Lipid Raft Association of SNARE Proteins Regulates Exocytosis in PC12 Cells*

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SNAP25 and SNAP23 are plasma membrane SNARE proteins essential for regulated exocytosis in diverse cell types. Several recent studies have shown that these proteins are partly localized in lipid rafts, domains of the plasma membrane enriched in sphingolipids, and cholesterol. Here, we have employed cysteine mutants of SNAP25/SNAP23, which have modified affinities for raft domains, to examine whether raft association of these proteins is important for the regulation of exocytosis. PC12 cells were engineered that express the light chain of botulinum neurotoxin; in these cells all of the SNAP25 was cleaved to a lower molecular weight form, and regulated when vesicles contain molecules such as neurotransmitters, adrenaline, or insulin. A large number of proteins have been identified that function in exocytosis (1, 2). Among these, SNARE1 proteins have emerged as potential membrane fusion catalysts (3, 4). Membrane fusion requires the interaction of Q-SNAREs present on the plasma membrane with R-SNAREs residing on the vesicle membrane. In neuronal and neuroendo-

crine cells, the Q-SNAREs that function in regulated exocytosis are syntaxin 1 and SNAP25, whereas the R-SNARE is VAMP/synaptobrevin (5).

Recently, there has been significant interest in the domain distribution of Q-SNAREs present at the plasma membrane. A number of studies have suggested that Q-SNAREs are partly localized in lipid rafts, lipid microdomains in the plasma membrane enriched in sphingolipids, and cholesterol (6–14). As rafts have been proposed to function in the regulation of numerous signal transduction (15) and membrane traffic pathways (16), these observations raise the intriguing possibility that rafts may regulate SNARE function and, hence, exocytosis. As yet, the importance of the raft association of SNARE proteins for exocytosis has not been examined.

Recent work from our group has reported that raft association of SNAP25 and its ubiquitous homologue, SNAP23, is mediated by the cysteine-rich domains of these proteins (17). We identified mutations within SNAP23 that decreased its raft association by ~2.5-fold and a point mutation in SNAP23 that increased raft association of this protein by 3-fold. Here, we have used these mutant proteins to directly test the importance of raft association of SNARE proteins for exocytosis. The results of this study show that an increased association of SNAP25/23 with rafts leads to a decrease in the extent of exocytosis. These results provide the first direct evidence that rafts regulate SNARE function and exocytosis and identify the central cysteine-rich region of SNAP25/23 as an important regulatory domain.

Exocytosis, the fusion of intracellular vesicles with the plasma membrane, mediates the secretion of molecules from the cell and the insertion of proteins and lipids into the plasma membrane. This membrane fusion event needs to be tightly regulated when vesicles contain molecules such as neurotransmitters, adrenaline, or insulin. A large number of proteins have been identified that function in exocytosis (1, 2). Among these, SNARE1 proteins have emerged as potential membrane fusion catalysts (3, 4). Membrane fusion requires the interaction of Q-SNAREs present on the plasma membrane with R-SNAREs residing on the vesicle membrane. In neuronal and neuroendo-

1 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SNAP25, synaptosomal-associated protein of 25 kDa; SNAP23, SNAP25 homologue of 23 kDa; HA, hemagglutinin; GFP, green fluorescent protein; BoNT/E, botulinum neurotoxin E; hGH, human growth hormone; Pipes, 1,4-pipera-

dinedithanesulfonic acid.

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Materials—Rat HA antibody and human growth hormone enzyme-linked immunosorbent assay kits were purchased from Roche Applied Science. Mouse HA antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). SNAP23 and SNAP25 antibodies were from Synaptic Systems (Göttingen, Germany). Anti-GFP was from Chemicon (Hampshire, UK). Digitonin was purchased from Merck Biosciences (Nottingham, UK). Triton X-100, n-octylglucoside, cadmium chloride, and all other reagents were of an analytical grade from Sigma.

