INTRODUCTION

In motor nerve endings, the neurotransmitter is released within 200 μseconds of the influx of calcium triggered by the action potential (Llinas et al., 1981). This release occurs at discrete specialized areas of the presynaptic plasma membrane, the active zones, where clusters of synaptic vesicles, the neurotransmitter-storing organelles, are docked (Couteaux and Pecot-Dechavassine, 1974) and where voltage-gated calcium channels are concentrated (Robitaille et al., 1990). According to the SNARE hypothesis (Rothman, 1994), the specificity of the docking and fusion of synaptic vesicles to the presynaptic plasma membrane requires the formation of a multimolecular complex involving proteins of both membranes, v- and t-SNAREs in the vesicular and target membranes respectively. Such a complex, as characterized from mammalian brain, contains two proteins of the presynaptic membrane, syntaxin and SNAP 25, and a synaptic vesicle membrane protein, VAMP/synaptobrevin (Söllner et al., 1993). Syntaxin was first identified as a protein that interacts with another synaptic vesicle protein, synaptotagmin, and presynaptic membrane calcium channels (see Seagar and Takahashi, 1998, for a review). The functional involvement of syntaxin, SNAP 25 and VAMP in presynaptic activity was demonstrated by the observation that they are specifically cleaved by clostridial neurotoxins (Schiavo et al., 1992; Blasi et al., 1993a, b) which are potent inhibitors of synaptic transmission. Genetic studies have shown that these three proteins, which display significant homology to proteins of the constitutive secretory pathway in yeast (reviewed by Bennett and Scheller, 1993), are required for evoked neurotransmitter release in Drosophila (Broadie et al., 1995; Littleton et al., 1998).

Despite compelling evidence linking the SNARE complexes to exocytosis, it is still not understood how and when they operate. A direct role in membrane fusion has been suggested by in vitro experiments (Weber et al., 1998), but questioned in the case of homotypic vacuole fusion in yeast (Ungermann et al., 1998a). Involvement of v- and t-SNAREs in the specific targeting and docking of synaptic vesicles to the active zones would require a specific localization of these proteins. Syntaxin and SNAP 25, the t-SNAREs, are not restricted to the nerve terminal membrane, but are also present in the axonal

VAMP (synaptobrevin) is present in the plasma membrane of nerve terminals

P. Taubenblatt1, J. C. Dedieu2, T. Gulik-Krzywicki2 and N. Morel1,*

1 Lab. Neurobiologie Cellulaire et Moléculaire and 2 Centre de Génétique Moléculaire, C.N.R.S., 91198 Gif sur Yvette, France

*Author for correspondence (e-mail: nicolas.morel@nbcm.cnrs-gif.fr)

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SUMMARY

Synaptic vesicle docking and exocytosis require the specific interaction of synaptic vesicle proteins (such as VAMP/synaptobrevin) with presynaptic plasma membrane proteins (such as syntaxin and SNAP 25). These proteins form a stable, SDS-resistant, multimolecular complex, the SNARE complex. The subcellular distribution of VAMP and syntaxin within Torpedo electric organ nerve endings was studied by immunogold labeling of SDS-digested freeze-fracture replicas (Fujimoto, 1995). This technique allowed us to visualize large surface areas of the presynaptic plasma membrane and numerous synaptic vesicles from rapidly frozen nerve endings and synaptosomes. VAMP was found associated with synaptic vesicles, as also shown by conventional electron microscopy immunolabeling, and to the presynaptic plasma membrane (P leaflet). Syntaxin was also detected in the nerve ending plasma membrane, without gold labeling of synaptic vesicles. Comparison of gold particle densities suggests that the presynaptic plasma membrane contains 3 VAMP molecules per molecule of syntaxin.

After biotinylation of intact synaptosomes, the synaptosomal plasma membrane was isolated on Strepavidin coated magnetic beads. Its antigenic content was compared to that of purified synaptic vesicles. VAMP was present in both membranes whereas syntaxin and SNAP 25 were highly enriched in the synaptosomal plasma membrane. This membrane has a low content of classical synaptic vesicle proteins (synaptophysin, SV2 and the vesicular acetylcholine transporter). The VAMP to syntaxin stoichiometry in the isolated synaptosomal membrane was estimated by comparison with purified antigens and close to 2, in accordance with morphological data. SDS-resistant SNARE complexes were detected in the isolated presynaptic membrane but absent in purified synaptic vesicles. Taken together, these results show that the presence of VAMP in the plasma membrane of nerve endings cannot result from exocytosis of synaptic vesicles, a process which could, as far as SNAREs are concerned, very much resemble homotypic fusion.

