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SNAP-25 and synaptotagmin involvement in the final Ca^{2+}-dependent triggering of neurotransmitter exocytosis

(neuroexocytosis/synaptic vesicle fusion/Ca^{2+}-dependent neurotransmission/synaptosomal membrane/soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors)

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ABSTRACT **In neurons, depolarization induces Ca^{2+} influx leading to fusion of synaptic vesicles docked at the active zone for neurotransmitter release. While a number of proteins have now been identified and postulated to participate in the assembly and subsequent disengagement of a vesicle docking complex for fusion, the mechanism that ultimately triggers neuroexocytosis remains elusive. Using a cell-free, lysed synaptosomal membrane preparation, we show that Ca^{2+} alone is sufficient to trigger secretion of glutamate and furthermore that Ca^{2+}-signaled exocytosis is effectively blocked by antibodies and peptides to SNAP-25, a key constituent of the vesicle docking complex. In addition, Ca^{2+} inhibits the ability of synaptotagmin, a synaptic vesicle protein proposed as a calcium sensor and triggering device, to associate with this docking complex. These results support a model in which Ca^{2+}-dependent triggering of neurotransmission at central synapses acts after ATP-dependent potentiation of the docking–fusion complex for membrane fusion.**

At chemical synapses, synaptic transmission is accomplished by exocytotic release of neurotransmitters stored in synaptic vesicles that fuse with the plasma membrane in response to Ca^{2+} influx within 200 μs of activation of voltage-gated calcium channels (1). This rapid signaling suggests that a subset of synaptic vesicles, likely those clustered at the "active zone" of presynaptic terminals, are primed and held poised for Ca^{2+}-signaled exocytosis (see ref. 2).

Several lines of evidence have identified three synaptic proteins, syntaxin, VAMP, and SNAP-25, which together with synaptotagmin form the core complex postulated to coordinate regulated vesicular fusion for neurotransmitter release (see refs. 3–5). *In vitro* studies with solubilized and recombinant proteins have begun to define the molecular relationships between these proteins (6–9). In an initial 7S complex, SNAP-25 binds both syntaxin 1A and VAMP to increase the strength and specificity of their interaction (8, 9). Binding of α-SNAP to this complex displaces synaptotagmin from syntaxin and provides entry of *N*-ethylmaleimide-sensitive fusion protein (NSF) to form a 20S prefusion complex that upon ATP hydrolysis subsequently leads to its disassembly (6). These observations have led to the proposal that neurotransmission represents a specialization of general membrane trafficking in which recognition between neural-specific vesicle- and target-soluble NSF attachment protein receptors (v- and t-SNAREs) targets synaptic vesicles to the plasma membrane and the ATP hydrolysis step mediated by NSF to drive exocytosis (10).

These *in vitro* studies, however, have yet to resolve the full roles played by these synaptic vesicle and plasma membrane proteins in the cascade of events required for neurotransmitter release. For example, it remains to be determined whether SNAREs serve simply as receptors in docking or whether they participate directly together with a calcium sensor or triggering device in the final Ca^{2+}-dependent events that distinguish synaptic transmission by neurons and secretion by neuroendocrine cells (2, 11). Recent evidence suggests that VAMP and syntaxin do, in fact, function downstream of docking in synaptic transmission at giant squid axon terminals (12) and *Drosophila* neuromuscular junctions (13). One limitation to resolving these questions biochemically in mammalian central synapses has been the unavailability of synaptic membrane preparations that allow access of peptides and antibodies to probe the function of these proteins during synaptic vesicle fusion and transmitter release. Here we have used a lysed synaptosomal membrane preparation to investigate the role of SNAP-25 (14) in ATP- and Ca^{2+}-activated steps of the synaptic release of glutamate. Our results indicate that SNAP-25 performs a postdocking role for Ca^{2+}-dependent exocytosis in neurons and that Ca^{2+} affects synaptotagmin, preventing it from associating with other components of the docking complex. These findings provide further evidence that Ca^{2+} alters interactions between components of the vesicle docking complex to trigger the final step of membrane fusion for synaptic transmission.

MATERIALS AND METHODS

Antibodies and Peptides. Rabbit polyclonal sera against the following proteins were used: SNAP-25 C terminus (14), VAMP and synaptotagmin (R. H. Scheller, Stanford University); MC17, lumenal domain of synaptotagmin (ref. 15 and P. De Camilli, Yale University) and affinity-purified guinea pig IgG (M. Buchmeier, Scripps Research Institute). Monoclonal antibodies (mAbs) used were SMI81 to SNAP-25 and SMI32 to neurofilament H (Sternberger Monoclonal), choline acetyltransferase (Chemicon), SY38 to synaptophysin (Boehringer Mannheim), transferrin receptor (Zymed), and HPC-1 to syntaxin (C. Barnstable, Yale University). The sequence of peptides used were: 12-residue C-terminal SNAP-25 peptide A12G, ANQRATKMLGSG; control peptide, DCGEGETLPQRT; 20-residue C terminal SNAP-25 peptide, SNKTRIDEANQRATKMLGSG; and control scrambled peptide, TDSSGREMIKANKQLANGTR (16). Peptides were purified by high performance liquid chromatography and Sephadex G-50 (Pharmacia) chromatography.

