

Mutations in the Effector Binding Loops in the C2A and C2B Domains of Synaptotagmin I Disrupt Exocytosis in a Nonadditive Manner*

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Ping Wang[‡], Chih-Tien Wang, Jihong Bai[‡], Meyer B. Jackson, and Edwin R. Chapman[§]

From the Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

The secretory vesicle protein synaptotagmin I (syt) plays a critical role in Ca^{2+} -triggered exocytosis. Its cytoplasmic domain is composed of tandem C2 domains, C2A and C2B; each C2 domain binds Ca^{2+} . Upon binding Ca^{2+} , positively charged residues within the Ca^{2+} -binding loops are thought to interact with negatively charged phospholipids in the target membrane to mediate docking of the cytoplasmic domain of syt onto lipid bilayers. The C2 domains of syt also interact with syntaxin and SNAP-25, two components of a conserved membrane fusion complex. Here, we have neutralized single positively charged residues at the membrane-binding interface of C2A (R233Q) and C2B (K366Q). Either of these mutations shifted the Ca^{2+} requirements for syt-liposome interactions from ~ 20 to $\sim 40 \mu\text{M}$ Ca^{2+} . Kinetic analysis revealed that the reduction in Ca^{2+} -sensing activity was associated with a decrease in affinity for membranes. These mutations did not affect syt-syntaxin interactions but resulted in a $\sim 50\%$ loss in SNAP-25 binding activity, suggesting that these residues lie at an interface between membranes and SNAP-25. Expression of full-length versions of syt that harbored these mutations reduced the rate of exocytosis in PC12 cells. In both biochemical and functional assays, effects of the R233Q and K366Q mutations were not additive, indicating that mutations in one domain affect the activity of the adjacent domain. These findings indicate that the tandem C2 domains of syt cooperate with one another to trigger release via loop-mediated electrostatic interactions with effector molecules.

Synaptotagmins are a family of proteins thought to function in the fusion and recycling of synaptic vesicles, large dense core granules, lysosomes, and potentially other trafficking organelles (1–3). Members of this gene family are anchored to the membranes of secretory organelles via an N-terminal trans-membrane domain and have a large cytoplasmic domain composed of two conserved motifs called C2 domains that, in many isoforms of synaptotagmin, form Ca^{2+} -sensing modules. The membrane-proximal C2 domain is C2A and the C-terminal C2

domain is C2B. The most abundant isoform in brain is synaptotagmin I (syt)¹, where the Ca^{2+} -sensing ability of each of its C2 domains appears to be critical for docked synaptic vesicles to fuse in response to stimulation (4, 5, 40) (but see also Refs. 7 and 8).

The key to the function of syt lies in understanding how Ca^{2+} triggers the activation of its tandem C2 domains. Insights into how metals activate C2 domains began with structural studies of the C2 domain of phospholipase C δ -1, where it was shown that metal binding opened two Ca^{2+} binding loops or “jaws,” resulting in significant changes in the electrostatic potential around the jaws (9). It was subsequently demonstrated that Ca^{2+} triggers the partial penetration of these jaws/loops into lipid bilayers (10–13). Whereas hydrophobic interactions between some side chains and the interior of the bilayer occur, the overall interaction of the C2 domains of syt with anionic lipid surfaces is highly sensitive to increases in ionic strength (10), consistent with an electrostatic mechanism for docking onto membranes. These loops dip into membranes at Ca^{2+} levels that trigger secretion and with kinetics that are rapid enough to mediate excitation-secretion coupling (13). These findings prompted a model in which the C2 domains of syt interact with lipids in the plasma membrane to help pull the bilayers together to accelerate Ca^{2+} -triggered membrane fusion (3).

Recent evidence suggests that syt may also regulate membrane fusion through interactions with components of the SNARE complex. This complex is composed of a vesicle SNARE (v-SNARE; synaptobrevin/VAMP) and two target membrane SNAREs (t-SNAREs; syntaxin and SNAP-25 (15)) that assemble into a parallel four-helix bundle (16). Zippering together of SNARE proteins is thought to pull the lipid bilayers together to mediate membrane fusion (17). Syt binds directly to syntaxin and SNAP-25 in a Ca^{2+} -promoted manner (18–21). *In vitro*, syt facilitates the assembly of SNARE complexes (5) and enhances the rate of SNARE catalyzed membrane fusion (22), but these effects were not modulated by Ca^{2+} , suggesting that additional factors are required to impart Ca^{2+} control to SNARE-catalyzed membrane fusion *in vivo*. In PC12 cells, mutations in SNAP-25 that selectively disrupt interactions with syt reduce secretion (23), and selective inhibitors of syt-t-SNARE interactions acutely block exocytosis (24), further supporting a functional role for syt-t-SNARE interactions during release. NMR studies suggested that syt binds syntaxin in the same manner that it engages lipid bilayers (25, 26). However, these studies

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[§] A Pew Scholar in the Biomedical Sciences. To whom correspondence should be addressed: Dept. of Physiology, University of Wisconsin, 1300 University Ave., SMI 129, Madison, WI 53706. Tel.: 608-263-1762; Fax: 608-265-5512; E-mail: chapman@physiology.wisc.edu.