Plasmids—All constructs used were N-terminally tagged. HA-SNAP25, HA-SNAP25 (F84C), and GFP-SNAP23 plasmids were described previously (17). HA-SNAP25 botulinum toxin E-resistant was obtained from R. Burgoyne. It contained the mutations R180W, I181E, and E183I. The F84C mutation was introduced by site-directed mutagenesis (Stratagene, La Jolla, CA). The plasmid encoding botulinum neurotoxin E (BoNT/E) light chain fused to GFP was a gift of R. Burgoyne (18). The pXGH5 plasmid (gift from R. Burgoyne) contains the human growth hormone cDNA under a metalloprotease inducible by the addition of CdCl2 in the growth medium.

Cell Culture and Transfection—PC12 cells were cultured in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum. Cells were transfected using Lipofectamine 2000 (Invitrogen). Transfected cells were analyzed 2 days posttransfection.

Growth Hormone Assays and Western Blotting—PC12 cells were transfected with 0.5 μg of human growth hormone (hGH) plasmid and 1 μg of test plasmid. The transfection medium was replaced 6 h later with fresh medium containing 1 μg/ml CdCl2. 48 h later, the cells were
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The association of proteins with detergent-resistant membranes is thought to reflect their association with lipid raft domains present in living cells (19). We recently showed that SNAP23 is significantly enriched in detergent-insoluble rafts compared with its homologue SNAP25 (17). This difference in raft association is because of an extra cysteine residue in the membrane-targeting domain of SNAP23. SNAP23 has 5 cysteines in this domain, whereas SNAP25 has 4 cysteine residues. Mutating specific cysteines in the membrane-targeting domains of SNAP25 and SNAP23 has a marked effect on the raft association of these proteins without affecting plasma membrane delivery (17). The aim of this work was to compare the ability of these raft-targeting mutants of SNAP25/23 to support exocytosis in PC12 cells.

Isolation of Botulinum Neurotoxin E-expressing PC12 Clones—To directly compare the effects of wild-type and a raft-targeting mutant of SNAP25 on exocytosis it was important to first inactivate endogenous SNAP25 protein. For this, PC12 cells were transfected with the light chain of BoNT/E fused to GFP. This toxin cleaves the C-terminal 26 amino acids from SNAP25 and causes a complete inhibition of exocytosis (20). A clone (number 38) was selected in which no intact SNAP25 was detectable (Fig. 1A). Cells were transfected with a plasmid encoding hGH, which is packaged into secretory vesicles and can be assayed to measure secretion specifically from transfected cells. ATP-stimulated hGH release in this clone was essentially absent and was not rescued by transfection of wild-type SNAP25 (Fig. 1B).

It has previously been shown that introducing mutations into the toxin-cleavage site of SNAP25 renders the protein resistant to cleavage by BoNT/E without affecting the exocytic function of SNAP25 (21, 22). In agreement with this, we found that a previously described BoNT/E-resistant form of SNAP25 was able to rescue exocytosis from cells expressing BoNT/E (Fig. 1B). This system is thus suitable for the analysis of the effects of SNAP25 mutants on exocytosis in PC12 cells.

SNAP25 (F84C) Displays a Reduced Ability to Restore Exocytosis in BoNT/E-expressing PC12 Cells—We previously described a cysteine mutant of SNAP25 (F84C) displaying a 3-fold increased affinity for detergent-resistant rafts compared with the wild-type protein (17). This increased raft association is because of the addition of an extra cysteine in the central domain of SNAP25B (Fig. 2A), which makes the number and distribution of cysteines in this protein identical to SNAP23. Lipid raft association of the BoNT/E-resistant forms of wild-type and F84C SNAP25 in BoNT/E-expressing PC12 cells was determined by detergent solubilization and sucrose gradient flotation. Fig. 2B shows that SNAP25 (F84C) is significantly more enriched in rafts than SNAP25 (wt) (6.6 ± 1.5% for toxin-resistant SNAP25, 30.9 ± 1.3% for toxin-resistant F84C in rafts, n = 3, p < 0.0002). Interestingly, the level of raft association of BoNT/E-resistant SNAP25 (6.6%) was reduced...
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compared with non-toxin-resistant SNAP25 (20%, see Refs. 7 and 17). We also detected a similar difference in the raft association of these two proteins in wild-type PC12 cells (data not shown), implying that mutations in the toxin-cleavage site of SNAP25 directly modulate raft association. Nevertheless, as the toxin-resistant forms of wild-type SNAP25 and the F84C mutant displayed significantly different levels of raft association, we were able to test the effect of these proteins (and hence of raft association of SNAP25) on release of hGH.