Preparation of Synaptosomes and Lysed Synaptosomal Membranes and Glutamate Release. Intact synaptosomes (P2′) or lysed synaptosomal membranes (LP1) were prepared from mouse cerebral cortices as described by Huttner *et al.* (17) except that 5 mM 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethane-

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Abbreviations: SNAREs, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors; trfR, transferrin receptor; LP1, lysed synaptosomal membrane preparation; P2′, intact synaptosomes.

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sulfonic acid (TES), pH 7.4, was used as buffer and was assayed for glutamate release by spectrofluoremetry as described by Nicholls and Sihra (18). P2′ or LP1 pellets were resuspended at 1.5–3.4 mg/ml in 1.5 ml of freshly prepared incubation medium [3.1 mM KCl/122 mM NaCl/1.2 mM $MgSO_4$/0.4 mM KH_2PO_4/5 mM $NaHCO_3$/20 mM Na-TES/10 mM D-glucose/16 μM albumin/12.5% (wt/vol) Ficoll, pH 7.4]. The membrane suspension was equilibrated for 2 min before the addition of 1 mM NAD^+ and KCl, $CaCl_2$, ATP, adenosine 5′-*O*-(thiotriphosphate) (ATPγS), EGTA, as indicated. After 3 min at room temperature, glutamate release was determined by adding 50 units of L-glutamate dehydrogenase and measuring NADH fluorescence at 28°C using a Jasco (Easton, MD) model FP-777 spectrofluorometer with excitation at 340 nm and emission at 450 nm. The highest point on each trace was chosen as the maximum extent of glutamate, which was normalized to express glutamate released in pmol per mg of protein. The baseline value for Ca^{2+}-independent, nonspecific release (in the presence of divalent cation, 1.2 mM Mg^{2+}) was 25–50 pmol per mg of protein, which represents 5–10% of the secretion in the presence of Ca^{2+} + ATP, or Ca^{2+} alone, respectively. As indicated, LP1 samples were incubated either with antibodies (3.3 μg/ml, final concentration), peptides (2 μM, final concentration), or left untreated for 1 h at 4°C prior to the fluorometric assay.

Immunodetection Methods. Western blots were probed with mAbs and polyclonal antibodies at the following dilutions: SNAP-25 SMI81, 1:2000; syntaxin, HPC-1 1:250; anti-VAMP, 1:1000; anti-synaptotagmin, 1:20,000; anti-transferrin receptor, 1:1000. After incubation for 1 h at room temperature, blots were washed and incubated with either secondary anti-mouse or anti-rabbit antibodies conjugated to peroxidase and processed for chemiluminescence detection (Enhanced Chemiluminescence Kit, Amersham). For quantitation, blots were also probed with ^{125}I-protein A, and the radioactive signal was detected using a Molecular Dynamics PhosphorImager.

To evaluate the exposure of lumenal domain of synaptotagmin as an index of synaptic vesicle exocytosis, 5 μl of the anti-lumenal synaptotagmin polyclonal antibody MC17 was added to 1 mg of LP1 membranes (1 mg/ml in glutamate release incubation buffer) on ice and brought to 1 mM EGTA, or 1.3 mM $CaCl_2$, or left untreated. After 5 min at room temperature, the membranes were pelleted in a microcentrifuge, washed three times with 3.1 mM KCl, 122 mM NaCl, and 5 mM Na-TES, pH 7.4, to remove the excess unbound antibodies, and resuspended in 1 ml before adding Triton X-100 (1% final concentration) to solubilize membrane proteins. Protein G-Sepharose beads (25 μl, Pharmacia) were added to 400 μg of protein in 500 μl of wash buffer, and the tubes were tumbled at 4°C for 2 h. After centrifugation, the beads were washed three times with the same buffer and resuspended in 20 μl of sample loading buffer, and the proteins were fractionated on 10% SDS/PAGE, blotted, and probed with anti-p65 rabbit polyclonal sera to synaptotagmin.