¹ The abbreviations used are: syt, synaptotagmin I; PS, 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; dansyl-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl); FRET, fluorescence resonance energy transfer; WT, wild type; GST, glutathione *S*-transferase; AED-ANS, 5-[2-(acetamido)ethyl]amino]naphthalene-1-sulfonic acid.

were largely based on a fragment of syntaxin that is completely dispensable for binding (20, 27). Thus, little is known concerning the interfaces that mediate assembly of syt with t-SNAREs (3).

Here, we report experiments aimed at testing a current model in which Ca^{2+} activates the C2 domains of syt by flipping an electrostatic switch. In this model, the change in electrostatic potential around the Ca^{2+} -binding loops of syt triggers interactions with effector molecules to drive secretion. To test this and to selectively tune the membrane binding affinity of syt, we neutralized positively charged residues in the membrane penetration loops of each C2 domain. R233Q in C2A and K366Q in C2B shifted the Ca^{2+} dependence for binding to membranes to higher $[\text{Ca}^{2+}]$ and reduced the affinity of syt for membranes. When expressed in PC12 cells, these mutant forms of syt reduced the rate of secretion. Unexpectedly, in each of the assays described in this study, the effects of the R233Q and K366Q mutations were not additive, indicating that mutations in one C2 domain can affect the properties of the adjacent C2 domain. Surprisingly, both of the charge neutralization mutations also selectively reduced SNAP-25 binding activity. These data suggest that residues 233 and 366, in the tips of the membrane penetration loops, lie at a junction between Ca^{2+} , membranes, and components of the SNARE complex. Perturbation of the electrostatic potential at the distal tips of these loops disrupts exocytosis, supporting the electrostatic switch model for activation of syt.

EXPERIMENTAL PROCEDURES

Recombinant Proteins—cDNA encoding rat syt I (28) (the Asp³⁷⁴ mutation in this clone was repaired with a Gly³⁷⁴ substitution as described (29, 30)) was kindly provided by T. C. Südhof (Dallas, TX), cDNAs encoding syntaxin 1A (18) and SNAP-25 (31) were kindly provided by R. Scheller (Stanford, CA) and M. Wilson (Albuquerque, NM).

Full-length syntaxin and SNAP-25 (24), the C2A domain of syt (residues 96–265), and the cytoplasmic domain of syt (C2A-C2B; residues 96–421) were subcloned into pGEX-2T (Amersham Biosciences), confirmed by DNA sequencing, and expressed and purified as described (32) with modifications. To remove tightly bound contaminants from the syt fusion proteins (33), bead-immobilized proteins were treated with RNase/DNase (10 $\mu\text{g}/\text{ml}$ in 50 mM HEPES, pH 7.4, 1 M NaCl, and 1 mM MgCl_2) for 10 min at room temperature. Samples were then washed three times in HEPES buffer (50 mM HEPES-NaOH, pH 7.4, 0.1 M NaCl), and in some cases the syt fragments were cleaved from the GST moiety using thrombin as described (32). Removal of the contaminant was confirmed by a complete lack of an absorbance at A_{260} .

Arginine 233, in Ca^{2+} /membrane binding loop-3 in the C2A domain of syt (10, 11), was neutralized by substitution with glutamine, in the context of the isolated domain or in C2A-C2B. Lysine 366, which lies in Ca^{2+} /membrane binding loop-3 of C2B (13), was also neutralized by substitution with glutamine, in the context of C2A-C2B and C2A(R233Q)-C2B.

Lipids—Synthetic 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (PS), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) (dansyl-PE), and brain phosphatidylinositol-4,5-bisphosphate were obtained from Avanti Polar Lipids. Lipids were dried under a stream of nitrogen and suspended in HEPES buffer. For fluorescence studies, large (~100-nm) unilamellar liposomes were prepared by extrusion as described previously (11).

1-3-Phosphatidyl[*N*-methyl-³H]choline-1,2-dipalmitoyl ([³H]PC) was purchased from Amersham Biosciences. Liposomes were prepared by sonication using a Microson ultrasonic cell disruptor (Misonics), and ³H-labeled liposome binding assays were carried out as described previously (11) in 100 μl of HEPES buffer using 6 μg of immobilized protein and 22 nm liposomes per data point. In all experiments, error bars represent the S.D. from triplicate determinations.