Key words: VAMP, Synaptobrevin, SNARE, Presynaptic membrane, Freeze-fracture, Synaptic vesicle

INTRODUCTION

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Despite compelling evidence linking the SNARE complexes to exocytosis, it is still not understood how and when they operate. A direct role in membrane fusion has been suggested by in vitro experiments (Weber et al., 1998), but questioned in the case of homotypic vacuole fusion in yeast (Ungermann et al., 1998a). Involvement of v- and t-SNAREs in the specific targeting and docking of synaptic vesicles to the active zones would require a specific localization of these proteins. Syntaxin and SNAP 25, the t-SNAREs, are not restricted to the nerve terminal membrane, but are also present in the axonal
membrane (Garcia et al., 1995). They were shown to be present in isolated brain synaptic vesicles, both by biochemical (Otto et al., 1997) and morphological (Kretzschmar et al., 1996) approaches. SNARE complexes are not exclusively assembled at the sites of exocytosis, but can result from the assembly of v- and t-SNAREs side by side in the same membrane as reported in the case of isolated synaptic vesicles (Otto et al., 1997) or during axonal transport (Shiff and Morel, 1997a,b). In the case of yeast vacuole homotypic fusion, a complex associating v- and t-SNAREs in the same vacuole membrane preexists (Ungeremann et al., 1998b). It must be actively disassembled by yeast NSF and SNAP homologues to allow the formation of a new SNARE complex from v- and t-SNAREs in opposing vacuoles and the fusion to proceed (Nichols et al., 1997).

Localization of SNARE antigens in the presynaptic membrane, in contrast to synaptic vesicles, has not been studied in great detail at the electron microscope level in differentiated nerve endings. Antigens are present at rather low density and only a limited surface of presynaptic membrane can be observed in conventional thin sections. Moreover, access to the cytoplasmic surface of the presynaptic membrane requires permeabilization or disruption of the nerve endings. These limitations can be overcome, in the case of membrane antigens, using a freeze-fracture approach, combined to immunolabeling as described by Fujimoto (1995). We have adapted this technique to study the distribution of SNARE antigens in Torpedo electric organ nerve terminals, fast acetylcholine releasing synapses very similar to neuromuscular junctions. After indirect immunogold labeling, the presence of syntaxin in the presynaptic membrane, and of VAMP in both synaptic vesicles and the presynaptic membrane was visualized.

MATERIALS AND METHODS

Materials

Torpedo marmorata were provided by the marine station of Arcachon (France). The anti-syntaxin monoclonal antibody 6D2 was kindly provided by Dr M. Takahashi, the monoclonal antibody to the synaptic vesicle antigen SV2 (Buckley and Kelly, 1985) by Dr K. Buckley. The anti-syntaxin monoclonal antibody HPC1 was purchased from Sigma. The anti-SNAP 25 antiserum was purchased from Alomone labs (Israel). Anti-SNAP 25 antibodies were affinity purified on a rat SNAP 25-GST fusion protein obtained from Alomone labs (Israel) from an anti-Torpedo SNAP 25 antiserum prepared in the laboratory. Antiserum to the synaptic vesicle acetylcholine transporter was a gift from Dr M. F. Diebler (Varoqui et al., 1996). Anti-VAMP monoclonal antibodies 19K2 and 19K7 were prepared in our laboratory (Shiff and Morel, 1997b). Anti-synaptophysin antibodies, raised against the EQEGYQPNYGQ synthetic peptide crosslinked to bovine serum albumin by glutaraldehyde, were affinity purified on this peptide by Fujimoto (1995). In most experiments, a thin layer of carbon (2 seconds evaporation) was deposited on the fractured specimens, before the platinum shadowing step. This results in an increased (2 to 3 times) immunolabeling of the replicas, probably as a consequence of a better protein adsorption on carbon than platinum films.