Interestingly, toxin-resistant SNAP25 (F84C) displayed a significantly reduced ability to restore exocytosis in BoNT/E-expressing PC12 cells (Fig. 2C). Although this effect was relatively modest we noted that transfection of the BoNT/E-resistant forms of SNAP25 and the F84C mutant displayed a decreased raft affinity compared with the wild-type protein because of the mutation of cysteines at amino acid positions 79 or 83 to phenylalanine (Fig. 3A and Ref. 17). As reported previously for wild-type PC12 cells, these mutants display a decreased raft association when expressed in BoNT/E-expressing PC12 cells (Fig. 2B; rafts association of wild-type SNAP25 was 49 ± 4.1%, SNAP25 (C79F) was 26.6 ± 0.4%, SNAP25 (C83F) was 22 ± 1.8%. Importantly, SNAP25 expression in BoNT/E-expressing cells did not allow the recovery of full-length endogenous SNAP25 (Fig. 2C). Also, immunoprecipitation of GFP-SNAP25 or the C79F mutant from PC12 cells co-precipitated similar amounts of both syntaxin 1A and syntaxin 4 but no SNAP25 or transferrin receptor (Fig. 3D). Thus, SNAP25 may support exocytosis in PC12 cells by SNARE pairing with syntaxin 1A and/or syntaxin 4.

We then examined the ability of SNAP25 to restore exocytosis in the toxin-expressing cells. All SNAP23 proteins were found to rescue exocytosis in toxin-expressing cells (Fig. 3E). Intriguingly, however, the C79F and C83F mutants supported substantially more exocytosis than wild-type SNAP23.

The Increased Level of Exocytosis Supported by the SNAP23 Cysteine Mutants Depends upon Membrane Integrity—Importantly, we found that the enhanced level of regulated exocytosis supported by the C79F and C83F mutants was not recapitulated in digitonin-permeabilized cells. Digitonin interacts with cholesterol in membranes and creates pores, allowing exocytosis to be stimulated directly by the addition of calcium. The extent of calcium-stimulated exocytosis was similar in cells expressing either wild-type SNAP23 or the cysteine mutants (Fig. 4A). Fig. 4B shows that digitonin treatment reduced the raft association of SNAP23; digitonin treatment of BoNT/E cells reduced the raft association of the endogenous SNAP23 by an average of 70% (n = 5, p < 0.0004). This result demonstrates that digitonin (as other cholesterol-binding agents) not only permeabilizes cell membranes but also disrupts raft structure. Thus, the effects of the mutant proteins on exocytosis depend upon membrane integrity, arguing strongly that their stimulatory effects on intact cells are a consequence of their altered localization within the membrane and not caused by intrinsic differences in the ability of the proteins to support exocytosis.

