Interaction of a synaptobrevin (VAMP)-syntaxin complex with presynaptic calcium channels

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Abstract Nerve terminal protein complexes implicated in exocytosis were examined by immuno-isolation from rat brain synaptosomes. Immunoprecipitation with anti-syntaxin or anti-VAMP antibodies revealed a syntaxin–SNAP25–VAMP–synaptotagmin complex. Anti-VAMP antibodies also trapped a distinct VAMP–synaptophysin complex. A similar fraction (about 70%) of N-type calcium channels ([^{125}I]ω conotoxin GVIA receptors), was immunoprecipitated by either anti-syntaxin or anti-VAMP antibodies, but not by anti-synaptophysin antibodies (<4%). The majority of N- but not L-type calcium channels ([^{3}H]PN200-110 receptors), appear to be associated with a synaptic vesicle prefusion complex.

Key words: Neurotransmitter release; Calcium channel; Synaptobrevin; Synaptophysin; Syntaxin; Synaptotagmin

1. Introduction

Presynaptic events in neurotransmission include the targeting, docking and calcium-dependent fusion of synaptic vesicles at the plasma membrane of nerve terminals. These processes appear to be mediated a multi-molecular complex that contains the synaptic vesicle membrane proteins VAMP (vesicle associated membrane protein or synaptobrevin) and synaptotagmin, associated with the plasma membrane proteins syntaxin, synaptosome associated 25 kDa protein (SNAP 25), and neurexin (reviewed in [1]). Cytoplasmic factors such as *N*-ethylmaleimide sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAP), interact with this complex [2] and may regulate vesicle fusion.

Neurotransmitter release is ultimately triggered by cytoplasmic calcium concentrations of the order of 100 μM, which are attained transitorily in close proximity to voltage gated calcium channels ([3], reviewed in [4]). Co-immunoprecipitation experiments and calcium channel purification have indicated that synaptotagmin and syntaxin can associate with N-type calcium channels [5,6]. Certain classes of calcium channel may therefore interact with synaptic vesicle docking complexes, locating calcium sensor proteins, such as synaptotagmin [7], within a microdomain of calcium entry. Coupling between ion influx and vesicle fusion occurs with a sub-millisecond latency and identification of components of the secretory machinery associated with calcium channels may thus provide an insight into the final stages of assembly of the prefusion complex.

A crucial step in the interaction between synaptic vesicles and the plasma membrane appears to be the formation of a VAMP–syntaxin complex, however, the association of VAMP with calcium channels has not been reported. We have therefore used a panel of antibodies to immunoprecipitate and detect the protein complexes that have been implicated in exocytosis, and demonstrated the interactions of a VAMP–syntaxin complex with N- but not L-type calcium channels in solubilized rat brain synaptic terminals.

2. Materials and methods

2.1. Materials

Omega conotoxin GVIA (ωCgTx) was purchased from the Peptide Institute (Osaka) and ^{125}I-iodinated as previously described [8]. [^{3}H]PN200-110 (70–85 Ci/mmol), Na^{125}I (2000 Ci/mmol), anti-human IgG peroxidase conjugates, ^{125}I-iodinated protein A, nitrocellulose membranes and ECL Western blotting detection kits were from Amersham. Anti-mouse and anti-rabbit IgG peroxidase conjugates were from Biosys. Cyanogen bromide activated Sepharose 4B, protein A-Sepharose CL-4B, CHAPS and wheat germ agglutinin (WGA) were from Sigma. Monoclonal antibodies against synaptotagmin (mAb1D12 [9]), syntaxin (mAb10H5 [10]) and synaptophysin (mAb171B5, kindly provided by Dr S. Fujita, Mitsubishi Kasei Institute for Life Science) and polyclonal anti-calcium channel antibodies BINt [6] and CR2 [11], were prepared as previously described. Antibodies were raised against synthetic peptides HYEQSADYYKGEE-(C) (residues 144–156 of α and β SNAP [12]), (C)-ANQRATKMLGSG (residues 195–206 of SNAP-25 [13]), and SATAATVPPAAPAGEGGPP-(C) (residues 2–20 of VAMP2 [14]), coupled to keyhole limpet hemocyanin. Rabbit IgG fractions were purified by Protein A-Sepharose CL-4B affinity chromatography.