Stopped-flow Rapid Mixing Experiments—Kinetics experiments were carried out using an Applied Photophysics SX.18MV stopped-flow spectrometer at 25 °C as described (11). Binding was monitored via fluorescence resonance energy transfer (FRET). The Trp residues in C2A (5 μM) or C2A-C2B (2 μM) were excited at 285 nm, and the emission

from the dansyl-PE acceptor (5% dansyl-PE, 25% PS, 70% PC) was collected using a 523-nm band pass filter. Proteins were loaded into one syringe of the stopped flow, and Ca^{2+} /EGTA plus liposomes were loaded into the other syringe. After mixing (dead time ~1.2 ms), a rapid increase in fluorescence was observed in samples containing Ca^{2+} . The on (k_{on}) and off rates (k_{off}) of syt-liposome complexes were calculated, assuming pseudo-first-order kinetics, according to the following equation.

$$k_{\text{obs}} = [\text{liposome}]k_{\text{on}} + k_{\text{off}} \quad (\text{Eq. 1})$$

Co-sedimentation Assays—Large (~100 nm) unilamellar liposomes were prepared by extrusion as described (11). 4 μM syt fragments were incubated with 22 nm liposomes composed of 25% PS/75% PC (2 mM total lipids) in 150 μl of HEPES buffer for 15 min at room temperature, in the presence of 1 mM Ca^{2+} or 2 mM EGTA. Samples were then centrifuged at $150,000 \times g$ for 40 min in a Beckman Optima MAX-E table top ultracentrifuge, and the supernatants and pellets were separated. Pellets were washed once with 150 μl of HEPES buffer and collected again via centrifugation. Equal fractions of the supernatants and pellets were subjected to SDS-PAGE, and proteins were stained with Coomassie Blue.

Measuring syt-t-SNARE Interactions—Binding of wild type and mutant C2A-C2B to bead-immobilized full-length GST-syntaxin and GST-SNAP-25 was monitored as described (24) but as a function of $[\text{Ca}^{2+}]_{\text{free}}$. Briefly, 15 μg of immobilized t-SNARE was incubated with 1.5 μM syt in 150 μl of HEPES-buffered saline for 2 h at 4 °C. Beads were washed three times in binding buffer, bound protein was solubilized by boiling in SDS sample buffer, and samples were separated by SDS-PAGE. Bound syt was detected using an anti-syt I monoclonal antibody (41.1) and Pierce SuperSignal enhanced chemiluminescence (ECL) reagents in the linear range.

Binding of syt to SNAP-25 was also monitored in solution using FRET. For these experiments, the native Trp residues in C2A-C2B were excited at 285 nm and served as the energy donors. The Cys residues of SNAP-25 were labeled with 1,5-IAEDANS (5-[[2-[(iodoacetyl)amino]ethylamino]naphthalene-1-sulfonic acid] (labeling was carried out as described for syt in Ref. 13; the stoichiometry for AEDANS to SNAP-25 was ~3.6:1) and served as energy acceptors (34). Fluorescence measurements were made at 24 °C using a PTI QM-1 fluorometer and Felix software. C2A-C2B (2 μM) and AEDANS-SNAP-25 (0.5 μM) were mixed in a cuvette using a castle-style stir bar, and the emission of the acceptor was collected from 430 to 550 nm (2-nm slits) as a function of $[\text{Ca}^{2+}]_{\text{free}}$. Emission spectra were corrected for blank, dilution, and instrument response.

Amperometry—Full-length syt (wild type (WT), R233Q, K366Q, and R233Q/K366Q double mutant) were subcloned into pIRES2EGFP and transfected into PC12 cells via electroporation, and their effects on secretion were monitored from green fluorescent protein-positive cells using carbon fiber amperometry as described (35). Briefly, cells were depolarized with 105 mM KCl for 6.5 s using a picospritzer. Release was monitored by applying a polarization potential (+650 mV) to a freshly cut 5- μm carbon fiber electrode (ALA Scientific Instruments) placed against the cell. A VA-10 npi amplifier (ALA Scientific Instruments) amplified and transferred the signal to a computer running pClamp8. The signal was digitized at 4 kHz and low pass-filtered at 1 kHz.

RESULTS

The distal tips of the Ca^{2+} -binding loops of syt I (shown in Fig. 6) rapidly penetrate into lipid bilayers composed of PS/PC. This was first shown by placing fluorescent reporters at positions 234 in C2A and 367 in C2B (10–12). These positions are flanked by positively charged residues, Arg²³³ and Lys³⁶⁶, hypothesized to participate in electrostatic interactions with the head groups of anionic phospholipids, an idea that was prompted by the observation that syt selectively binds to membranes that contain negatively charged lipids (14, 36). To test this, we neutralized Arg²³³ and Lys³⁶⁶ by substitution with glutamine. We then determined the effects of these mutations on syt-effector interactions and on secretion.