Isolation of presynaptic plasma membranes

Synaptosomes were prepared from Torpedo electric organ as previously described (Morel et al., 1977). Nerve terminal plasma membranes were isolated after biotinylation of intact synaptosomes. Synaptosomes, in suspension in the Torpedo physiological medium (Morel et al., 1977), were incubated for 60 minutes at 4°C with EZ-LinkTM Sulfo-NHS-LC-Biotin (Pierce, 0.4 mM final concentration). Biotin was then quenched by incubation for an additional 15 minutes with glycine (30 mM final concentration). Synaptosomes were then concentrated by centrifugation (10000 g max for 20 minutes) and the synaptosomal pellet was carefully surface washed to remove unbound biotin. Synaptosomes were disrupted by resuspension (2 mg protein/ml) in a hypoosmotic buffer (20 mM Tris buffer, pH 7.2, 1 mM PMSF, protease inhibitor cocktail (0.5 μL/mL, ref. P8340) (from Sigma), freezing and thawing and several passages through a 26 gauge needle. Outwardly biotinylated membranes were isolated on streptavidin coupled to magnetic beads (Dynabeads M280, Dynal). Antigens in bound membranes were directly solubilized in 1% SDS and concentrated under vacuum (Speedvac).

Synaptic vesicles were purified from frozen electric organ by fractionation in two successive sucrose gradients as reported by Dieler and Lazere (1985).

Freeze-fracture immunolabeling

Small pieces of Torpedo electric organ were preincubated in calcium free Torpedo physiological medium. Synaptosomes were concentrated (about 50 mg protein/ml) by centrifugation (10000 g max for 20 minutes). They were freeze-fractured by the ‘sandwich freezing’ procedure (Guli-Krzywicki and Costello, 1978) as previously described (Morel et al., 1980). The fracture surfaces were replicated with platinum and carbon. Replicas were detached from the frozen material by immersion in phosphate-buffered saline (PBS) and digested by 2% SDS (20 minutes for synaptosomes, 6 to 18 hours for electric organ), and immunolabeled according to the method of Fujimoto (1995). In most experiments, a thin layer of carbon (2 seconds evaporation) was deposited on the fractured specimens, before the platinum shadowing step. This results in an increased (2 to 3 times) immunolabeling of the replicas, probably as a consequence of a better protein adsorption on carbon than platinum films.

RESULTS

The distribution of VAMP and syntaxin in peripheral cholinergic nerve endings was studied on freeze-fractured Torpedo electromotoneuron nerve endings using the SDS-digested freeze-fracture replica labeling technique described by Fujimoto (1995). The specificity of the anti-VAMP and anti-syntaxin antibodies used in this study was first tested by western blot analysis (Fig. 1). In boiled samples, anti-VAMP antibodies 19K2 and 19K7 labelled a single band at 19 kDa whereas the anti-syntaxin 6D2 antibody stained a single band at 35 kDa, as expected. In unboiled samples, two additional bands (at 65 and 50 kDa) were detected by the 19K2 anti-VAMP antibody, one of which is also labeled by the anti-syntaxin 6D2 antibody. This 65 kDa band corresponds to the SDS resistant SNARE complex already described (Hayashi et al., 1994; Pellegrini et al., 1995). The SDS resistant protein complexes were not labeled by the anti-VAMP 19K7 antibody. By progressive proteolysis of VAMP, we have previously
shown (unpublished results), that the 19K7 epitope is located in the middle of VAMP (and hidden in the SDS-resistant SNARE complex) while the 19K2 epitope is situated at the N terminus of the protein (and accessible to the antibody even in protein complexes).

VAMP/synaptobrevin was first identified as an abundant membrane protein of synaptic vesicles in \textit{Torpedo} electric organ (Trimble et al., 1988) and rat brain (Baumert et al., 1989). Using the anti-\textit{Torpedo} VAMP monoclonal antibody 19K2 (Shiff and Morel, 1997b), VAMP was detected at the electron microscope level in fixed permeabilized \textit{Torpedo} electric organ (Fig. 2). Staining was restricted to nerve terminals, no binding being detected elsewhere in the electric organ. In nerve endings, virtually all synaptic vesicles are labeled, usually by several gold particles, whereas no gold particles were found associated with the nerve terminal plasma membrane.