2.2. Membrane preparation

Rat brains were dissected and the cerebella discarded before homogenization in a glass/Teflon Thomas apparatus in buffer A (0.32 M sucrose, 5 mM Tris-base adjusted to pH 7.4 with HCl) containing the protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 2 mM EDTA, 1.2 μM benzamidine and 1 mM iodoacetamide. After a 10 min centrifugation at 750 × *g*, the supernatant was recovered and spun at 17,000 × *g* for 60 min. The pellet (P2) was resuspended in buffer B (150 mM NaCl, 25 mM Tris-base adjusted to pH 7.4 with HCl) containing 0.2 mM PMSF and 2 mM EDTA and stored until use in liquid N_2.

2.3. Immunoprecipitation assays

Protein A-Sepharose CL4B was swollen in buffer A containing 0.5% BSA and 0.4% CHAPS and incubated with antibodies for 1 h at 4°C. Following centrifugation, the pellet was washed with 0.5% BSA in buffer B then with 0.5% BSA in buffer A.

Membranes (2 mg/ml) were solubilized with 1% CHAPS in buffer A and solubilized membranes (60 μg) were added to the Protein A–antibodies complex in a final volume of 100 μl and incubated for 3 h at 4°C. The pellet was washed twice with buffer B containing 0.5% BSA and 0.4% CHAPS, and once with buffer B.

[^{125}I]ωCgTx receptor immunoprecipitation was performed as previously described [6]. For DHP receptor immunoprecipitation, rat brain membranes at a concentration of 1 mg protein/ml in buffer C (1 mM

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$CaCl_2$, 170 mM Tris, adjusted to pH 7.4 with HCl) were incubated with 2 nM [^{3}H]PN200-110, washed by centrifugation and resuspended at 2 mg protein/ml in buffer C containing 10% glycerol (v/v), and either 1% CHAPS or 1% digitonin. After 30 min, insoluble material was removed by centrifugation at 100,000 × *g* for 30 min, the supernatant was diluted 1.7-fold with buffer C and mixed for 60 min with 1 ml of WGA-Sepharose. The column was washed with 10 vols. of buffer D (25 mM HEPES, 0.15 M NaCl, 0.4% CHAPS or 0.1% digitonin, glycerol 10%, 1 mM $CaCl_2$ adjusted to pH 7.4 with Tris) and eluted with buffer D containing 200 mM *N*-acetyl-D-glucosamine. All buffers contained 0.4 mM phenylmethylsulfonyl fluoride and 2 mM benzamidine, and were maintained at 4°C. Fractions containing [^{3}H]PN200-110 receptors were identified by liquid scintillation counting and the % of bound ligand was determined by filtration through GF/B filters treated with 0.3% polyethylenimine (PEI). Peak fractions were then pooled and stored in liquid N_2 for immunoprecipitation assays.

WGA-Sepharose CL-4B fractions containing 5.5 fmol of [^{3}H]PN200-110 labeled DHP receptor in buffer C were incubated with antibodies for 1 h at 4°C; after centrifugation and washing in buffer D, immunoprecipitated radioactivity was determined by liquid scintillation counting.

2.4. Electrophoresis and Western blotting

Proteins were denatured at 100°C for 5 min in SDS-PAGE sample buffer. SDS-PAGE was performed on 12% acrylamide gels which were electroblotted to nitrocellulose membranes. For ECL detection, membranes were blocked in 10% non-fat milk powder in buffer B and incubated with antibodies overnight at 4°C. After washing in buffer B containing 0.05% Tween 20, ECL detection with anti-IgG peroxidase was carried out. For detection with [^{125}I]protein A, membranes were saturated in 5% BSA in buffer B and incubated with antibodies overnight at 4°C. After washing in buffer B containing 0.05% Tween 20, membranes were incubated for 1 h at room temperature with [^{125}I]protein A (100K cpm/0.5 ml) in buffer B containing 5% BSA and 10% milk. After washing in buffer B, 0.05% Tween 20, bands were excised and bound [^{125}I]protein A was measured in a gamma counter.

