The \( V_0 \) Sector of the V-ATPase, Synaptobrevin, and Synaptophysin Are Associated on Synaptic Vesicles in a Triton X-100-resistant, Freeze-thawing Sensitive, Complex*

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**Abstract**

Synaptic vesicles (SVs),\(^1\) the vesicular carriers responsible for secretion of non-peptide neurotransmitters at synapses, are a very useful experimental model for investigating fundamental mechanisms in vesicular traffic (1–4). Studies carried out on SVs have converged with studies on neurotoxins, genetic studies in yeast, and cell-free studies of vesicular transport, and allowed to formulate a first hypothetical model of docking and fusion (5). The model predicts that small proteins located at the cytosolic surface of the vesicular carrier, v-SNAREs, interact with small proteins located at the cytosolic surface of the target membrane, t-SNAREs, to form the so-called SNARE complex, and that this interaction is one of the key steps leading to fusion (5, 6). In the case of SVs, v-SNAREs are the synaptobrevins and t-SNAREs are syntaxin and SNAP-25 (5).

Formation of SNARE complexes, and in particular of the synaptic SNARE complex, must be highly regulated. The presence of the same v-SNAREs on different types of secretory organelles, and of t-SNAREs all along the plasmalemma of neurons, clearly indicates that SNAREs are not themselves responsible for the spatiotemporal regulation of exocytosis (7–9). A variety of other factors which control formation and dissociation of the SNARE complex have already been identified (for review, see Ref. 10).

Studies on purified SVs have demonstrated that at least a significant fraction of synaptobrevin is in a complex with synaptophysin, and that this complex is mutually exclusive with the interaction of synaptobrevin with syntaxin 1 and with SNAP-25 to form the-SNARE complex (11–13). Thus, the synaptophysin/synaptobrevin interaction may have a regulatory role on SNARE complex formation (11–13). Conversely, the interaction of synaptophysin with synaptobrevin may be important for the biogenesis of a new SV at each exo-endocytic cycle, and this process may be coupled to dissociation of synaptobrevin from the t-SNAREs after exocytosis. Given the key role of synaptobrevin in vesicle docking, it is possible that the synaptobrevin-synaptophysin complex may in turn interact with other vesicle components to ensure their incorporation into the vesicular carrier. At present, only very little information is available on the protein-protein interactions in vesicle membranes which may be important for SV biogenesis (14).

In order to obtain new insights into the protein interactions established by synaptobrevin during the exo-endocytic cycle of SVs, we have studied proteins which are coprecipitated together with synaptobrevin 2 from a Triton X-100 extract of synaptosomes. We have found that the binding partners of synaptobrevin 2 are drastically different dependent upon whether fresh or frozen-thawed Triton X-100 extracts are used for the immunoprecipitations. The bulk of synaptobrevin 2 is associated with synaptophysin in fresh brain synaptosomal extracts and with t-SNAREs in frozen-thawed extracts, suggesting that freeze-thawing may mimic physiological steps leading to docking and fusion. In addition, a significant fraction of the c subunit and other subunits of the \( V_0 \) sector of the V-ATPase are associated with synaptobrevin 2 in fresh extracts.
EXPERIMENTAL PROCEDURES

Antibodies—Antibodies raised in rabbits specific for synaptophysin (MC1), synaptobrevin 1 (MC9), synaptobrevin 2 (MC 23), SNAP-25 (MC21), cellubrevin (MC16), rbsec1, and synaptotagmin 1 (8907) were described previously (7, 8, 15, 16). The following antibodies were generated: monoclonal antibodies against synaptobrevin 2 (CI 69.1) (11), synaptophysin (CI 72), and synaptophysin 1 (CI 41.1) (17), and polyclonal antibodies against syntaxin 1 (R31) from Dr. R. Jahn (Yale University, New Haven, CT); monoclonal antibody against synaptotagmin 1 (HPC-1) from Dr. C. Barnstable (Yale University, New Haven, CT); monoclonal antibody against the B subunit of V-ATPase (Ac116) of V-ATPase from Dr. T. Südhof (University of Texas Southwestern, Dallas, TX); rabbit serum against the subunit Ac39 of V-ATPase from Dr. T. Südhof (University of Texas Southwestern, Dallas, TX); rabbit serum against the subunit Ac39 of V-ATPase from Dr. N. Nelson (Hoffmann-La Roche).