Analysis of the isolated C2A domain revealed that the R233Q mutation shifted the $[\text{Ca}^{2+}]_{1/2}$ for binding liposomes composed of PS/PC from 19 ± 0.8 to 42 ± 0.5 μM Ca^{2+} (Hill coefficients of 2.6 ± 0.2 to 3.6 ± 0.2 ; Fig. 1A). Similar results were reported in a previous study (4). At saturating Ca^{2+} , the total level of

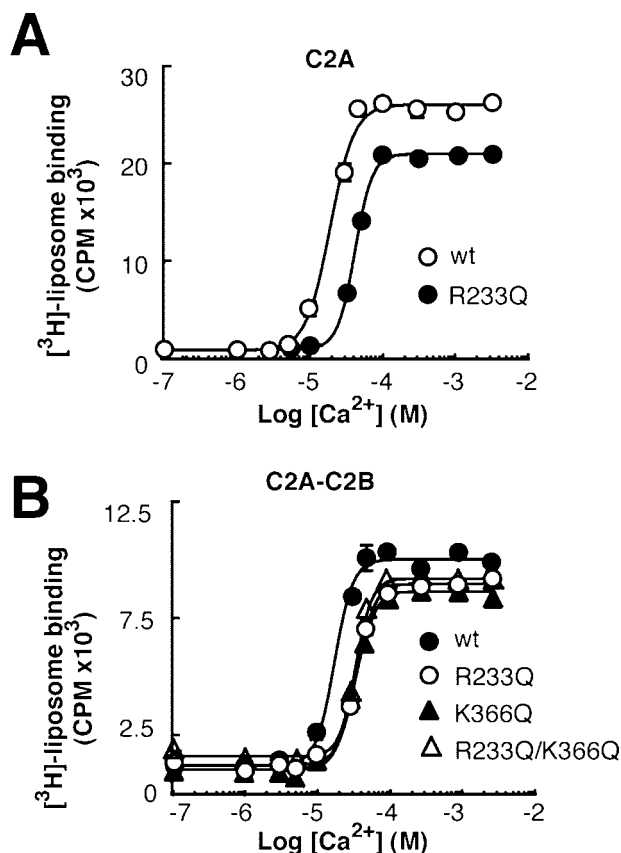


FIG. 1. Mutations in the membrane binding loops of either C2 domain of syt shift the Ca^{2+} dependence for binding PS/PC. A, WT or R233Q mutant C2A domain was assayed for binding PS/PC liposomes as described under "Experimental Procedures" but as a function of the indicated Ca^{2+} concentration. The EC_{50} values for the WT and mutant were 19 ± 0.8 and 42 ± 0.5 μM Ca^{2+} , and the Hill coefficients were 2.6 ± 0.2 and 3.6 ± 0.2 , respectively. These $[\text{Ca}^{2+}]_{1/2}$ values are significantly different ($p = 0.000014$). The change in the Hill coefficient is less marked but still significant ($p = 0.012$). B, the R233Q mutant was assayed as described for A but in the context of C2A-C2B; WT C2A-C2B served as a control. For comparison, an analogous mutation was generated in the C2B domain (K366Q) of C2A-C2B. To determine whether the R233Q and K366Q mutations were additive, the double mutant was generated and analyzed. The EC_{50} values (μM)/Hill coefficients were as follows: WT syt, $18 \pm 1.0/3.3 \pm 0.3$; R233Q, $37 \pm 0.7/3.4 \pm 0.2$; K366Q, $35 \pm 0.4/3.2 \pm 0.2$; R233Q/K366Q, $36 \pm 0.9/3.8 \pm 0.4$. In each case, the differences in the $[\text{Ca}^{2+}]_{1/2}$ values were statistically significant ($p = 0.00012$, 0.00011 , and 0.00025 for WT versus the R233Q, K366Q, and R233Q/K366Q double mutant). The differences in Hill coefficients were not significant for WT versus R233Q ($p = 0.4$), K366Q ($p = 0.4$), and the R233Q/K366Q double mutant ($p = 0.19$).

binding was slightly reduced in the R233Q mutant, suggesting that the affinity of Ca^{2+} -loaded C2A-liposomes interactions might be impaired (for more details, see below).

In the context of C2A-C2B, the C2B domain of syt also binds tightly to liposomes composed of PS/PC (13). This finding raises the question of whether an adjacent wild type C2B domain can compensate for the impaired interaction of R233Q-C2A with membranes. As shown in Fig. 1B, the R233Q mutation shifted the Ca^{2+} dependence of the intact cytoplasmic domain of syt (designated C2A-C2B) from 18 ± 1.0 to 37 ± 0.7 μM ; thus, C2B failed to rescue this defect under steady-state conditions. Interestingly, the K366Q mutation resulted in a virtually identical shift as did the R233Q mutation ($\text{EC}_{50} = 35 \pm 0.4$ μM Ca^{2+}), consistent with the idea that C2A and C2B dock onto membranes via similar electrostatic interactions (13). We then determined whether the effects of R233Q in C2A and K366Q in C2B were additive and found that this was not the case; the double mutant had an EC_{50} of 36 ± 0.9 μM (Fig. 1B). With the