3. Results and discussion

CHAPS extracts of rat brain synaptosomes were incubated with protein A-Sepharose CL-4B beads coated with antibodies against syntaxin or VAMP (Fig. 1). Protein complexes, recovered after centrifugation and washing, were separated by SDS-PAGE and analyzed by immunoblotting. Immunoprecipitation with a monoclonal antibody that recognizes syntaxin 1a and b, resulted in co-immunoprecipitation of synaptotagmin, SNAP25 and synaptobrevin, but not synaptophysin (Fig. 1, lane 1). The same proteins were identified when immunoprecipitation was performed with anti-VAMP antibodies, however, synaptophysin was also recovered (Fig. 1, lane 2). α or β SNAP ($\alpha\beta$SNAP) were not detected in the immunoprecipitated complexes, although immunoreactivity was identified in solubilized synaptosomes (Fig. 1, lane 5).

These experiments, in agreement with previous reports [1,15,16], revealed a protein complex containing synaptotagmin, syntaxin, SNAP25 and VAMP. None of these proteins were detected when immunoprecipitation was performed with control mouse IgG (Fig. 1, lane 3). Control rabbit IgG led to the staining of a single band with slightly higher mobility than SNAP-25, corresponding to rabbit immunoglobulin light chain (Fig. 1, lane 4).

An inconsistency was, however, apparent. Immunoprecipitation with anti-syntaxin antibodies isolated a complex containing VAMP but not synaptophysin, whereas anti-VAMP antibodies revealed VAMP to be associated with both syntaxin and synaptophysin. This observation suggests that anti-VAMP antibodies recovered two distinct protein complexes: a docking

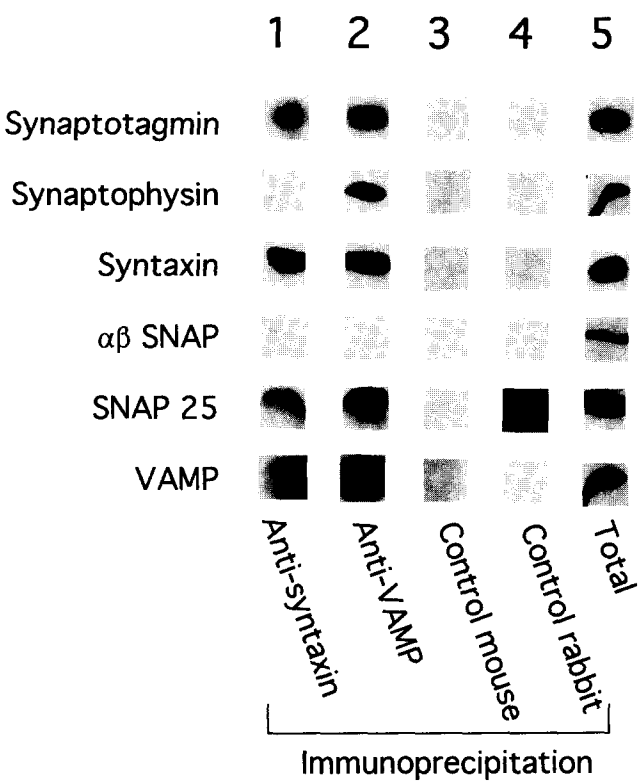

Fig. 1. Co-immunoprecipitation of synaptic proteins implicated in vesicle docking and fusion. Rat brain synaptosomes were solubilized with 1% CHAPS and high speed supernatants were subjected to immunoprecipitation with anti-syntaxin (lane 1), anti-VAMP (lane 2), and control antibodies (lanes 3,4). Immunoblots of immunoprecipitated proteins (lanes 1–4) or the initial detergent extract (lane 5) were probed with antibodies against synaptotagmin, synaptophysin, syntaxin, $\alpha\beta$SNAP, SNAP-25, and VAMP.

complex containing syntaxin but not synaptophysin, and a second complex containing synaptophysin but not syntaxin. In agreement with this interpretation, anti-synaptophysin antibodies immunoprecipitated VAMP but not syntaxin (not shown).