Immunoprecipitation from Brain Synaptosomes—Rat brain synaptosomes (P2 fraction) were prepared following standard procedures (18). The pellet (P2) was resuspended in 120 ml of 0.32 m sucrose and spun again at 9200 × g for 15 min. The pellet corresponding to the synaptosomal fraction was then resuspended in solubilization buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin) at a final protein concentration of 5 mg/ml. Triton X-100 was then added (final concentration: 1%), and the resulting extract was incubated for 1 h at 4–6°C under rotation and centrifuged at 200,000 × g for 15 min. The supernatant was used for immunoprecipitation either immediately or after freezing overnight at −20°C followed by thawing at 4°C. For immunoprecipitation, the fresh or frozen-thawed synaptosome Triton X-100 extract was incubated overnight at 4–6°C under rotation, with protein G-Sepharose Fast Flow beads (Pharmacia Biotech Inc.) precoated with the following antibodies: anti-synaptobrevin 2 (CI 69.1), anti-syntaxin 1 (HPC-1), anti-synaptophysin (CI 72), mouse control IgGs. The beads were then pelleted for 1 min at 14,000 × g and washed with solubilization buffer containing 0.5% Triton X-100. Finally, the beads were resuspended in gel sample buffer and boiled for 5 min. Gel sample buffer consisted of 60 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.07% bromphenol blue, and 10 mM N-ethylmaleimide (nonreducing conditions) or 40 mM dithiothreitol (reducing conditions).

Immunoprecipitations from PC12 Cells—PC12 cells (19) grown as monolayer cultures were washed once with Krebs-Ringer buffer. They were then resuspended in solubilization buffer (see above) at a protein concentration of 1–2 mg/ml. The extract was incubated for 1 h at 4–6°C and then centrifuged. The supernatant was used for immunoprecipitation either immediately or after freezing overnight at −20°C followed by thawing at 4°C. For immunoprecipitation, the fresh or frozen-thawed synaptosome Triton X-100 extract was incubated overnight at 4–6°C under rotation, with protein G-Sepharose Fast Flow beads (Pharmacia Biotech Inc.) precoated with the following antibodies: anti-synaptobrevin 2 (CI 69.1) antibody from Dr. T. Südhof (University of Texas Southwestern, Dallas, TX); rabbit serum against the subunit Ac39 of V-ATPase from Dr. N. Nelson (Hoffmann-La Roche). The pellets (P2) were resuspended in 120 ml of 0.32 M sucrose and spun again at 9200 × g for 15 min. The pellet corresponding to the synaptosomal fraction was then resuspended in solubilization buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin) at a final protein concentration of 5 mg/ml. Triton X-100 was then added (final concentration: 1%), and the resulting extract was incubated for 1 h at 4–6°C under rotation and centrifuged at 200,000 × g for 15 min. The supernatant was used for immunoprecipitation either immediately or after freezing overnight at −20°C followed by thawing at 4°C. For immunoprecipitation, the fresh or frozen-thawed synaptosome Triton X-100 extract was incubated overnight at 4–6°C under rotation, with protein G-Sepharose Fast Flow beads (Pharmacia Biotech Inc.) precoated with the following antibodies: anti-synaptobrevin 2 (CI 69.1), anti-syntaxin 1 (HPC-1), anti-synaptophysin (CI 72), mouse control IgGs. The beads were then pelleted for 1 min at 14,000 × g and washed with solubilization buffer containing 0.5% Triton X-100. Finally, the beads were resuspended in gel sample buffer and boiled for 5 min. Gel sample buffer consisted of 60 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.07% bromphenol blue, and 10 mM N-ethylmaleimide (nonreducing conditions) or 40 mM dithiothreitol (reducing conditions).