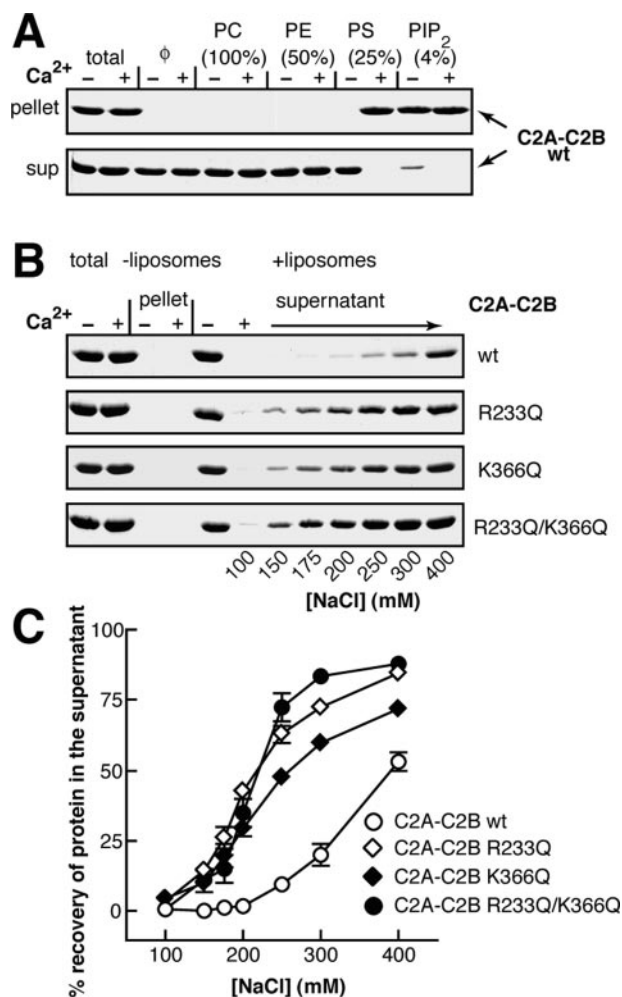


FIG. 2. Effect of ionic strength on the co-sedimentation of WT and mutant versions of C2A-C2B with liposomes. A, the interaction between C2A-C2B and liposomes was assayed by co-sedimentation as described under "Experimental Procedures"; co-sedimentation is mediated by syt-membrane interactions as well as syt-syt interactions (37). The total amounts of C2A-C2B used for each assay point are shown in the left two lanes. Co-sedimentation of C2A-C2B was not observed in samples lacking liposomes (indicated as ϕ), or using liposomes composed of 100% PC or 50% PE, 50% PC. In contrast, efficient co-sedimentation of C2A-C2B was observed in samples containing liposomes composed of 25% PS, 75% PC; co-sedimentation was strictly Ca^{2+} -dependent. Co-sedimentation was also observed in samples that contained 4% phosphatidylinositol-4,5-bisphosphate (PIP_2)/96% PC, but in this case, significant co-sedimentation was observed in the absence of Ca^{2+} ; co-sedimentation was complete in the presence of Ca^{2+} . B, the sensitivity of C2A-C2B/25% PS plus 75% PC interactions to ionic strength was assayed using the co-sedimentation assay described for A. In the absence of liposomes, sedimentation was not observed in either EGTA or Ca^{2+} . In the presence of liposomes, sedimentation was not observed in EGTA but was complete in the presence of Ca^{2+} . Sedimentation in the presence of Ca^{2+} was assayed at increasing concentrations of NaCl; the same percentage of supernatant from each sample was loaded onto the gel. With increasing [NaCl], WT and the indicated mutant C2A-C2B fragments were stripped from the pellet and recovered in the supernatant fraction. C, quantification of the effect of [NaCl] on C2A-C2B-PS/PC co-sedimentation. The Coomassie-stained gels from B were quantified using a UVP BioImaging Systems gel documentation system and Labworks 4.0 software; percentage of recovery of protein in the supernatant is plotted against [NaCl].

exception of the experiment on isolated C2A, these mutations selectively shifted the apparent affinity for Ca^{2+} , leaving the Hill coefficient unchanged.

To further characterize the effects of the R233Q and K366Q mutations, we assayed for membrane binding activity using a C2A-C2B-liposome co-sedimentation assay. Sedimentation in-