VAMP interacts directly with syntaxin [17] and with synaptophysin [18]. However, our data imply that syntaxin and synaptophysin are not recovered in the same complex. Consequently the interactions of these two proteins with VAMP may be mutually exclusive. Association of VAMP with syntaxin occurs at the plasma membrane, whereas complexes containing VAMP and synaptophysin are enriched in synaptic vesicle fractions [18]. These findings suggest that protein complexes containing VAMP and synaptophysin may occur in undocked synaptic vesicles, and that dissociation from synaptophysin may subsequently allow VAMP to bind to a syntaxin–SNAP25 complex, thus mediating vesicle docking at the plasma membrane.

The interactions of VAMP, synaptophysin and syntaxin with N-type calcium channels were examined in experiments illustrated in Fig. 2. N-Type calcium channels in synaptic membranes were prelabeled with a specific radioligand [^{125}I]ω-conotoxin GVIA ([^{125}I]ωGVIA) and the ability of antibody-coated protein A-Sepharose CL4B beads to immunoprecipitate CHAPS-extracted calcium channels was tested. Anti-syntaxin and anti-VAMP antibodies retrieved a similar fraction (about

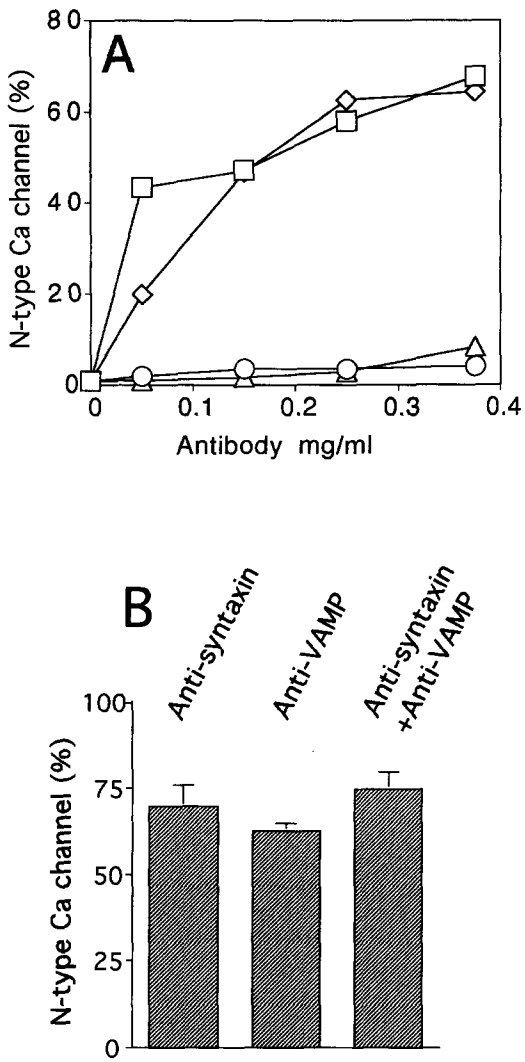

Fig. 2. Interaction of a syntaxin–VAMP complex with N-type calcium channels. (A) N-Type calcium channels in synaptosomes were prelabeled with 0.1 nM [^{125}I]ωGVIA, extracted with CHAPS, and incubated with protein A-Sepharose 4BCL beads coated with antibodies against syntaxin (□), VAMP (◇), synaptophysin (△) and control IgG (○). (B) N-Type calcium channels were immunoprecipitated with antibodies against syntaxin (300 μg/ml), VAMP (300 μg/ml) or a mixture of the two antibodies (600 μg/ml). Immunoprecipitated radioactivity is represented as a percentage of total channel-bound radioligand estimated by filtration over PEI treated GF/B filters.