Microsequencing of Gel Bands—The band corresponding to p10 was excised, digested with CNBr, and then with trypsin (20). p16 and p22 were digested with lysyl endopeptidase (Lys-C) (20). The peptides generated were separated by gel exclusion (20). Two peptides from p10, one from p16, and one from p22 were microsequenced on an Applied Biosystems Sequencer.

Miscellaneous Procedures—Brain synaptic vesicles were prepared according to the procedure of Hutten et al. (18). Protein concentrations were determined using the method of Peterson (21). SDS-polyacrylamide gel electrophoresis was performed according to Schagger and von Jagow (22), a procedure which maximizes separation of low molecular weight proteins. Immunoblotting was performed as described above.

RESULTS

Monoclonal antibodies directed against synaptobrevin 2 (CI 69.1) and control mice IgGs were used to immunoprecipitate synaptobrevin-associated proteins in Triton X-100 extracts of rat brain synaptosomes. Immunoprecipitates were analyzed by SDS-PAGE run in either reducing or nonreducing conditions to allow visualization of bands hidden by the heavy and light chains of IgGs. In agreement with previous data, several known proteins were found to coimmunoprecipitate specifically with synaptobrevin 2 and not with control antibody as demonstrated by Fig. 1a. Surprisingly, however, the pattern of coprecipitating bands was completely different dependent on whether the Triton X-100 used as the starting material was fresh or frozen-thawed.

As shown in the nonreduced gels of Fig. 1, when freshly made Triton X-100 extract was used, synaptophysin (Fig. 1, a and b, see also Fig. 3) and a protein migrating at approximately 80 kDa were the major bands visible by protein staining in the immunoprecipitates. In addition, a 10-kDa protein (p10) and several other minor bands of higher molecular mass were visible. When frozen-thawed extract was used, the major proteins which coprecipitated with synaptobrevin 2 were SNAP-25 and syntaxin 1 (Fig. 1, a and b, and Fig. 3), in addition to a band of approximately 85 kDa. Less abundant bands of 16, 18, and 22 kDa (p16, p18, p22) were visible. Synaptophysin, p10, and the band of 80 kDa were virtually absent from the immunoprecipitate obtained from frozen-thawed extract (Fig. 1, a and b, and Fig. 3). By Western blotting, rbsec1 (26) was not detected in any of the immunoprecipitates while synaptotagmin 1 (6, 27) was detected in the immunoprecipitate formed from frozen-thawed extract (Fig. 3).
The 80-kDa band, visible in the nonreduced immunoprecipitates formed from fresh extracts, was reactive for synaptophysin by Western blots and disappeared in reduced gels with a corresponding increase of the synaptophysin band (compare the two lanes A in Fig. 1b). Thus, the 80-kDa band represents a synaptophysin dimer in agreement with previous reports that synaptophysin can dimerize (28). The 85-kDa band visible in the nonreduced immunoprecipitates obtained from frozen-thawed extracts reacted with anti-syntaxin 1 antibodies by Western blotting and most likely represents the 7 S SNARE complex. It was shown previously that even in reducing conditions the 7 S synaptobrevin 2-SNAP-25-syntaxin 1 complex does not dissociate in SDS unless boiled for at least 5 min (29). The close proximity of the three SNAREs may facilitate the formation of disulfide bridges in nonreducing conditions. The migration of p10, p16, p18, and p22 was unaffected by the reducing or nonreducing conditions of SDS-PAGE. p10, p16, and p22 were investigated further.

The bands corresponding to these proteins were excised and processed for microsequencing. Sequences of one peptide each derived from p16 and p22 perfectly matched the sequences of the rat myelin basic proteins of 14 and 19 kDa (30), respectively (not shown). The identity of p16 and p22 as myelin basic proteins was further confirmed by Western blotting (not shown). The association of myelin basic proteins with SNARE complexes had already been documented (6). It must represent an in vitro artifact since myelin basic proteins and synaptic SNAREs are localized in different cell types (7, 8).