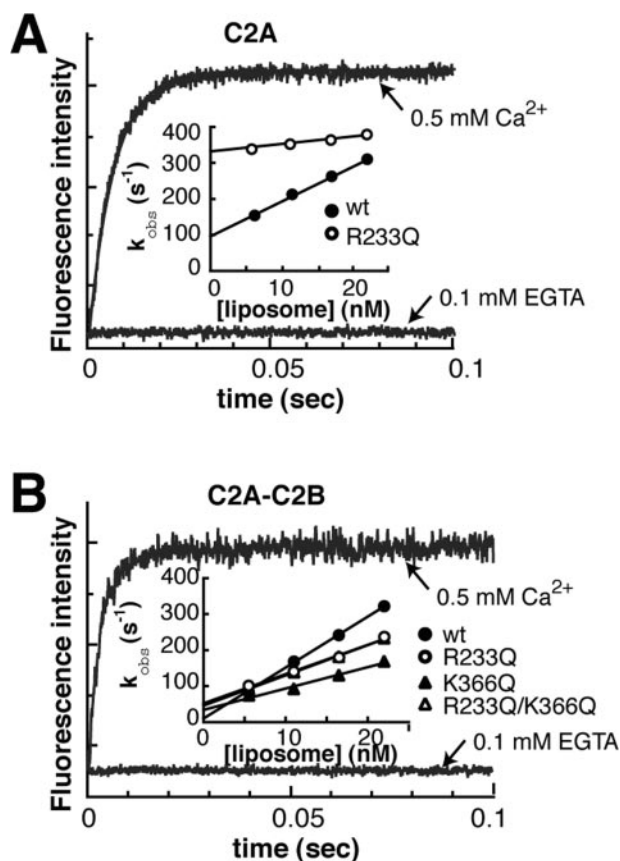


FIG. 3. R233Q and K366Q mutations affect the kinetics of Ca^{2+} -triggered PS/PC interactions. A, effects of the R233Q mutation on the kinetics of C2A-membrane interactions, monitored via FRET between native tryptophan 259 in C2A and a dansyl-PE acceptor present on target liposomes. Kinetics were resolved using a stopped-flow spectrometer in either 0.1 mM EGTA or 0.5 mM Ca^{2+} as described (11). Representative fluorescence traces using WT C2A are shown. The Ca^{2+} -triggered increase in fluorescence was well fitted with a single exponential function to yield k_{obs} ; k_{obs} was plotted versus [liposome] to yield k_{off} and k_{on} , as seen in the inset. The dissociation constants K_d for WT and R233Q C2A were calculated by dividing k_{off} by k_{on} ; results are listed in Table I. Experiments were carried out in 100 mM NaCl. B, effects of the R233Q and K366Q mutations on the kinetics of C2A-C2B-membrane interactions. Experiments were carried out as in A but using the intact cytoplasmic domain of syt; representative fluorescence traces using WT C2A-C2B are shown in the inset (note that these experiments were carried out in both 100 and 150 mM NaCl). Rate constants for both wild type and mutants are summarized in Table I.

involves both the membrane binding and oligomerization activity of C2A-C2B (37). As shown in Fig. 2A, WT C2A-C2B co-sediments with liposomes that contain PS/PC in a strictly Ca^{2+} -dependent manner. In contrast, C2A-C2B exhibits significant levels of co-sedimentation with phosphatidylinositol-4,5-bisphosphate (PIP₂)/PC membranes in the absence of Ca^{2+} (Fig. 2A); co-sedimentation is complete in the presence of Ca^{2+} . As negative controls, C2A-C2B did not co-sediment with liposomes composed of PC or PE/PC (Fig. 2A). Co-sedimentation assays were repeated for WT and the charge neutralization mutants as a function of increasing ionic strength (Fig. 2B). Consistent with previous reports, membrane interactions are highly sensitive to [NaCl] (10). The R233Q and K366Q single and double mutant versions of C2A-C2B were more readily stripped from the liposomes by increasing the ionic strength (Fig. 2C). These results are consistent with the idea that Arg²³³ and Lys³⁶⁶ participate in the docking of the cytoplasmic domain of syt onto membranes.

The effects of the charge neutralization mutations on the affinity of syt-PS/PC interactions were first addressed by meas-

TABLE I
Kinetics of syt-PS/PC interactions

The data shown in the table correspond to the experiments described in the legend to Fig. 3. Rate (k_{on} , k_{off}) and dissociation (K_d) constants were determined using FRET, as described under "Experimental Procedures."

	k_{on} $\times 10^{10} M^{-1} s^{-1}$	k_{off} s^{-1}	K_d nM
100 mM NaCl			
C2A	0.97 ± 0.03	95.2 ± 4.2	9.8 ± 0.7
C2A R233Q	0.25 ± 0.04	320.0 ± 6.5	128.0 ± 27.0
100 mM NaCl			
C2A-C2B	2.08 ± 0.12	12.9 ± 8.7	0.6 ± 0.4
C2A-C2B R233Q	1.72 ± 0.20	20.8 ± 15.0	1.2 ± 0.7
C2A-C2B K366Q	1.31 ± 0.08	29.7 ± 6.2	2.3 ± 0.6
C2A-C2B R233Q/K366Q	1.19 ± 0.14	36.8 ± 10.3	3.1 ± 1.2
150 mM NaCl			
C2A-C2B	1.42 ± 0.02	8.77 ± 2.3	0.6 ± 0.2
C2A-C2B R233Q	0.82 ± 0.06	51.4 ± 8.4	6.3 ± 0.5
C2A-C2B K366Q	0.60 ± 0.06	34.3 ± 9.6	5.7 ± 0.8
C2A-C2B R233Q/K366Q	0.84 ± 0.02	48.8 ± 3.5	5.8 ± 0.4

uring the on and off rates of WT and R233Q mutant C2A-liposome interactions in response to Ca^{2+} . FRET from a naturally occurring tryptophan residue (in position 259) to a dansyl-PE acceptor that has been incorporated into the liposomes was used to monitor binding in real time using a stopped-flow rapid mixing approach. Representative kinetic traces are shown in Fig. 3A; k_{obs} was plotted as a function of the liposome concentration (Fig. 3A, inset), and the rate constants are summarized in Table I. The R233Q mutation increased the off-rate and decreased the on-rate, resulting in a ~13-fold reduction in affinity of C2A for membranes (Table I).