70%) of N-type calcium channels at saturating concentrations, whereas anti-synaptophysin antibodies and control IgG immunoprecipitated less than 4% (Fig. 2A). The absence of reactivity with anti-synaptophysin antibodies did not result from their inability to immunoprecipitate their antigen, as these antibodies recovered complexes containing synaptophysin and VAMP (not shown).

Immunoprecipitation of an equivalent fraction of N-type calcium channels by anti-syntaxin or anti-VAMP antibodies is compatible with the interaction of a syntaxin–VAMP complex with calcium channels. Furthermore the fraction of immunoprecipitated calcium channels did not significantly increase when anti-syntaxin and anti-VAMP antibodies were added together (Fig. 2B). These results are thus consistent with the association of a vesicle docking complex containing syntaxin and VAMP, but not synaptophysin, with N-type calcium channels. No conclusions could be reached as to the association of SNAP-25 with the N-type calcium channel. Although anti-SNAP25 antibodies reacted with their cognate antigen in immunoblots, they did not immunoprecipitate SNAP25 efficiently.

Neurons express multiple classes of voltage gated calcium channels (reviewed in [19]). In order to determine whether interactions with the exocytotic complex are specific to certain types of calcium channel, the immunoprecipitation of L-type calcium channels, labeled with a 1,4-dihydropyridine antagonist [^{3}H]PN200-110, was compared with that of N-type calcium channels in parallel experiments. Immunoprecipitation of >50% of N-type channels was observed both with BINt antibodies [6] that recognize the α_{1A} and α_{1B} subunits of P/Q- and N-type calcium channels, respectively, and with anti-syntaxin, anti-VAMP, and anti-synaptotagmin antibodies. CR2 antibodies [9] recognize the α_{1C} subunit, and immunoprecipitate significant fractions of brain L-type calcium channels after solubilization in digitonin (74% of [^{3}H]PN200-110 receptors) or CHAPS (35% of [^{3}H]PN200-110 receptors). Only low levels of L-type channel immunoprecipitation (<8%) were observed with anti-syntaxin, anti-VAMP and anti-synaptotagmin antibodies, in the presence of either detergent (Fig. 3). Our results are therefore consistent with the preferential interaction of synaptic vesicle docking complexes with N- rather than L-type calcium channels.

The present data and previous reports suggest that synaptic vesicle docking is mediated by a syntaxin–SNAP25–VAMP–synaptotagmin complex [2,16,20,21], which can also include presynaptic calcium channels [5,6,22]. A recent report has indicated that syntaxin can bind directly to a cytoplasmic loop of the α_{1B} calcium channel subunit [23]. Are all syntaxin–SNAP25–VAMP–synaptotagmin complexes in synaptic terminals bound to calcium channels, or can at least two distinct populations, namely associated and unassociated with calcium channels, be detected? Density gradient centrifugation of solubilized synaptosomes (not shown) indicated that syntaxin–SNAP25–VAMP–synaptotagmin complexes could be demonstrated in fractions that did not contain N-type calcium channels, which is consistent with the second hypothesis. In order to estimate the relative abundance of the two complexes, sequential immunoprecipitation experiments were performed, and the syntaxin in each pellet was detected by probing immunoblots of immunoprecipitated complexes with anti-syntaxin antibodies. Solubilized synaptosomes were first incubated with beads coated with either control or anti-calcium channel antibodies (Fig. 4, lanes 2–4) to recover channel-associated syntaxin. After centrifugation to remove beads and bound proteins, the supernatants were incubated with anti-VAMP antibodies to trap residual VAMP–syntaxin complexes (Fig. 4, lanes 5–7). In agreement with the data presented in Fig. 3, syntaxin was found to be associated with $\alpha_{1A/B}$ but not α_{1C} calcium channel subunits. Furthermore after depletion of $\alpha_{1A/B}$ subunits and associated syntaxin–VAMP by antibody concentrations that remove at least 70% of N-type calcium channels, substantial amounts of syntaxin–VAMP complexes still remained in the supernatant (Fig. 4, lane 7). Quantification of immunoblots with [^{125}I]protein A suggested that only a small fraction (<10%) of the total syntaxin in P2 membranes was