Sequences from two peptides of p10 matched the sequence of the c subunit of rat V-ATPase also called ductin (31) (Fig. 2). In agreement with this identification, Western blotting demonstrated that V-ATPase c and other transmembrane subunits of V-ATPase including Ac39 and Ac116 were present in anti-synaptobrevin 2 immunoprecipitates obtained from fresh Triton X-100 extract but not from frozen-thawed extract (Fig. 3). The c subunit of the V-ATPase precisely comigrated with p10. A cytosolic subunit of the catalytic domain of V-ATPase, the B subunit, was absent from the immunoprecipitates (Fig. 3). Thus, our data suggest the existence of a Triton X-100-resistant complex between synaptobrevin 2, synapticophysin, and the V0 sector of the V-ATPase which does not include the cytosolic V1 sector of V-ATPase. Quantification of the autoradiograms showed that 57% of the total synapticophysin and 56% of the total c subunit of V-ATPase are associated with synaptobrevin 2 in fresh Triton X-100 extract. Furthermore, the two other subunits of the V0 sector of the V-ATPase, Ac39 and Ac116, were enriched in roughly similar proportion as the c subunit (see legend of Fig. 3). The putative stoichiometric ratio of the c, Ac39, Ac116, and 19-kDa subunits in the V0 domain is 6:1:1:1 (32), thus explaining why the c domain is more easily detectable than other V-ATPase subunits in Coomassie Blue-stained gels of the immunoprecipitates. It was shown previously that the Ac39 subunit can bind in vitro to synapticophysin (33). It is therefore possible that the interaction between V-ATPase subunits and synaptobrevin 2 may be indirect and mediated by synapticophysin.

We confirmed the specificity of the association between synaptobrevin 2, synapticophysin, and the V-ATPase by performing immunoprecipitation experiments using monoclonal antibodies against synapticophysin (Cl 7.2) and syntaxin 1 (HPC-1) (Fig. 4). In agreement with the data shown above and with previous studies, syntaxin 1 was not found in anti-synaptobrevin 2 immunoprecipitates and synapticophysin was not found in anti-syntaxin 1 immunoprecipitates. Furthermore, the V-ATPase c subunit was present together with synaptobrevin 2 in anti-syntaxin 1 immunoprecipitates obtained from fresh, but not from frozen-thawed extracts, and was not found in any anti-syntaxin 1 immunoprecipitate. Therefore, the freeze-thawing treatment which induces dissociation of synaptobrevin 2 from synapticophysin and V-ATPase also induces dissociation of synapticophysin from V-ATPase. Note that the synapticophysin monomer is less abundant in anti-synaptophysin immunoprecipitates obtained from fresh, but not from frozen-thawed extracts, and was not found in any anti-syntaxin 1 immunoprecipitate. Therefore, the freeze-thawing procedure enhances dimer formation. Freeze-thawing must also induce a conformational change in syntaxin 1, because the recovery of syntaxin 1 (both monomer and SNARE complex) in anti-syntaxin 1 (antibody HPC1) immunoprecipitates was consistently greater when frozen-thawed extract was used.

The c subunit of the V-ATPase was found to be associated with synaptobrevin 2 and synapticophysin also in freshly prepared Triton X-100 extracts of PC12 cells. Fig. 5 shows presence of the c subunit in anti-synaptobrevin 2 (Cl 69.1) (lane C) and anti-synapticophysin immunoprecipitates (Cl 7.3) (lane E), but not in anti-syntaxin 1 (HPC1) (lane D) or control immunoprecipitates (lane B).

SVs are known to contain a functionally active proton pump
Fig. 4. Western blots demonstrating the presence of the c subunit of V-ATPase in anti-synaptophysin (antibody Cl 7.2) immunoprecipitates obtained from Triton X-100 synaptosomal extracts. The c subunit is absent from anti-syntaxin 1 (antibody HPC-1) immunoprecipitates obtained from both extracts. Lanes A correspond to the starting material. The gel was run in nonreduced conditions. The greater recovery of syntaxin 1 in the bead fraction from frozen-thawed extracts was reproducible and due to the greater reactivity of syntaxin 1 with the HPC-1 antibody after freeze-thawing.

