RESEARCH COMMUNICATION

Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin

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Vesicle-associated membrane protein (VAMP) (or synaptobrevin), a type II membrane protein of small synaptic vesicles, is essential for neuroexocytosis because its proteolysis by tetanus and botulinum neurotoxins types B, D, F and G blocks neurotransmitter release. The addition of cross-linking reagents to isolated small synaptic vesicles induces the formation of 30 and 50 kDa complexes containing the isoform 2 of VAMP (VAMP-

2). Whereas the 30 kDa band is a VAMP-2 homodimer, the 50 kDa species results from the cross-linking of VAMP-2 with synaptophysin. This heterodimer also forms in detergent-solubilized vesicles and involves the N-terminal part of VAMP-2. The implications of the existence of a synaptophysin–VAMP-2 complex in the processes of vesicle docking and fusion with the presynaptic membrane are discussed.

INTRODUCTION

Neurons communicate by quantal release of neurotransmitters, discharged into the synaptic cleft from small synaptic vesicles (SSV) of diameter ~ 50 nm within < 1 ms of the rise of Ca^{2+} concentration as a result of the opening of voltage-gated Ca^{2+} channels of the presynaptic membrane [1–5]. Several protein components thought to be involved in docking and fusion of SSV with the presynaptic membrane have been identified [4,6], although the actual process of exocytosis remains obscure. VAMP (also referred to as synaptobrevin) plays an important role in the process. Indeed, when it is cleaved by the zinc-endopeptidase activity of tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) types B, D, F and G, neuroexocytosis is blocked [7–10]. In addition, VAMP is a member of the putative membrane fusion complex [11,12]. VAMP has been suggested to play the role of vesicle receptor (SNARE) (SNAP, soluble NSF attachment protein) in the process of vesicle docking to the active zones of the presynaptic membrane, where syntaxin and SNAP-25 (synaptosomal associated protein of 25 kDa) would play the corresponding role of target membrane receptors (t-SNAREs). The interaction between v- and t-SNAREs is suggested to be mediated by previously identified cytosolic components: *N*-ethylmaleimide-sensitive fusion protein (NSF) and α/β and γ SNAPs [12].

Knowledge of the protein–protein interactions by which VAMP engages the vesicle membrane might be relevant to the docking–fusion process itself and thus might lead to further understanding of the mechanism of neuronal signal transduction. This has been recently investigated by immunoprecipitation of small synaptic vesicles dissolved in detergents, and it was found that VAMP co-immunoprecipitates with other vesicle proteins including synaptophysin [13]. Here, we provide evidence for a direct interaction between VAMP-2 and synaptophysin which form a stable heterodimeric complex.

EXPERIMENTAL

Materials

Synaptic vesicles were isolated from rat and chicken cerebral cortex omitting the glass-bead chromatography step [14] and were finally resuspended in 4 mM Hepes (sodium salt), 320 mM sucrose, pH 7.3, and used immediately. Some vesicles were solubilized in 1% (v/v) Triton X-100 for 30 min at 4 °C and then centrifuged at 20000 *g* for 15 min also at 4 °C. Disuccinimidyl suberate (DSS) was from Boehringer Mannheim (Germany); 3,3'-dithio-bis(propionic acid *N*-hydroxysuccinimide ester) (DTSP) and γ -maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS) were from Sigma (St. Louis, MO, U.S.A.).

Affinity-purified polyclonal antibodies against VAMP isoforms 1 and 2, syntaxin and cysteine string protein (CSP) were a kind gift of Dr. O. Rossetto (University of Padova). They were obtained by immunizing New Zealand white rabbits with antigenic peptides coupled to haemocyanin and subsequently affinity purifying the antisera with the immobilized antigen. Peptides of sequence corresponding to rat VAMP-1 (Ac-SAPAQPPAEGT-EGAAPGGGPPGPPNTTTSNRRL-COOH) and rat VAMP-2 (Ac-SATAATVPPAAPAGEGGPPAPPPNLTTSNRRL-COOH) [15], with an acetylated N-terminal Ser as was found to be the case *in vivo* [11], were used for immunization and affinity purification. The VAMP-isoform-specific antibodies do not cross-react, as determined by immunoblotting of recombinant VAMP-1 and VAMP-2 (results not shown). The full characterization of these antibodies will be reported elsewhere (O. Rossetto et al., unpublished work). A mouse monoclonal antibody against rat synaptophysin was purchased from Sigma, and the one specific for rat synaptotagmin was kindly provided by Dr. M. Popoli (University of Milan). Peptides corresponding to segments 38–48 and 62–71 of rat VAMP-1 and -2, and those mentioned above, were a kind gift of Dr. L. Lozzi (University of Siena). They were synthesized with an automated Applied

Abbreviations used: BoNT, botulinum neurotoxin; CSP, cysteine string protein; DSS, disuccinimidyl suberate; DTSP, 3,3'-dithio-bis(propionic acid *N*-hydroxysuccinimide ester); GMBS, γ -maleimidobutyric acid *N*-hydroxysuccinimide ester; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome associated protein of 25 kDa; SNARE, SNAP receptor; SSV, small synaptic vesicles; t, target; TeNT, tetanus neurotoxin; v, vesicle; VAMP, vesicle-associated membrane protein.