The distribution of VAMP was also studied after ultra-rapid freezing of \textit{Torpedo} electric organ, in the absence of any chemical fixation or detergent permeabilization of the tissue (Fig. 3). The frozen tissue was fractured and a film of platinum (Pt) and carbon (C) was deposited on the fractured surface. The frozen tissue, covered by the Pt/C replica, was allowed to thaw in PBS and was then digested by SDS. Membrane proteins, exposed after fracture of membranes and embedded in the Pt/C film, remain (at least some of them) associated with the replica and can be visualized using specific antibodies and indirect immunogold labeling. In Fig. 3, upper panel, the fracture crossed a nerve ending. In the presynaptic cytoplasm, fractured synaptic vesicles are visible, either as concave or convex structures. The interior of concave profiles is covered by the replica, leaving the P cytoplasmic leaflet (P face) of the vesicle membrane exposed to the antibodies. The internal surface of synaptic vesicle membrane is accessible to antibodies in the case of convex profiles (E face). Gold particles are mainly associated with the concave profiles of synaptic vesicles (about half of them are labeled) whereas the majority of convex vesicle fractures are unstained. This demonstrates that VAMP
is pinched off with the P face of the synaptic vesicle membrane during the freeze-fracture and that the anti-VAMP antibody (mAb 19K7 in this particular experiment) binds to a cytoplasmic epitope. This was expected since VAMP is anchored in the vesicle membrane by a single C terminus membrane spanning domain, with most of the protein in the cytoplasm and only two amino acids in the intravesicular space (Trimble et al., 1988; Baumert et al., 1989). A large surface of the E (external) leaflet of the presynaptic membrane is exposed on the left and is free of gold particles. The synaptic cleft and the postsynaptic membrane, covered with numerous intramembrane particles corresponding to the acetylcholine receptor molecules, are devoid of gold particles, as expected. In Fig. 3, bottom panel, an alignment of synaptic vesicles docked at the presynaptic membrane was exposed by the fracture. These anchored vesicles are labeled by the anti-VAMP antibody, as are undocked vesicles. A large surface of the P face of the presynaptic membrane is visible near this active zone, where small groups of large intramembrane particles are present. VAMP was unambiguously detected in the cytoplasmic leaflet of the presynaptic plasma membrane. It was not restricted to special domains of the membrane, but, as observed in several experiments, more or less evenly distributed throughout the cytoplasmic surface of the nerve terminal membrane.

Similar experiments were performed using concentrated synaptosomes, allowing the study of large numbers of fractured isolated nerve endings. A typical synaptosome, labeled by the anti-VAMP 19K2 antibody, is shown in Fig. 4. Most concave fractures of synaptic vesicles are decorated by gold particles. The P face of the synaptosomal membrane is also labeled, in contrast to the E leaflet (not shown). Estimates of the gold particle density were made. Considering a mean synaptic vesicle surface of $2.10^{-2}$ μm$^2$ (vesicle diameter of 80 nm), the gold particle density (from about 500 concave vesicle fractures in this experiment) would be around 230 grains/μm$^2$. In the same synaptosomes, the density of gold particles in the presynaptic membrane P face is lower, about 85 grains/μm$^2$.

The distribution of syntaxin was studied under similar conditions (Fig. 5). In these freeze-fracture experiments,
syntaxin is expected to behave in much the same way as VAMP since it also contains a single transmembrane domain at its C terminus, most of the protein being in the cytoplasm (Inoue et al., 1992; Bennett et al., 1992). Several typical presynaptic membrane profiles are shown. P faces are convex and stained by gold particles. A small E face fragment at the right of the figure is not labeled. It can be seen in the synaptosome fractured along an equatorial plane that synaptic vesicles are not labeled. A few gold particles are associated with the cytoplasmic side of the synaptosomal membrane cross-section. Syntaxin therefore behaves as a presynaptic membrane antigen, even if the density of gold particles is rather low (about 30 gold part./μm²) when compared to that of VAMP staining.

Using the SDS-fracture immunogold labeling technique and antibodies directed against cytoplasmic epitopes, we have been unable to detect synaptophysin and SV2, even in synaptic vesicles (not shown). Very low immunogold labeling of the vesicular acetylcholine transporter was associated with synaptic vesicles (about 5% of synaptic vesicles in the best experiments, not shown). Using affinity purified anti-SNAP 25 antibodies reacting with monomeric SNAP 25 but not the SDS resistant SNARE complex, we were unable to detect SNAP 25 in the synaptosomes (not shown).