For other immunoprecipitations, membrane fractions were incubated in the presence or absence of 1.3 mM $CaCl_2$/100 μM ATP/30 mM KCl, as indicated, for 30 min followed by solubilization with 1% Triton X-100. Extracts were clarified by centrifugation (13,300 × *g*), dialyzed against 10 mM Hepes, pH 7.0/100 mM KCl/1 mM $MgCl_2$/1 mM DTT/0.5% Triton X-100, clarified again by centrifugation, and made to a final concentration of 1% glycerol, 1% polyethyleneglycol 6000. Aliquots of 100 μg of protein were incubated with the SMI81 SNAP-25 mAb coupled to protein G-Sepharose beads (Pharmacia) for 3 h at 4°C with constant mixing. After washing the beads four times with 10 mM Hepes, pH 7.0/100 mM KCl/1 mM DTT, proteins were eluted and fractionated on 12% SDS/PAGE, blotted, and probed with antibodies.

RESULTS

Biochemical Characterization of Synaptosomes and Synaptosomal Membrane Preparations. To monitor the efficiency of hypotonic lysis of synaptosomes in preparing LP1 membranes, the accessibility of intracellular synaptic proteins to trypsin digestion was compared with the extracellular plasma membrane protein, transferrin receptor (trfR). As shown in Fig. 1, synaptotagmin was susceptible to trypsin cleavage in LP1, but was resistant to digestion in P2′, indicating that osmotic shock effectively ruptured the synaptosomal membrane to expose the cytoplasmic domain to extracellular protease. Similar results were obtained with the plasma membrane-associated proteins SNAP-25 and syntaxin (data not shown). In contrast to synaptic proteins, trfR was digested comparably in both P2′ and LP1 preparations, although more effectively than synaptotagmin (65 kDa) because of its greater molecular mass (95 kDa). Electron microscopic examination showed that following hypotonic lysis and centrifugation the LP1 membrane fraction was largely depleted of synaptic vesicles and composed of remnants of synaptosomal membranes, which showed changes in continuity that may reflect regions permeable to proteases, and that the remaining few vesicles situated at the plasma membrane often opposed apparent postsynaptic densities (data not shown).

Ca^{2+}-Dependent Exocytotic Release of Glutamate from LP1 Lysed Synaptosomal Membrane Preparations. As demonstrated previously (18), glutamate release from intact P2′ synaptosomal preparations of mouse cortex exhibited Ca^{2+}-dependent release superimposed on release in response to K^+-mediated depolarization (Fig. 2 *A* and *C*). In intact synaptosome preparations, K^+-dependent release in the absence of exogenous Ca^{2+} may be attributed to induction of nonexocytotic release of cytosolic glutamate via reversal of the plasma membrane transporter (19). Addition of ATP to depolarized synaptosomes had no effect on glutamate release due to impermeability of the intact membranes in this preparation.

In contrast to P2′, glutamate release from LP1 membranes was stimulated by the addition of Ca^{2+} alone (Fig. 2*B*). Consistent with a relatively high Ca^{2+} concentration required to activate a low affinity Ca^{2+}-dependent mechanism of neuroexocytosis (20), 0.32 mM and 0.65 mM $CaCl_2$ stimulated 23.7 and 49.1% of the release obtained with 1.3 mM $CaCl_2$ (data not shown). Secretion of glutamate from LP1 membranes was also independent of K^+ stimulation used to depolarize intact synaptosomes. No release of glutamate was obtained with 30 mM KCl alone (Fig. 2*D*), and the amount of release did not

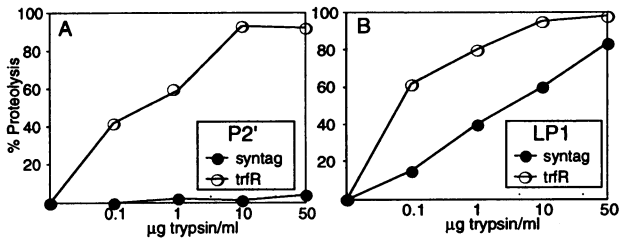

FIG. 1. Susceptibility of synaptic proteins in LP1 to digestion by trypsin. Aliquots (150μg) aliquots of protein were incubated with trypsin as indicated, and the level of proteolysis was compared relative to Triton X-100-solubilized proteins ±digestion with 50 μg/ml trypsin (not shown). After 30 min of digestion on ice, proteins were precipitated with 10% trichloroacetic acid, and analyzed by SDS/PAGE and Western blotting with antibodies to synaptotagmin and trfR. The mean percentages of synaptotagmin and trfR proteolysed by increasing amounts of trypsin in P2′ (*A*) and LP1 (*B*) preparations were determined from duplicate blots with less than 10% variability. Quantitiation using ^{125}I-labeled protein A confirmed that 50% of synaptotagmin was degraded in LP1 at 0.1–1 μg/ml trypsin, whereas 90% of synaptotagmin in P2′ was resistant to 50 μg/ml trypsin.