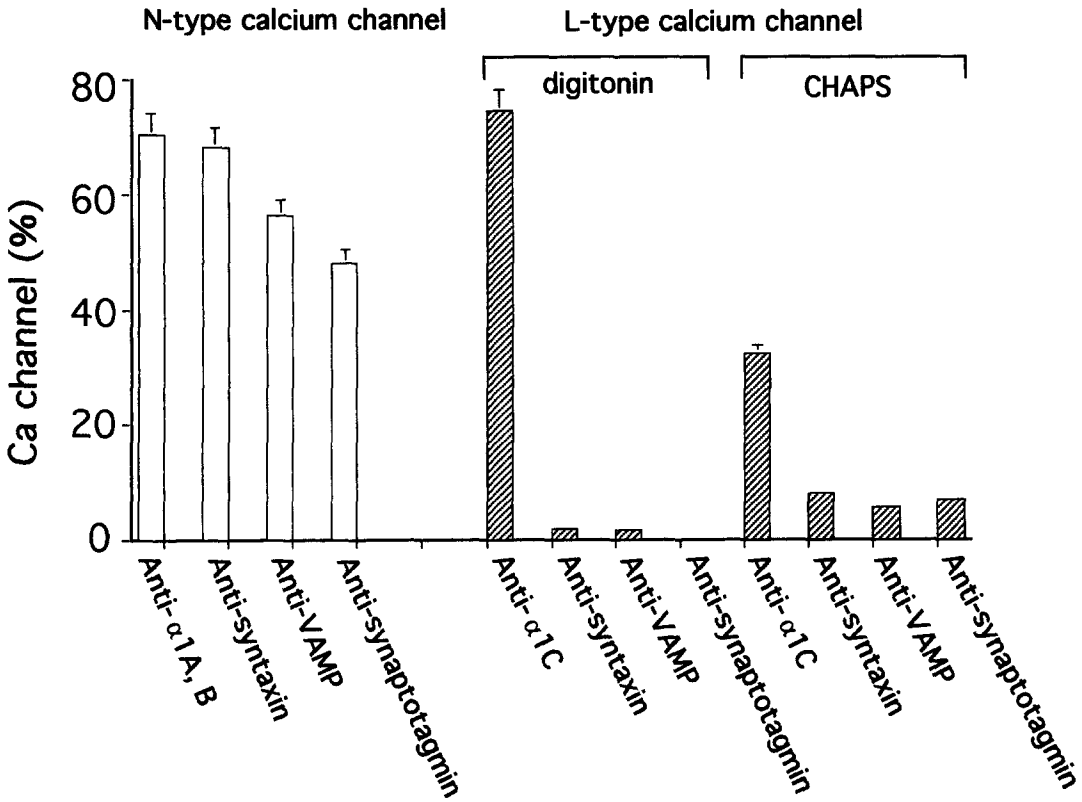

Fig. 3. Association of N- but not L-type calcium channels with synaptic proteins involved in exocytosis. N- and L-type calcium channels in rat brain synaptosomes were labeled with [^{125}I]ωGVIA and [^{3}H]PN200-110, respectively. CHAPS extracts were incubated with the indicated antibodies and the fraction of immunoprecipitated calcium channels estimated by gamma or beta counting. Experiments with L-type calcium channels were also performed after solubilization in digitonin, a detergent which stabilizes [^{3}H]PN200-110–receptor complexes more efficiently than CHAPS. Immunoprecipitated radioactivity is represented as a percentage of the total channel-bound radioligand estimated by filtration over PEI treated GF/B filters.

associated with calcium channel $\alpha_{1A/B}$ subunits, whereas about 50% of syntaxin occurred in a complex containing VAMP. In similar experiments, αβSNAP was not detected in the protein complex immunoprecipitated by antibodies against calcium channel $\alpha_{1A/B}$ subunits (not shown).