To determine whether an adjacent C2B domain could compensate for the loss in affinity of R233Q-C2A, kinetics experiments were performed on the R233Q mutation in the context of C2A-C2B. For this analysis, we also included the K366Q and R233Q/K366Q double mutant versions of C2A-C2B. A representative kinetics trace is shown in Fig. 3B. As described above for the isolated C2A domain, k_{obs} was plotted as a function of the liposome concentration (Fig. 3B, inset), and the kinetics data are summarized in Table I. Because C2A-C2B-membrane interactions are sensitive to ionic strength (Fig. 2B), these experiments were carried out in 100 and 150 mM NaCl. At 100 mM NaCl, the reduction in affinity caused by the mutations was only ~2-fold, indicating that C2B can compensate for some of the loss in Ca^{2+} -triggered membrane binding activity of R233Q C2A. We note that inclusion of the C2B domain increased the affinity of syt for PS/PC by ~16-fold. This result quantitatively confirms earlier studies demonstrating that, in C2A-C2B, C2A and C2B both contribute to membrane-binding activity (13, 24), whereas isolated C2B binds membranes weakly (37). We also note that the effects of the mutations in C2A-C2B were much more pronounced at 150 mM NaCl, resulting in a ~10-fold reduction in affinity (Table I). These results suggest that ionic strength has strong effects on the structure and biochemical properties of syt.

Our kinetic measurements demonstrate that R233Q, K366Q, and R233Q/K366Q mutant C2A-C2B have similar dissociation constants for binding PS/PC. A key finding is that the effects of the two mutations were not additive, indicating that a mutation in one C2 domain is "dominant" and can affect the properties of the adjacent C2 domain. Together, the data above support a model in which Ca^{2+} -loaded syt docks onto PS/PC membrane via loop-mediated electrostatic interactions. Moreover, the Ca^{2+} /membrane binding kinetics of syt can be tuned via mutations in these membrane-penetration loops. Consistent with previous studies, the affinity of Ca^{2+} -C2A-C2B liposome (25% PS, 75% PC) complexes lies in the low nanomolar