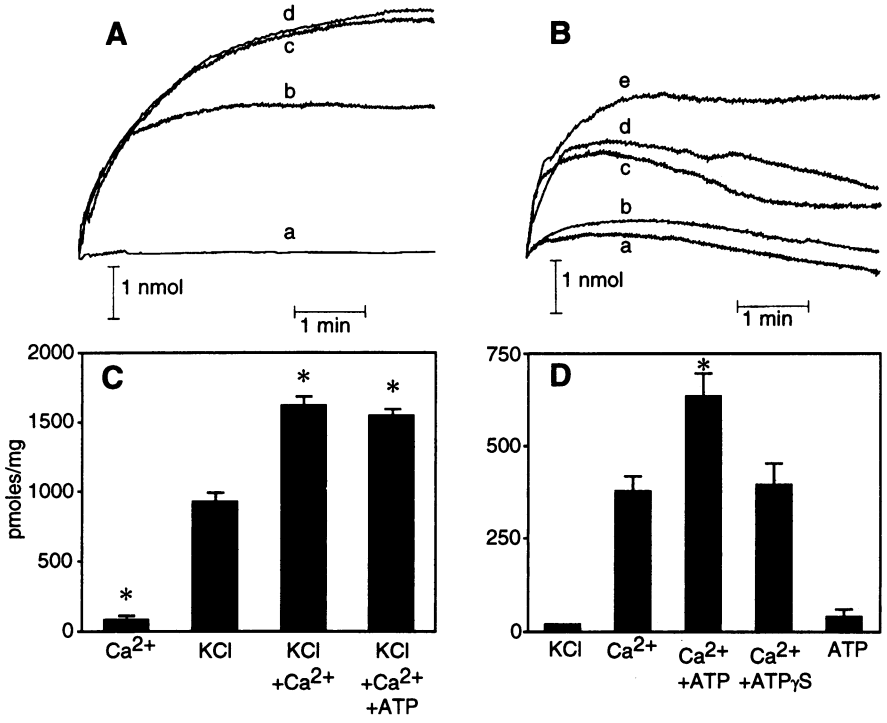

FIG. 2. Ca^{2+}- and ATP-dependent release of glutamate from washed P2′ and LP1. *A* and *B* represent tracings from representative experiments of glutamate release evoked from P2′ and LP1 preparations, respectively. (*A*) Traces: a, control (no KCl, no $CaCl_2$); b, 30 mM KCl; c, 30 mM KCl, 1.3 mM $CaCl_2$, 100 μM ATP; d, 30 mM KCl and 1.3 mM $CaCl_2$. (*B*) Traces: a, 1 mM EGTA; b, control (no $CaCl_2$, no ATP); c, 1.3 mM $CaCl_2$; d, 1.3 mM $CaCl_2$, 100 μM ATPγS; e, 1.3 mM $CaCl_2$, 100 μM ATP. The declining signal in the spectrofluoremetric trace in *B* is likely due to slow re-oxidation of reduced NADH by enzymes released from damaged mitochondria (18) present in LP1 preparation. *C* and *D* show average results obtained from P2′ and LP1 membrane preparations, respectively, expressed as pmol of glutamate released per mg of protein ± SD (at least three different experiments, except for KCl alone in *D*, which is the mean of two determinations). Asterisks indicate values significantly different ($P < 0.05$, Student's *t* test) from KCl treatment alone (*C*), and between Ca^{2+} + ATP, and Ca^{2+} alone, and Ca^{2+} + ATP (*D*).

differ between samples treated with Ca^{2+} in the presence (340 pmol/mg of protein) or absence (353 pmol/mg of protein) of KCl. Together with the evidence from proteolysis and electron microscopic analysis, this indicates that intact synaptosomes contribute little, if any, to glutamate release from the LP1 membrane preparation.

In agreement with divalent cation selectivity for synaptic transmission (see ref. 21), Sr^{2+} and Ba^{2+}, but not Mg^{2+}, stimulated glutamate release but were comparably less efficient than Ca^{2+} (Fig. 3*A*). Ca^{2+}-stimulated release of glutamate was also dependent on maintaining physiological pH, again consistent with the release reflecting bona fide neuroexocytosis and not nonspecific leakage from synaptic vesicles. Decreasing the pH to 6.0, which counteracts the electrochemical gradient required for transport of glutamate into the vesicle and results in leakage through the transporter (22), caused extensive Ca^{2+}-independent efflux of glutamate from vesicular stores that was only minimally affected by Ca^{2+} (Fig. 3*B*). This indicates that while glutamate leakage occurs from purified synaptic vesicles in the absence of an ATP regenerating system (23), such leakage does not appear to contribute significantly to glutamate secretion from those vesicles that fractionate with the LP1 synaptic membrane.