Fig. 5. The c subunit of V-ATPase is in a complex with synaptobrevin 2 and synaptophysin but not with syntaxin 1 in PC12 cells. Western blots for synaptophysin, syntaxin 1, synaptobrevin 2, and the c subunit V-ATPase of immunoprecipitates (bead fractions) obtained from Triton X-100 extracts of PC12 cells. Lanes A, starting material. Lanes B–E, bead fractions from immunoprecipitations with control mouse IgGs (lane B), anti-synaptobrevin 2 (Cl 69.1, lane C), anti-syntaxin 1 (HPC-1, lane D), and anti-synaptophysin (Cl 7.3, lane E) antibodies.

Fig. 6. The c subunit of V-ATPase copurifies with other proton pump subunits and SV markers during preparation of a purified SV fraction. Western blots of subcellular fractions for the proteins are indicated on the right. The fractions are as follows (18): H, homogenate; P1, 800 g pellet; P2 (synaptosomal fraction), 9,200 g pellet; P3, 100,000 g supernatant; P3 (microsomal fraction), 100,000 g pellet; LP1, 25,000 g pellet of lysed synaptosomes; LP2 (crude SV fraction), 165,000 g pellet of lysed synaptosomes; LS2 (synaptosomal fraction), 165,000 g supernatant of lysed synaptosomes; CP, controlled pore glass purified SVs. Note the strong co-enrichment in the PL2 and CPG-3 fractions of the c subunit of V-ATPase together with other V-ATPase subunits (Ac116 and B subunits) and with the SV markers synaptobrevin and synaptophysin.

A Synaptic V-ATPase-Synaptobrevin-Synaptophysin Complex

A c subunit of V-ATPase was also found in the cytosolic fraction of synaptosomes LS2, in agreement with previous findings (38), suggesting a dynamic association-dissociation between the cytosolic and transmembrane portions of the V-ATPase (Fig. 6). These findings are not in contrast with the expected localization of the V-ATPase, and in particular of the c-subunit, in other membranes of brain including membranes of non-neuronal cells. They suggest, however, that the bulk of the brain V-ATPase in the brain is localized on SVs. Accordingly, immunofluorescence staining of frozen sections of rat brain stem with antibodies directed against the B subunit of the V-ATPase, demonstrated that V-ATPase immunoreactivity is concentrated in nerve terminals in a pattern which resembles synaptobrevin immunoreactivity (Fig. 7). Available antibodies directed against the c subunit and other ATPase subunits did not react in immunofluorescence experiments.

DISCUSSION

In this study we have demonstrated that in brain the subunits comprised in the $V_0$ sector of V-ATPase (subunit c, Ac39, and Ac116) (for review see Nelson (34)) are involved in a Triton X-100-resistant interaction with synaptophysin and synaptobrevin 2. The interactions among these components, which are likely to reflect a physiological association in SV membranes (35), are highly sensitive to freeze-thawing of the Triton X-100 extract, indicating that they can be disrupted by a mild denaturing step. Freeze-thawing, in contrast, promotes a massive association of synaptobrevin 2 with t-SNAREs to form SNARE complexes. Freeze-thawing may induce a conformational change in synaptobrevin 2 which mimics a physiological change occurring in vivo and which is part of the events leading to exocytosis.

The Ac39 and Ac116 subunits of V-ATPase had already been shown to be highly enriched in mammalian SVs (33, 34, 39) where the proton pump plays a crucial role in the uptake of neurotransmitters. However, the presence of the V-ATPase c subunit on mammalian SVs had never been demonstrated. This subunit, which is represented by a small polypeptide with 4 putative transmembrane regions, is thought to represent the hydrophilic channel thorough which protons are translocated (for review, see Ref. 36). The demonstration of the localization of the c subunit on SVs within the context of a V-ATPase is of
significant interest because alternative functions and localizations have been proposed for this molecule. The c subunit is identical with the so-called mediatophore, a synaptic protein from the Torpedo electric organ which has been reported to form an acetylcholine-permeable transmembrane channel (40). The mediatophore was originally thought to be localized in the presynaptic plasmalemma and to be responsible for nonexocytotic, quantal release of acetylcholine, although more recent studies in the nervous system of Torpedo have suggested a predominant localization in SVs (41). The same proteins were also found to be identical with ductin, a putative subunit of gap junction channels (36). Based on findings suggesting that ductin can be present in membranes in two opposite orientations, it has been proposed that the orientation of ductin in the membrane specifies whether the proteins will be assembled into a V-ATPase or into a GAP junction (42). Our data indicate a colocalization of ductin with other subunits of the V₀ sector of the V-ATPase in the antisyntaphobrevin 2 or antisynaptophysin immunoprecipitates and favors therefore the model in which ductin is a subunit of the ATPase. However, additional roles for this protein cannot be excluded. A possible function of the V₀ sector of the V-ATPase, independent from the V₁ sector, has been discussed (36). Given its pore-forming properties (36), its unusual mechanism of membrane incorporation (42), and its link to syntaphobrevin demonstrated here, a role of the c subunit/ductin in the membrane interactions which lead to fusion cannot be ruled out completely.