DISCUSSION

SNARE proteins are partly associated with detergent-resistant raft domains present in several distinct cell types (6–14). Disruption of raft domains by the cholesterol-binding agent methyl-β-cyclodextrin decreased ATP or depolarization-induced exocytosis in PC12 cells (7, 8), leading to the hypothesis that raft (or cholesterol-rich) domains of the plasma membrane were sites for fusion and exocytosis. Recent studies, however, argue against this hypothesis. For example, cyclodextrin treatment of pancreatic β cells enhances insulin secretion (13). Also,
disruption of lipid rafts by the action of an exogenously added sphingomyelinase activates Glut4 translocation in 3T3-L1 adipocytes (25). Moreover these studies do not provide a direct measure of the role of raft domains in regulating SNARE function. In the present study we tested this directly by de-localizing from or enriching SNARE proteins in rafts. We showed that increasing the association of SNAP25 to lipid rafts resulted in a decrease in regulated exocytosis. Conversely, reducing SNAP23 partitioning into lipid rafts increased exocytosis. The equalizing of exocytosis levels in digitonin-permeabilized cells expressing wild-type or mutant SNAP23 proteins shows that membrane integrity is required to maintain the differences in the ability of these proteins to support exocytosis.

Recent work has shown that SNAP25A/B and SNAP23 differ in their ability to support exocytosis in chromaffin cells (26). In particular, exocytosis in SNAP23-expressing cells lacked a release component arising from a primed vesicle pool, and SNAP25B supported a larger primed vesicle pool than SNAP25A. Although these results may reflect differences in protein-protein interactions of the SNARE homologues, we speculate that the distinct affinities of these proteins for rafts impacts on their function in exocytosis in these cells. The central cysteine-rich domain of SNAP25 has previously been implicated in the plasma membrane targeting of SNAP25 (27) and in the efficient concentration of SNAP25 at the plasma membrane (28). We now show that the distinct cysteine-rich domains of SNAP25 and SNAP23 are also directly related to the efficiency of exocytosis.

It is striking that such a difference in the efficiency of exocytosis (and in raft association) is because of a single mutation in the cysteine-rich domain of SNAP25 and SNAP23. These results suggest that the cysteine-rich domain of SNAP25 and SNAP23 is directly related to the efficiency of exocytosis.
central cysteines are modified by S-acylation (29, 30), and this study stresses the importance of the addition or removal of a single acyl chain residue within a domain already containing four or five other fatty acids at steady state. This suggests a possible modulation of SNAP25 and SNAP23 function by inducible palmitoylation/depalmitoylation. It is indeed known that chemical deacylation does not remove SNAP25 from membranes (31), and we showed recently that four cysteine residues (as compared with five) do not compromise the plasma membrane association of SNAP23 (17).

The importance of the exact position of a single cysteine within the central domain is revealed by the study of the two SNAP23 mutants. Although the raft association of SNAP23 (C79F) and SNAP23 (C83F) is comparable, the C79F mutant is significantly more efficient than C83F at supporting exocytosis. Thus, the distinct cysteine-rich domains of these proteins may be important for some other aspect of exocytosis not related to raft association, or, alternatively, these different cysteine-rich domains may target to distinct raft-like domains present in the plasma membrane. This is of particular importance because the position of the cysteines (and phenylalanines) within the two SNAP23 mutant proteins is similar to that found within the two isoforms of SNAP25. Recent studies have shown a difference in the ability of SNAP25A and B to support exocytosis (26, 32). Interestingly, SNAP25B seems to be more efficient than SNAP25A, and this difference is reflected in the present study by the SNAP23 (C79F) (whose central domain is comparable to SNAP25B) and SNAP23 (C83F) (whose central domain is comparable to SNAP25A) mutants. This could suggest that the position of one cysteine within SNAP25 may actually contribute to the functional differences between the two isoforms.

In contrast to previous models (7, 14), our results implicate rafts as negative regulators of neuronal exocytosis. Many levels of regulation exist to ensure that exocytosis in neuronal and neuroendocrine cells is tightly regulated. There is a wealth of published data describing different protein-protein interactions that may regulate SNARE function and exocytosis, but little is known about the regulation of SNARE function imposed by compartmentalization of the plasma membrane. In this regard, the results presented here provide clear evidence that SNAP25/23 association with lipid rafts inhibits exocytosis; as these SNARE proteins are highly enriched in rafts purified from unstimulated cells, this suggests that lipid rafts are important physiological regulators of SNARE function.

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