To evaluate further whether Ca^{2+} treatment promotes fusion of synaptic vesicles with the plasma membrane, we determined if this led to exposure of the lumenal domain of synaptotagmin (24, 25). As shown in Fig. 3*C*, Ca^{2+} treatment of LP1 membranes increased the amount of synaptotagmin that was immunoprecipited with a lumenal-specific antibody. Quantitative measurement using ^{125}I-protein A demonstrated a nearly 4-fold increase of immunoreactive synaptotagmin compared with untreated controls. In contrast, addition of EGTA only resulted in about 75% more immunoprecipitable synaptotagmin than untreated preparations, which may represent disruption of vesicular components vulnerable to chelation of protein-bound Ca^{2+}. Comparing the amount of total

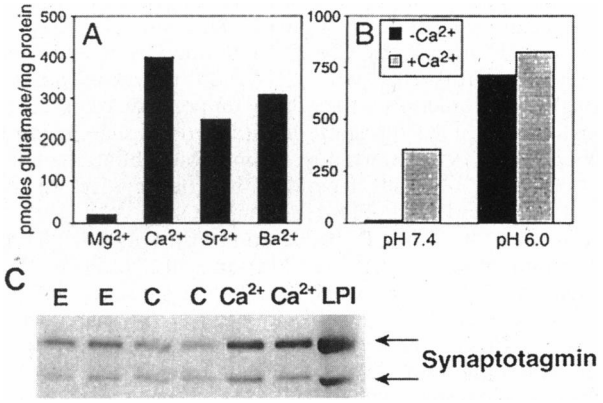

FIG. 3. Effect of divalent cations and pH on glutamate release and Ca^{2+}-dependent exposure of the lumenal domain of synaptic vesicle protein synaptotagmin. *A* demonstrates divalent cation selectivity for glutamate release from LP1 membranes performed as described in Fig. 2 but in the presence of 1 mM concentrations of $MgCl_2$, $CaCl_2$, $SrCl_2$, or $BaCl_2$. *B* compares glutamate release from LP1 with the incubation buffer set at pH 7.4 and 6.0. (*C*) A Western blot of synaptotagmin immunoprecipitations using a lumenal domain specific antibody on duplicate LP1 samples with either no addition (C); 1 mM EGTA (E), or 1.3 mM $CaCl_2$ (Ca^{2+}). The lane marked LP1 indicates the position of synaptotagmin and represents one-third (133 μg of protein) of the Triton X-100-soluble LP1 protein used for immunoprecipitations. The two bands, p65 and p45, correspond to intact and a proteolytic cleavage fragments of synaptotagmin obtained during tissue homogenization/cell fractionation (47).

and immunoprecipitable synaptotagmin showed that Ca^{2+} treatment resulted in exposure of lumenal epitopes of approximately 12% of the synaptotagmin in the LP1 preparation (not shown). This suggests that only a relatively small proportion of synaptotagmin associated with synaptic vesicles or other vesicular compartments is externalized by Ca^{2+}-stimulated membrane fusion and exocytosis. These results, demonstrating cation selectivity and pH requirement for Ca^{2+}-dependent release, and the Ca^{2+}-dependent increased exposure of the lumenal domain of synaptotagmin strongly support the authenticity of the secretory event monitored in this preparation.

Addition of ATP resulted in a 2-fold increase of Ca^{2+}-dependent release from LP1 membranes, whereas ATPγS had no effect (Fig. 2 *B* and *D*). In the absence of Ca^{2+}, however, ATP alone did not stimulate release of glutamate (Fig. 2*D*). Thus, while ATP hydrolysis potentiated glutamate release, it was not sufficient, which suggests that an ATP hydrolysis-driven mechanism occurs either before or concurrent with a Ca^{2+}-dependent trigger for neuroexocytosis. Moreover, because the glutamate content of the LP1 fraction (2.1 nmol/mg of protein) was less than P2′ (10.4 nmol/mg), the relative amount of glutamate released from LP1 membranes in the presence of Ca^{2+} and ATP was 4- to 5-fold greater than the Ca^{2+}-dependent release from P2′ synaptosomes, indicating that the LP1 preparation is enriched for synaptic vesicles competent for release.

SNAP-25 Antibody and Peptide Block of Neuroexocytosis. With the accessibility of the LP1 preparation to antibodies and peptides, we examined the involvement of SNAP-25 in glutamate release. The addition of affinity-purified polyclonal antibody directed against the carboxyl terminus of SNAP-25 (14) caused a 60–70% decrease in Ca^{2+}-dependent glutamate release, whereas a comparable concentration of control IgG had no effect (Table 1). Furthermore, the SNAP-25 mAb SMI81, whose epitope lies amino-terminal of the peptide-directed sera (P.P.M. and M.C.W., unpublished observations), also inhibited release by 77–82%, while a control mAb to neurofilament protein had little effect. Moreover, a mAb to synaptophysin (SY 38) also had no effect, either at the same (3.3 μg/ml) or twice the concentration (6.6 μg/ml), resulting in 101 and 99% of the release of untreated controls (average of duplicate samples, not shown). This suggests that the inhibition of release by SNAP-25 antibodies results from specifically interfering with SNAP-25 function, possibly through its interaction with other components required for exocytosis and not from steric hindrance of vesicle fusion by antibody networks. Importantly, comparable inhibition of both Ca^{2+} and Ca^{2+} + ATP-dependent neuroexocytosis was obtained with these antibodies (see Table 1).