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Biosystem synthesizer model 431A with FastMoc chemistry and were purified by reverse-phase h.p.l.c. on a C₈ Ultra-Sphere preparative column (Beckman).

Cross-linking

SSV or solubilized SSV (20 µg) was treated with DSS, DTSP or GMBS (from freshly prepared 10 mg/ml stock solutions in dimethylsulphoxide) at a final concentration of 0.2 mg/ml for 60 min at 25 °C. In some samples, BoNT/B (final concentration of 100 nM) was added for 2 h at 37 °C before addition of cross-linker. In other samples, VAMP-2 peptides corresponding to segments 1–32, 38–48 and 62–71 were added to a final concentration of 0.18 mM. Some samples, cross-linked with DTSP, were further incubated with 10 mM dithiothreitol for 15 min at 37 °C. The samples were electrophoresed in 10–18% (w/v) gradient Laemmli SDS polyacrylamide gels and transferred to nitrocellulose membranes as before [8]. These were incubated with affinity-purified polyclonal antibodies against VAMP-2, VAMP-1, CSP, syntaxin and monoclonal antibodies against synaptophysin and synaptotagmin. Blots were stained using goat anti-(rabbit IgG) or anti-(mouse IgG) conjugated with alkaline phosphatase (Boehringer Mannheim, Germany) and photographed and/or scanned with a dual-wavelength Shimadzu CS-630 densitometer.

RESULTS AND DISCUSSION

VAMP is one of the most prominent components of SSV [16] and is essential for neuroexocytosis to occur [7,17,18]. SSV can be purified to homogeneity from brain cortex [14] and their SDS/PAGE profile is well characterized. Cross-linking agents have been used for identifying cell-surface receptors and membrane complexes [19]. They were used recently on SSV to identify supra-molecular complexes of synaptophysin [20–22]. The aim of this study was to identify VAMP partners on the SSV membrane,

using homo- and heterofunctional cross-linkers that stabilize already present complexes. Figure 1 shows that the addition of DSS, DTSP or GMBS induces the formation of VAMP-containing 30 and 50 kDa complexes. Immunoblotting with isoform-specific antibodies shows that only VAMP-2 is implicated, whereas VAMP-1-specific antibodies only detected the 18 kDa monomer band and several multimers (Figures 1 and 2a, results for GMBS not shown). We cannot exclude the possibility that VAMP-1 does interact with synaptophysin but cannot be cross-linked to it. However, the fact that cross-linked products of VAMP-1 can be detected (lane 1 of Figure 1) but none of them contains synaptophysin rules against this possibility. Moreover, synaptophysin-VAMP-1 covalent adducts cannot be detected upon incubation with any of the different cross-linkers used here. The specific involvement of VAMP-2 found here correlates with the finding that VAMP-2 is found in the 20 S complex isolated from brain [11], and that VAMP-2, but not VAMP-1, immunoprecipitates with antibodies specific for the N-type Ca²⁺ channels [23].

From the electrophoretic mobility of the cross-linked complexes, the interacting proteins were likely to be of sizes 12–17 and 30–40 kDa. These wide ranges take into account the anomalous electrophoretic mobility of VAMP, which has a molecular mass of 13 kDa but which migrates in SDS/PAGE to a position indicating a size of 18 kDa. Possible partners for VAMP-2 would then be VAMP-2 itself or a yet unidentified low-molecular-mass component for the smaller complex. For the larger one, CSP, synaptophysin and syntaxin (syntaxin is found as a contaminant in SSV) could be involved. Immunoblotting with antibodies specific for these proteins showed that synaptophysin is present in protein bands of apparent molecular mass 50, 72, 100 and 110 kDa, whereas the other candidates were not part of the cross-linked products (Figure 1). This result is in agreement with previous reports that synaptophysin can be cross-linked to form various multimers [20–22] and immunoprecipitates together with

Figure 1 Cross-linking of SSV

SSV (20 µg) isolated from rat brain cortex was incubated with the cross-linking reagent DSS (0.2 mg/ml) for 1 h at 25 °C. Untreated control samples (SSV) were electrophoresed and stained with silver (Ag) or blotted onto nitrocellulose and revealed with antibodies against VAMP-2 and synaptophysin. Cross-linked SSV samples (SSV + DSS) were blotted and stained with antibodies anti-(VAMP-2) and anti-synaptophysin or anti-(VAMP-1) (lane 1), anti-syntaxin (lane 2), anti-CSP (lane 3) and anti-synaptotagmin (lane 4). The apparent molecular masses (kDa) of bands of interest are shown.

