already fused with the plasma membrane). The homotypic component of fusion is likely to depend on syntaxin 3 on the granule membrane, and cleavage of this Q-SNARE might account for the 37% reduction in the extent of fusion caused by treatment of the granules with BoNT/C. Further, this figure might represent the contribution to the dequenching signal of granule-granule fusion. The fact that granule-granule fusion is less sensitive to Ca^{2+} than granule-plasma membrane fusion might reflect the situation in the cell, where compound exocytosis is seen predominantly at high levels of stimulation by secretagogues (4).

Our results strongly support the notion that the role of syntaxin 3 is to mediate fusion between zymogen granules. It has been suggested that some homotypic fusion events, such as fusion between endoplasmic reticulum-derived vesicles in yeast (34), occur through the homo-oligomerization of Q-SNAREs between the two interacting membranes. The evidence for this phenomenon is the demonstration that inactivation of the Q-SNARE on one membrane completely blocks fusion. In contrast, homotypic fusion between yeast vacuoles operates at maximal efficiency when both interacting membranes contain R- and Q-SNAREs (35). In our experiments, cleavage of syntaxin 3 on one population of zymogen granules reduced fusion by about 50%, and cleavage of the Q-SNARE on both membranes was required to abolish fusion. These results are more compatible with a model in which syntaxin 3 interacts with a different binding partner on the other membrane.

Our experiments show that synaptobrevin 2 plays only a minor role in exocytotic membrane fusion in the acinar cell (16%, in the case of granule-plasma membrane fusion). A partial (30%) inhibition of exocytosis in permeabilized acini after TeNT treatment has also been reported previously (18). The situation is very different in the pancreatic beta cell, where there is a much stronger immunofluorescence signal for synaptobrevin 2 (36) and where Ca^{2+}-stimulated exocytosis is abolished by TeNT treatment (37). We have looked for other synaptobrevin isoforms on the granule membrane (for example, the recently identified VAMP 7/TeNT-insensitive VAMP (28) and VAMP 8/endobrevin (38)) but failed to find them (data not shown). We are therefore left with the problem of identifying the major R-SNARE involved in exocytotic membrane fusion in the acinar cell. Whether another so far unidentified synaptobrevin isoform is responsible or whether a totally new mechanism needs to be proposed remains to be established.

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