Preincubation of the LP1 preparation with a peptide (A12G) corresponding to the carboxyl 12 residues of SNAP-25, which includes the BoNT/A cleavage site (26, 27), similarly inhibited both Ca^{2+} − and Ca^{2+} + ATP-dependent glutamate release (Table 1). Consistent with a dose response for the peptide block, less inhibition was obtained at half-concentration of peptide (1 μM), resulting in 38 and 54% release of controls in the presence of Ca^{2+} and Ca^{2+} + ATP, respectively (data not shown). Similar results were also obtained using a larger peptide to the carboxyl-terminal 20 residues of SNAP-25 (16), which blocked release by 47% at 2 μM and by 87% at 20 μM, in contrast to a scrambled peptide that resulted in no inhibition with 97 (2 μM) and 96% (20 μM) of release of Ca^2-stimulated controls (duplicate samples, data not shown). These findings suggest that in addition to its role in strengthening interactions between syntaxin and VAMP for synaptic vesicle docking at the target membrane (8) and for recruiting α-SNAP and NSF (10), SNAP-25 is also required for Ca^{2+}-triggered fusion between the opposing membrane bilayers at central synapses.

Synaptotagmin Is Excluded from the Core Complex in Presence of Ca^{2+}. To examine whether Ca^{2+} affects interactions between these proteins, the 7S core complex composed of synaptotagmin, VAMP, syntaxin, and SNAP-25 in solubilized extracts (6, 28–30) was immunoprecipitated with the SNAP-25 mAb. As shown in Fig. 4, while there was no apparent dissociation of VAMP or syntaxin, addition of Ca^{2+} to LP1 membranes and to K^+-depolarized P2′ synaptosomes resulted in significant (80–90%) loss of synaptotagmin from SNAP-25 immunoprecipitable complexes. Moreover, K^+-depolarization of P2′ synaptosomes in the absence of exogenous Ca^{2+}, which elicits Ca^{2+}-independent release of cytosolic glutamate (ref. 19 and see Fig. 2), did not lead to a detectable loss of synaptotagmin in SNAP-25 immunoprecipitable complexes (data not shown). Importantly, addition of Ca^{2+} to LP1 membranes after Triton X-100 solubilization (Fig. 4*c*) also did not affect the co-precipitation of synaptotagmin, consistent with the requirement of membrane or phospholipid association for Ca^{2+} binding by synaptotagmin (31).

Table 1. Effect of antibodies to SNAP-25 and peptides of SNAP-25 on Ca^{2+}-, and Ca^{2+} + ATP-dependent release of glutamate from LP1 membranes.

	Ca^{2+}-dependent		Ca^{2+} + ATP-dependent	
	pmol/mg protein	% control	pmol/mg protein	% control
Control (no additions)	376 ± 41	100 ± 10	633 ± 60	100 ± 9
SNAP-25 Ab 7293	120 ± 30*	32 ± 8	262 ± 43*	41 ± 9
Control IgG	383 ± 45	101 ± 10	640 ± 96	101 ± 15
Mab SM181	63 ± 11*	17 ± 3	115 ± 31*	18 ± 5
Control mAb	335 ± 45	89 ± 9	550 ± 62	87 ± 10
SNAP-25 (A12G)	92 ± 23*	24 ± 6	240 ± 69*	38 ± 11
Control peptide	373 ± 68	99 ± 18	576 ± 72	91 ± 11

Synaptosomal LP1 membranes incubated with either antibodies (3.3 μg/ml), peptides (2 μM), or left untreated for 1 h at 4°C prior to stimulation to evoke glutamate release with either 1.3 mM $CaCl_2$ or 1.3 mM $CaCl_2$ and 100 μM ATP. Each value is the mean ± S.D. of at least three independent determinations. Similar results were obtained using half the concentration of SNAP-25 antibodies (1.65 μg/ml) with the polyclonal and monoclonal SNAP-25 antibodies each suppressing Ca^{2+}-dependent release by 55 and 49%, respectively, and Ca^{2+} + ATP evoked release by 65 and 73% (not shown).

*One-way analysis of variance followed by a Scheffe's *post hoc* test indicates significant difference ($P = 0.0001$) from controls with no additions and those treated with control reagents for both Ca^{2+}-dependent [$F(6, 23) = 46.654$], and Ca^{2+} + ATP activated [$F(6, 23) = 39.154$] release.