The synaptosomal plasma membrane was isolated and its antigenic content compared to that of highly purified synaptic vesicles (Fig. 6). We took advantage of the excellent functional preservation of Torpedo synaptosomes which maintain a physiological membrane potential (Meunier, 1984) and are able to synthesize, store in synaptic vesicles and release acetylcholine (Morel et al., 1977; Israël and Lesbats, 1981; Dolezal et al., 1993) for several hours. Intact synaptosomes were biotinylated using a non permeant biotin derivative to label their plasma membrane. After mechanical disruption of the synaptosomes, biotinylated membranes were isolated on streptavidin-coated magnetic beads. Synaptic vesicles, with a high acetylcholine content, are purified directly from electric organ by taking benefit of the much lower equilibrium density of acetylcholine filled vesicles as compared to depleted ones (Diebler and Lazereg, 1985). VAMP was easily detected in both membranes (samples in Fig. 6a were adjusted to give bands of VAMP of similar intensities). Syntaxin and SNAP 25, present in the synaptosomal membrane, are weakly detected in synaptic vesicles. In contrast, classical synaptic vesicle markers such as synaptophysin, SV2 and the vesicular acetylcholine transporter VACt are hardly detected in synaptic vesicles.

In order to compare the amount of VAMP in the synaptosomal plasma membrane to that of classical synaptic vesicle proteins, the synaptosomal membrane content of various presynaptic antigens was estimated by comparison to that of known amounts of synaptic vesicles (Fig. 6b). Taking VAMP as 100, synaptophysin (29±15, mean ± s.e.m. from 3 independent experiments), SV2 (17±10) or the vesicular acetylcholine transporter (27±5) are present in the synaptosomal membrane at significantly (P<0.05, paired t-test) lower proportions than VAMP. The t-SNAREs syntaxin and SNAP 25 are highly enriched in the synaptosomal membrane as compared to synaptic vesicles (1089±146 and 775±82, respectively). The proportion of VAMP present in the synaptosomal membrane is therefore 3 times larger than that of other classical synaptic vesicle proteins.

The VAMP to syntaxin stoichiometry in the isolated synaptosomal membrane was estimated by comparing the
staining intensity of these antigens with that of known amounts of purified VAMP and syntaxin (Shiff and Morel, 1997a) treated in parallel in the same western blots (not shown). In 2 independent membrane preparations, we found 7 and 8 ng VAMP for 6 and 7 ng syntaxin respectively, which gives (taking 19 and 35 kDa for the VAMP and syntaxin molecular masses, respectively) 2 molecules of VAMP per syntaxin molecule in the synaptosomal plasma membrane.

We looked for the presence of SNARE complex associating VAMP, syntaxin and SNAP 25 in isolated synaptosomal membrane and purified synaptic vesicles (Fig. 7). SNARE complex has been shown to be SDS-resistant in unboiled samples (Hayashi et al., 1994; Pellegrini et al., 1995). Therefore we have directly solubilized isolated presynaptic membranes or purified synaptic vesicles in SDS, just before proteins were submitted to polyacrylamide gel electrophoresis. Probing the blots with either the anti-syntaxin 6D2 or the anti-VAMP 19K2 antibodies revealed the presence of a SDS-resistant SNARE complex in the 65 kDa region in unboiled synaptosomal membrane. This complex was absent in unboiled

![Fig. 5. Syntaxin distribution in freeze-fractured synaptosomes. Binding of mAb 6D2 was visualized as in Fig. 4. Cytoplasmic (PF) and external (EF) leaflets of the synaptosomal membrane. Bar, 0.5 μm.](image)

![Fig. 6. Comparison of the antigenic content of the synaptosomal plasma membrane and synaptic vesicles. After biotinylation of intact synaptosomes, the biotinylated synaptosomal membrane (Biot) was isolated on streptavidin coated beads. In A, its antigenic content was compared to that of synaptic vesicles (SV, 2 μg protein) and to background binding of unbiotinylated synaptosomal membrane to streptavidin beads (C). In B, presynaptic antigens in the synaptosomal plasma membrane were quantified by comparison with their content in increasing amounts of synaptic vesicles. Results (mean ± s.e.m. from 3 independent experiments) are expressed by comparison with VAMP taken as 100. Synaptophysin (physin); vesicular acetylcholine transporter (VACHT).](image)
DISCUSSION