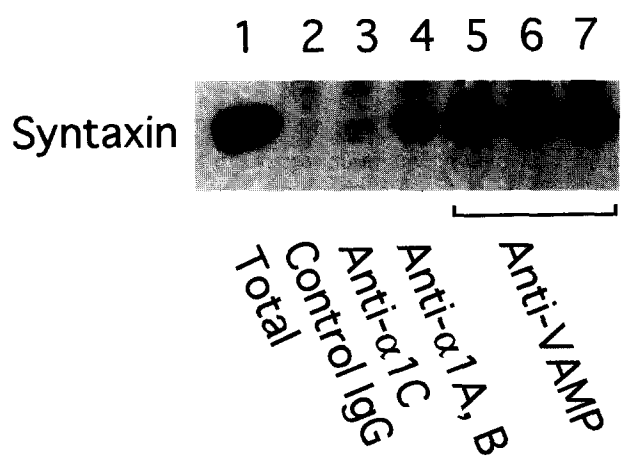

Fig. 4. Association of syntaxin with calcium channel $\alpha_{1A/B}$ subunits and VAMP. CHAPS extracts of synaptosomes (lane 1) were incubated with beads coated with control rabbit IgG (lane 2), antibodies against the calcium channel subunits α_{1C} (lane 3) or $\alpha_{1A/B}$ antibodies (lane 4). Immune complexes were recovered by centrifugation. The respective supernatants (lanes 5–7) were then incubated with beads coated with anti-VAMP antibodies. Immunoprecipitated syntaxin was detected by immunoblotting.

Multiple protein complexes appear to be involved in the docking and fusion of synaptic vesicles with the presynaptic plasma membrane. Initial docking of vesicles at the plasma membrane may involve VAMP dissociating from an intrinsic vesicle protein complex with synaptophysin, allowing VAMP to bind to syntaxin, in a process facilitated by SNAP25 and regulated by n-sec1/Munc18 [16,21,24]. Synaptotagmin may then associate with the complex, although the proposed mechanisms by which this occurs are conflicting.

αγSNAP/NSF has been shown to bind to a syntaxin–SNAP25–VAMP (SNARE) complex, and initiate dissociation upon hydrolysis of ATP by NSF [2]. Addition of αSNAP in vitro displaces synaptotagmin from its binding site on the SNARE complex [15]. The latter observation has lead to two alternative hypotheses. Either synaptotagmin initially associated with the complex is displaced by αSNAP, allowing NSF association and ATP hydrolysis which may trigger vesicle fusion, by analogy to constitutive vesicular transport [15]. Alternatively after ATP-dependent priming of the SNARE complex, synaptotagmin may displace NSF/SNAP, and constitute a fusion clamp which is subsequently removed by a calcium transient, triggering exocytosis (reviewed in [25]).

Although synaptotagmin is present in both calcium channel-associated and unassociated SNARE complexes, we have been unable to detect αβSNAP in either. Possibly association of

NSF/SNAP with SNARE complexes in synaptosomes is transitory as dissociation is immediately triggered by ATP hydrolysis, thus precluding our ability to trap a complex by immunoprecipitation. However, attempts to stabilize these complexes, by permeabilizing synaptosomes, then performing solubilization and immunoprecipitation in the constant presence of ATPγS, were also unsuccesful (not shown).

Our results are compatible with a model in which, after priming by ATP hydrolysis, the complex is stabilized by synaptotagmin before association with calcium channels. Based on quantification of syntaxin, we have estimated that more than 80% of SNARE complexes are not associated with channels. Interaction with the calcium channel may, however, be necessary for calcium sensors, such as synaptotagmin, to encounter the calcium concentrations required to trigger rapid neurotransmitter release. As N-type calcium channels are much less abundant than SNARE complexes, but at least 70% of ωGVIA receptors are associated with both syntaxin and VAMP, the availability of calcium channels is possibly the limiting factor for formation of the final exocytotic complex.

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