In a previous study, a search for syntaphophysin binding proteins in synaptic membrane had led to the identification of physophillin (43). Physophillin was subsequently found to be identical with the Ac39 subunit of the V-ATPase (33). A possible connection between the Ac39 subunit of the V-ATPase and syntaphophysin was also suggested by a study on SV protein complexes formed in various detergents (14). Our data could be explained by an indirect interaction of syntaphobrevin with the V-ATPase mediated by syntaphophysin. The association of the c subunit of the V-ATPase with a syntaphophysin-containing complex is of special interest because this protein was shown to coassemble with connexin. Syntaphophysin and connexins are structurally related (44).

The association of the syntaphobrevin 2-synaptophysin complex with the V-ATPase could be important in the biogenesis of SVs by ensuring inclusion of the proton pump into the vesicles. Since many other organelles of the secretory and endocytic pathway contain the V-ATPase, it will be of interest to determine whether an interaction between V-SNAREs and the proton pump is generally found on other organelles. Such an interaction would provide a simple mechanism to couple generation of a transport vesicle to inclusion of the pump in the carrier.

A striking observation made in our study is that a cycle of freeze-thawing has a drastic effect on the V-ATPase-synaptophysin-synaptobrevin 2 complex and on SNARE complex formation. Most likely, the effect of freeze-thawing can be explained by the transient increase in the ionic strength which occurs during freezing, when the growth of ice crystal displaces solutes in the residual liquid phase. This result may explain the variable recovery of SNARE complexes observed in published studies. We note that the first evidence for the interaction of synaptobrevin 2 with syntaxin 1 and SNAP-25 came from studies performed with frozen-thawed bovine brain Triton X-100 extract (6). The abundance of synaptobrevin-syntaxin 1-SNAP-25-SNARE complexes observed in many published studies cannot reflect endogenous complexes because only minor pools of these SNAREs are colocalized in the cell (the v/t-SNARE complexes of docked vesicles) (7). NSF and its yeast homologue Sec18 have the structure of molecular chaperones (45). Therefore, it cannot be excluded that even the massive presence of Golgi SNARE complexes observed in extracts of Sec18 yeast mutants exposed to the restrictive temperature, may reflect a postlysosome phenomenon due to abnormal folding states of the SNAREs (46).

Our data favor a model in which the bulk of brain syntaphobrevin is localized on SVs in a complex with syntaphophysin and in which the V₀ sector of the V-ATPase is associated with this complex. Since the presence of syntaphobrevin in the complex is incompatible with its interaction with the t-SNAREs (11) and this study) and since the bulk of t-SNAREs is localized on the plasmalemma (7, 9), dissociation of this complex must precede fusion. Freeze-thawing may promote a conformational change which bypasses the need for the physiological regulators which catalyze this transition in situ. Since NSF and α-SNAP do not bind the v-SNARE (47, 48), it seems unlikely that NSF and α-SNAP could have such an activity. Identifying such factors is an important priority for future work. NSF, which is required for the dissociation of the SNARE complex (5, 6) may help in regenerating a syntaphobrevin conformation (45) compatible with the interaction with syntaphophysin and the V₀-ATPase.

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