The SDS-fracture immunolabeling technique (Fujimoto, 1995) was applied to the study of presynaptic antigens in nerve endings of *Torpedo* electric organ. Synaptosomes or small pieces of electric organ were rapidly frozen in the absence of any chemical fixation (Morel et al., 1980). Upon freeze-fracture and Pt/C replication, the tissue was digested by SDS. Proteins in the fractured membranes remain adsorbed on the replica and are immunolabeled in experimental conditions very similar to that encountered when probing antigens blotted onto nitrocellulose. Direct comparisons of biochemical and ultrastructural data can therefore be made. Before their use for SDS-fracture immunolabeling, the specificity of purified antibodies can be tested in western blot on SDS solubilized antigens. Monoclonal anti-VAMP antibodies 19K2 and 19K7 both gave similar results with western blot as well as freeze-fracture immunolabeling experiments while only antibody 19K2, and not antibody 19K7, was able to bind to VAMP in formaldehyde fixed tissues. In the present work, the main advantage of the SDS-fracture immunolabeling technique was to allow antibodies and 15 nm gold conjugates to have an easy access to large surface areas of the presynaptic membrane. This permitted the visualization of VAMP in the freeze-fractured presynaptic membrane. VAMP was not detected in nerve ending membrane by classical immunolabeling at the electron microscope level (see Fig. 2), in spite of the fact that VAMP in synaptic vesicles was labeled with a similar efficiency with both techniques. A large proportion of synaptic vesicle surface can be observed in both techniques but only a limited presynaptic membrane surface area is contained in conventional thin sections.

The SDS-fracture label technique does not allow the detection of all membrane antigens. During the fracture, the membrane is split in two leaflets and membrane proteins are pinched off with the cytoplasmic P or the external E leaflet depending on their interactions with their intra or extra-cellular environment. For a P face embedded protein, only cytoplasmic epitopes will be exposed at the surface of the replica while extra-cellular epitopes will be detected for E face associated antigens. Both VAMP and syntaxin are membrane proteins, with a single C-terminal transmembrane domain and most of the protein in the cytoplasm (Trimble et al., 1988; Baumert et al., 1989; Inoue et al., 1992; Bennett et al., 1992). It was therefore not surprising to find most of the immunogold particles associated with P faces, demonstrating that both proteins were pinched off with the cytoplasmic membrane leaflet and that antibodies were directed to cytoplasmic epitopes.

The efficacy of immunolabeling in the SDS-fracture label technique can be estimated in special circumstances, such as gap-junctions where connexins are highly concentrated in patches which can be visualized as intramembrane particles. As an example, in Fig. 4b of Dunia et al. (1998), a direct comparison of immunogold labeling of connexin 63 and intramembrane particles densities gives about 15% of labeling assuming that each intramembrane particle corresponds to one antigen. In the present work, we observed that the deposition of a thin layer of carbon on the fractured surface before the platinum shadowing resulted in a 2- to 3-fold increase in the immunogold labeling of syntaxin and VAMP. Assuming that, with the carbon layer, about 30% of the antigen molecules are labeled, we can estimate (from the gold particle density associated with the synaptic vesicle P faces, 230 grains/μm²) that there are about 15 VAMP molecules per synaptic vesicle (mean surface area: 2×10⁻² μm²). Similar calculations for the synaptosomal membrane give 280 VAMP molecules/μm² (85 VAMP associated gold particles/μm²) and 100 syntaxin molecules/μm² (30 syntaxin associated gold particles/μm²). This estimate of the VAMP to syntaxin stoechiometry in the synaptic vesicle is in good agreement with that obtained from immunoblots of isolated synaptosomal membrane (2 VAMP molecules/syntaxin molecule, see Results).