DISCUSSION

Previous studies using permeabilized and semi-intact cell preparations have identified ATP- and Ca^{2+}-dependent activities required to prime the secretory apparatus and signal norepinephrine release from secretory granules in PC12 and adrenal chromaffin cells (32, 33). Our evidence using synaptosomal LP1 membranes supports a role for SNAP-25 in the final Ca^{2+}-triggered event played downstream of vesicle docking and ATP-dependent steps that mediate fast synaptic transmission by neurons. The ability to distinguish this late function of SNAP-25 in exocytosis and changes in synaptotagmin binding to the docking complex, likely results from retaining membrane association, and thereby the structural configuration of the docking complex between opposing vesicle and plasma membranes.

Because addition of Ca^{2+} alone is sufficient to evoke glutamate release from LP1 membranes, it is reasonable that this reflects the final signaling mechanism in synaptic neurotransmitter release. In fact, Ca^{2+}-stimulated release from LP1 membranes consistently amounted to 15–17% of the available glutamate, which is comparable with the 12% increased exposure of the lumenal domain of synaptotagmin measured

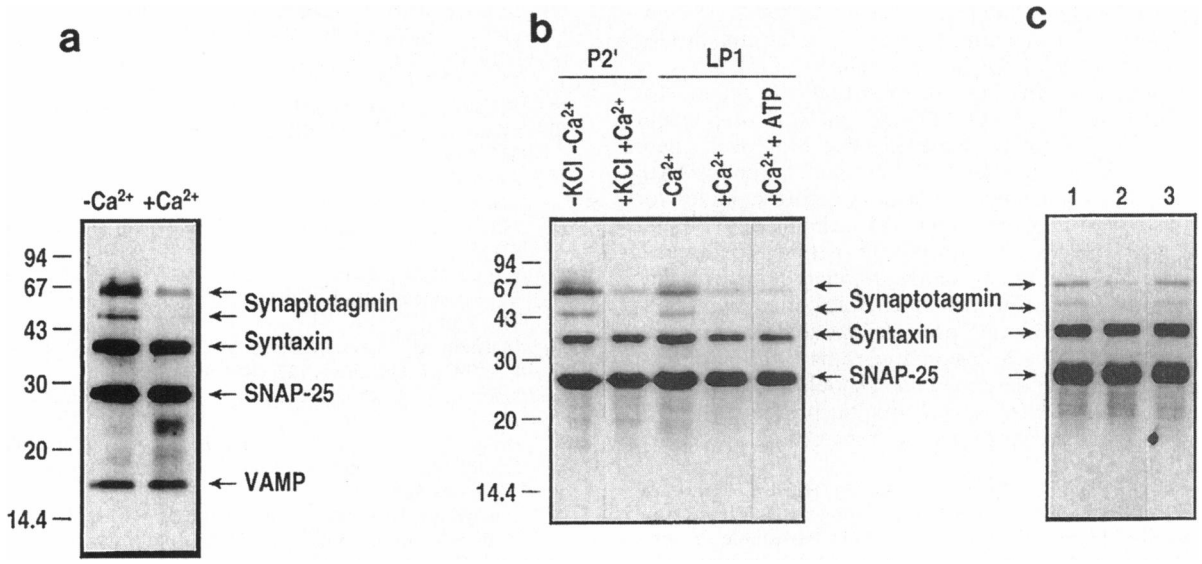

FIG. 4. Effect of Ca^{2+} on synaptotagmin binding to the docking–fusion complex. Triton X-100 extracts of P2′ and LP1 were immunoprecipitated with SNAP-25 mAb SMI81 and probed with antibodies to synaptotagmin, syntaxin, SNAP-25, and VAMP. *a* shows the effect of 1.3 mM $CaCl_2$ on LP1 membrane proteins. *b* compares proteins immunoprecipited after incubation of P2′ ± 1.3 mM $CaCl_2$/30 mM KCl and LP1 ± 1.3 mM $CaCl_2$ and 100 μM ATP. *c* compares SNAP-25 immunoprecipitable proteins obtained from LP1 membranes treated with no $CaCl_2$, lane 1; 1.3 mM $CaCl_2$ before solubilization with Triton X-100, lane 2; and $CaCl_2$ in the presence of Triton X-100, lane 3. Blots in *b* and *c* were not probed for VAMP. In *a* the smudge under SNAP-25 in the $+Ca^{2+}$ lane is nonspecific background not seen in other blots (e.g., *b* and *c*) and does not reflect a product of proteolysis.

under similar conditions. This limited extent of exocytosis could reflect a low probability of release ($p_r < 0.1$) observed for central synapses (34, 35). Moreover, the increased release promoted by the addition of ATP suggests that an additional population of docked vesicles are equipped with an ATP-dependent activity, which may reflect priming, that enables them to fuse in response to the final Ca^{2+}-dependent signal. This preparation, however, is unlikely to accommodate further steps in vesicular trafficking that may compromise assays of permeabilized synaptosomes that contain both an extensive reserve pool as well as scant number of releasable synaptic vesicles (36).