Figure 2 Effect on SSV cross-linking of BoNT/B and VAMP-2-derived peptides

(a) Cross-linking of SSV carried out after incubation with BoNT/B and BoNT/C, after solubilization in 1% (w/v) Triton X-100 (TX-100) and cross-linking using the reducible cross-linking reagent DTSP revealed with antibodies against VAMP-2. The molecular masses (kDa) of bands of interest are shown. 2-ME, 2-mercaptoethanol. (b) Inhibition of cross-linking as a result of the addition of the N-terminal peptide of VAMP-2 (amino acids 1–32) at a concentration of 0.18 mM for 30 min before the reaction. Immunoblots were scanned with a Shimadzu CS-930 densitometer. Data represent means $\pm$ S.D. ($n = 3$).

VAMP [13]. On this basis, it was proposed that synaptophysin may form at the active zone a hexameric gap-junction-like pore that would mediate neurotransmitter release [16,20].

Synaptotagmin, although not of the correct size to be part of a 50 kDa complex, was also tested as it has been implicated as a Ca^{2+} -sensitive clamp [12] and might consequently show interactions with VAMP-2. This proved not to be the case (Figure 1). Figure 2(a) shows that the profile of SSV cross-linked with the thiol-cleavable DTSP reagent under reducing conditions is identical with the control. This indicates that the formation of the cross-linked products was actually caused by reaction with the cross-linker and not by a non-specific alteration of the SSV proteins.

The intensity of the 50 kDa complex and its formation in chicken SSV (results not shown) suggest that VAMP-2 and synaptophysin exist independently in the absence of any cross-linkers. To rule out the possibility that the cross-linked product arises as a consequence of a lateral movements of the two components in the restricted space of the membrane, the cross-linking reaction was carried out after detergent solubilization. As shown in Figure 2(a), VAMP-2 and synaptophysin do cross-link also in solution, thus suggesting that they form long-living complexes in the SSV of physiological significance.

The VAMP-2 region mainly responsible for the formation of the 30 and 50 kDa complexes could be the cytosolic portion of VAMP-2 or its membrane domain, or both. To discriminate among these possibilities, SSV was treated, before addition of cross-linkers, with BoNT/B, using BoNT/C as a control. The zinc-endopeptidase activity of BoNT/B leads to the release from

the vesicle of the VAMP-2 fragment 1–76, whereas BoNT/C cleaves syntaxin [7,24]. Figure 2(a) shows that the formation of the 50 and 30 kDa bands is prevented by BoNT/B, but not by BoNT/C, thus indicating that it is the cytosolic portion of VAMP-2 that is mainly implicated in the interaction with synaptophysin.

In order to determine which part of VAMP 2 is involved in the binding of synaptophysin, cross-linking was effectuated in the presence of different VAMP-2 peptides. Two peptides from the central conserved region (38–48 and 62–71) showed no competitive inhibition of cross-linking (results not shown), whereas the 32-residue-long N-terminal segment of VAMP-2, the one used to raise isoform-specific antibodies, decreased cross-linking to $\sim 30\%$ of the control (Figure 2b). This result is in agreement with the fact that VAMP-1 does not interact with synaptophysin (see above), as the two isoforms differ in their proline-rich N-terminal parts. Moreover, there are now many examples of intracellular specific protein–protein recognitions based on proline-rich regions [25].

Synaptophysin has been previously described as a putative hexameric pore-forming membrane protein of synaptic vesicles. It is a glycosylated 34 kDa polypeptide with four transmembrane domains that shows the propensity to form channels in membranes [20]. Synaptophysin and its homologue synaptoporin (58% amino acid identity, also called synaptophysin 2) [26] have both been compared with the connexins. Although the sequence of the connexins shows only 5% identity with the synaptophysins [26], the topographical structure of the connexins also shows four transmembrane domains. Moreover, electrophysiological studies

show that the conductance values for synaptophysin channels are similar to those for the connexin gap junctions [20].

In conclusion, this study shows that VAMP-2 interacts strongly via its N-terminal end with synaptophysin, a putative pore-forming protein, and forms an heterodimer stable to detergent solubilization of the synaptic vesicle membrane. If VAMP-2 is indeed the v-SNARE responsible for vesicle docking to the active zone of the presynaptic membrane, its interaction with synaptophysin would bring it close to the region of neurotransmitter release. At this stage VAMP-2 must dissociate from synaptophysin as the latter protein is not found in the 20 S complex [11].

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