VAMP is present in the synaptosomal membrane, at a density that is only 3 times lower than in synaptic vesicles. VAMP could have been brought to the presynaptic membrane as a consequence of a massive exocytosis. In this case, the incorporated vesicular membrane would amount to one third of the synaptosomal membrane surface area. This seems unlikely since the present experiments were done in resting conditions. No difference was noticed with or without added external calcium, using synaptosomes or electric organ. Moreover, we have previously shown that exocytotic pits remained infrequent, even after stimulation of synaptosomes, and that no significant modification in the number of synaptic endings of was applied to the study of presynaptic antigens in nerve samples of purified synaptic vesicles. Only VAMP and syntaxin monomers were detected in boiled samples. 

![Fig. 7. SDS-resistant SNARE complexes are present in isolated synaptosomal plasma membrane (SPM) and not in synaptic vesicles (SV). Samples were directly solubilized in the SDS cracking buffer, boiled (+) or not (-) for 2 minutes and immediately submitted to gel electrophoresis. Synaptic vesicle samples (0.5 and 5 μg protein/lane for VAMP and syntaxin respectively) were adjusted to contain the same amounts of VAMP or syntaxin (probed by mAbs 19K2 and 6D2) as synaptosomal plasma membrane.](image-url)
vesicles could be found (Morel et al., 1980). In addition, all synaptic vesicle proteins should have been incorporated in similar proportions in the presynaptic membrane. After isolation of the synaptosomal plasma membrane, it turned out that synaptophysin, SV2 and the vesicular acetylcholine transporter, three well established proteins of the membrane of cholinergic synaptic vesicles, were present in much lower amounts than VAMP in the presynaptic membrane (Fig. 6).

The density of VAMP molecules in the synaptosomal membrane is higher than that of syntaxin. A small proportion of presynaptic plasma membrane syntaxin and VAMP was recovered in an SDS resistant complex of 65 kDa (Fig. 7). One must envisage the possibility that syntaxin and VAMP form, with SNAP 25, a side by side ternary SNARE complex in the presynaptic membrane as already reported for isolated synaptic vesicles (Otto et al., 1997) and yeast vacuoles (Ungermann et al., 1998b). However, it cannot be excluded that a few synaptic vesicles remain docked to the presynaptic plasma membrane via classical SNARE complexes during its isolation. In contrast to results on brain synaptic vesicles (Otto et al., 1997), SDS resistant SNARE complexes were not detected in purified cholinergic synaptic vesicles. Only synaptic vesicles filled with acetylcholine are purified from Torpedo electric organ (Diebler and Lazereg, 1985) since they are isolated according to their equilibrium density which is directly related to their neurotransmitter content. No empty recycling synaptic vesicles or endosomes are present in these fractions in contrast to brain synaptic vesicles which are isolated according to their size on porous glass beads columns or according to their antigenic determinants by immunoinosilation (Walach-Solimena et al., 1995). It should be stressed that we were not able to detect syntaxin associated with synaptic vesicles in our SDS freeze-fracture label experiments and that only low amounts of syntaxin and SNAP 25 are detected on immunoblots of purified synaptic vesicles. In PC12 cells, syntaxin and SNAP 25 recently were found associated with endosomes and almost excluded from synaptic vesicles (Salem et al., 1998).

In the present work, we found that VAMP is present in the nerve terminal plasma membrane, under resting conditions, in larger amounts than classical synaptic vesicle constituents such as synaptophysin, SV2 and the vesicular acetylcholine transporter. Both immunochemical and morphological data show that VAMP molecules are 2 to 3 times more numerous than syntaxin molecules in the presynaptic membrane. This renders unlikely a role of VAMP in the specific targeting and tethering of synaptic vesicles to the presynaptic membrane. Morphological analysis of Drosophila SNAF deficient mutants revealed that syntaxin and VAMP function downstream of vesicle docking (Broadie et al., 1995; Littleton et al., 1998), possibly by promoting membrane fusion (Weber et al., 1998). The presence of the t-SNAREs in synaptic vesicles (Otto et al., 1997), even if in small amounts, and of VAMP in the presynaptic plasma membrane suggests that, as far as SNAREs are concerned, exocytosis of synaptic vesicles should be similar to homotypic fusion (Nichols et al., 1997). Besides SNARE complex, VAMP has been shown to associate to synaptic vesicle proteins such as synaptophysin (Edelmann et al., 1995) and the membrane sector of the vacuolar H+ATPase (Galli et al., 1996). Targeting of VAMP to the nerve endings and to synaptic vesicles appears to occur in two separate steps, encoded by different signals (West et al., 1997).

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