These studies demonstrate, as recently shown for VAMP and syntaxin in invertebrate synapses (12, 13), that SNAP-25 has a role beyond its participation in docking of synaptic vesicles to the plasma membrane in neurons and that these SNAREs have definite roles in the final steps of neurotransmission. Studies using botulinum neurotoxins that block neurotransmitter release (26, 37) have provided direct evidence for the function of these proteins in exocytosis, but could not distinguish precisely the function played by SNAP-25 or other SNAREs in the release process. *In vitro* binding studies have shown that SNAP-25 may help direct docking of synaptic vesicles to the active zone by selectively strengthening interactions between neural-specific isoforms of syntaxin and VAMP (8). However, because both peptide-directed antisera and mAb, which recognize distinct epitopes, as well as peptides corresponding to the carboxyl terminus, inhibit glutamate release from docked vesicles in LP1 membranes, SNAP-25 must also function downstream of docking. Since comparable inhibition was obtained for release with Ca^{2+} alone or potentiated by ATP, this additional role of SNAP-25 in exocytosis must also be played after an ATP-mediated step, which may represent priming of docked vesicles.

The participation of SNAP-25 at a step after docking is consistent with the observation that inhibition of neurotransmission by BoNT/A or tetanus toxin does not deplete the density of synaptic vesicles docked to the plasma membrane (38), as would be expected if the block were solely at vesicle recruitment and docking. Current evidence suggests in fact that SNAP-25 complexed with other SNAREs is not susceptible to BoNT/A and that toxin cleavage at the carboxyl terminus of "free" SNAP-25 does not prevent assembly but that it does reduce the stability of the fusion complex and its ability to promote membrane fusion (39). Because both antibodies and peptides to the carboxyl terminus effectively block glutamate release, this region of the SNAP-25 polypeptide must be exposed for protein interactions, even when engaged in the core complex, that are likely to reflect conformational transitions in the final step of Ca^{2+}-triggered vesicle fusion. For example, this region of SNAP-25 is required for, although not sufficient for, binding to the v-SNARE VAMP (9, 40). However, *in vitro* binding assays indicate that the carboxyl-terminal SNAP-25 peptides are insufficient to block VAMP binding to SNAP-25 (M.C.W. and P.P.M., unpublished observations), suggesting that this sequence could also function by interacting with additional constituents of the fusion machinery. Recently, Gutiérrez *et al.* (16) have shown that both the SNAP-25 mAb and the 20-mer carboxyl-terminal peptide inhibit Ca^{2+}-dependent release of noradrenaline in permeabilized chromaffin cells. While these studies could not pinpoint the stage where this block occurred in neuroendocrine cells, taken together with the evidence presented here, it appears likely that SNAP-25 is involved in the same final Ca^{2+}-dependent step common to exocytosis from both synaptic vesicles and secretory granules.

Ca^{2+} binding causes a conformational change in the structure of synaptotagmin, the constituent of the core complex postulated to act as a triggering device (41). Our results show that under conditions of Ca^{2+}-dependent exocytotic release, synaptotagmin is unable to bind effectively with other constituents of the complex. Interestingly, Ca^{2+} suppresses the *in vitro* interaction of syntaxin with the second C2 (PKC-B) domain of synaptotagmin, which contrasts with the Ca^{2+}-dependent binding of syntaxin to the first PKC-A domain (42). One possiblity, therefore, is that the Ca^{2+}-dependent dissociation of synaptotagmin from SNAP-25 immunoprecipitated complexes is regulated through this PKC-B domain. This could represent a role of synaptotagmin in Ca^{2+}-activated events required either for triggering fusion as a positive activator or

as a negative acting "clamp" in Ca^{+2}-regulated vesicular release (43, 44), or for initiating disassembly of the complex for recycling components of the fusion machinery (45).

Taken together, our findings are consistent with a model for the sequential action of ATP-driven and Ca^{2+}-dependent events in synaptic vesicle release (5, 46). Moreover, these results demonstrate that SNAP-25, like syntaxin and VAMP (12, 13), functions not only to assemble a core complex for docking of vesicles and recruiting NSF priming activity, but also acts after these steps, possibly by helping to direct a Ca^{2+}-evoked conformational change in synaptotagmin to initiate membrane fusion. Further biochemical analysis of cell-free synaptosomal membrane preparations that preserve the integrity of synaptic vesicle fusion apparatus with the plasma membrane, such as that described here, should prove useful in resolving precisely how these presynaptic proteins serve to regulate these processes for synaptic transmission.

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