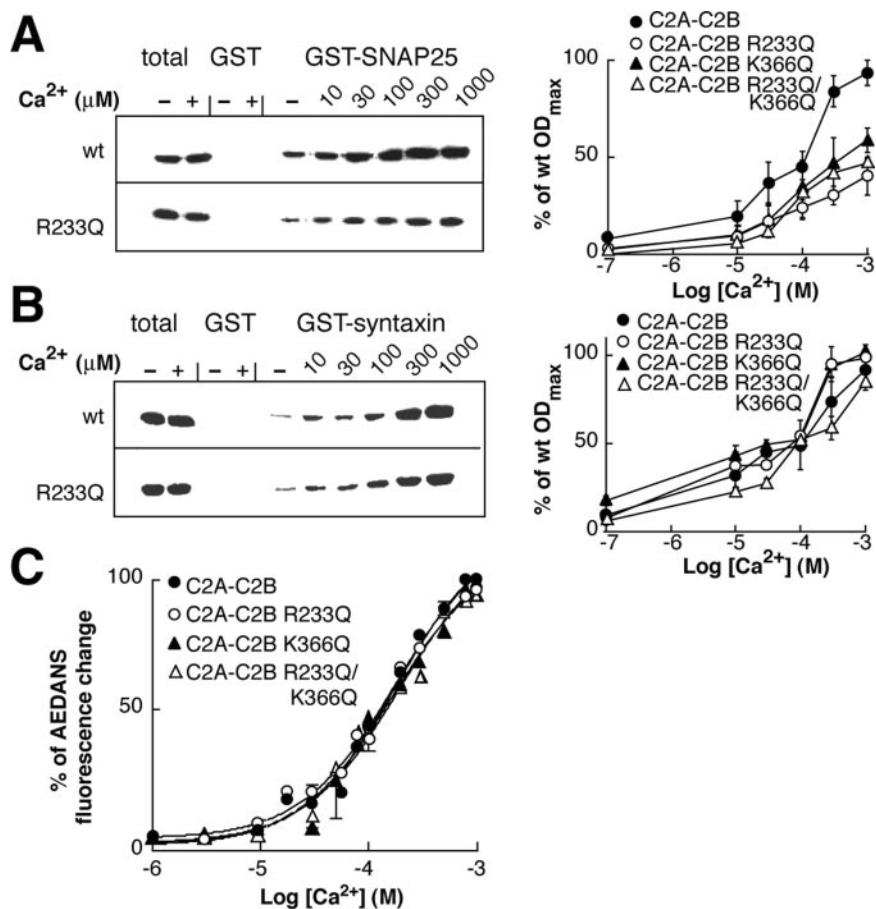


FIG. 4. R233Q and K366Q mutations decrease the SNAP-25 binding activity of C2A-C2B but have no effect on syntaxin binding activity. A, the interaction between SNAP-25 and C2A-C2B was monitored using a GST pull-down assay, as described under "Experimental Procedures." SNAP-25 was immobilized as a GST fusion protein and incubated with either WT or mutant versions of C2A-C2B, in the presence of the indicated [Ca²⁺]. GST alone served as a negative control. 13% of each sample was subjected to SDS-PAGE. Total corresponds to 0.1 μ g of wild type or mutant C2A-C2B. Proteins were visualized by immunoblotting with a monoclonal antibody against C2A-C2B (41.1) and ECL within the linear range. Representative blots, for both wild type and R233Q mutant, are shown in the left panel. Results for the K366Q and double mutant were similar to the R233Q mutant (data not shown). The blots were quantified as described in the legend to Fig. 2C, and percentage of maximum binding is plotted versus [Ca²⁺] (right panels). [Ca²⁺]_{1/2} values for each construct were similar: wild type, R233Q, K366Q and double mutant are 120 ± 20 , 102 ± 20 , 115 ± 30 , 110 ± 40 μ M, respectively. B, interaction of syntaxin with WT and mutant versions of C2A-C2B. Experiments were carried out as in A. Representative blots for WT and the R233Q mutant are shown in the left panel; similar results were obtained for K366Q and the R233Q/K366Q double mutant (data not shown). Binding was quantified, and the results are shown in the right panel. As estimated from the plots, the [Ca²⁺]_{1/2} values were as follows: WT, 100 ± 10 μ M; R233Q, 108 ± 30 μ M; K366Q, 117 ± 20 μ M; and R233Q/K366Q double mutant, 103 ± 20 μ M. C, the Ca²⁺ sensitivity of SNAP-25 and C2A-C2B interaction was monitored using FRET. Tryptophan residues in C2A-C2B were excited at 285 nm and served as energy donors. An AEDANS attached to SNAP-25 served as an energy acceptor, and its emission was collected from 430 to 550 nm. The emission spectra was corrected and integrated, and the percentage change was calculated by normalizing to the maximum fluorescence signal and plotted versus [Ca²⁺]. The curves were fitted with sigmoidal curves. The [Ca²⁺]_{1/2} values were as follows: WT, 178 ± 1.1 μ M; R233Q, 176 ± 1.1 μ M; K366Q, 165 ± 1.2 μ M; and R233Q/K366Q double mutant, 187 ± 1.2 μ M.

range and thus might provide significant energy to the fusion reaction (11, 13).

We also explored the effects of the R233Q and K366Q mutations on interactions with the t-SNAREs syntaxin and SNAP-25. t-SNAREs were immobilized on beads and used as an affinity matrix to bind soluble WT and mutant C2A-C2B as a function of [Ca²⁺] (Fig. 4, A and B). For both t-SNAREs, the [Ca²⁺] for binding of WT, R233Q, K366Q, and R233Q/K366Q were similar (100–120 μ M Ca²⁺). These data were confirmed using FRET to monitor C2A-C2B-AEDANS-SNAP-25 interactions in solution (Fig. 4C).

In addition to measuring the Ca²⁺-sensitivity of syt-t-SNARE interactions, we examined the maximal extent of binding. Previous studies indicated that the R233Q mutation either decreases (38) or has no effect on syt-syntaxin interactions (4). In our experiments, WT as well as each of the mutant versions of C2A-C2B bound to syntaxin to similar extents. In contrast, the R233Q, K366Q, and R233Q/K366Q mutant versions of

C2A-C2B all exhibited ~50% reductions in SNAP-25 binding activity. These findings suggest that Arg²³³ and Lys³⁶⁶ might lie at an interface between Ca²⁺, membranes, and SNAP-25; this model is addressed under "Discussion" (see Fig. 6). The key findings here are that the loop mutations are not specific for perturbation of membrane binding; they can also disrupt interactions with SNAP-25. Also, the effects of the R233Q and K366Q mutations on syt-SNAP-25 interactions were not additive, further supporting the idea that the tandem C2 domains of syt can influence one another.

In the final series of experiments we expressed full-length WT, R233Q, K366Q, or R233Q/K366Q mutant syt in PC12 cells using a bicistronic vector that also encodes enhanced green fluorescent protein. This approach is likely to result in the displacement of native syt molecules at release sites with copies of WT or mutant syt I (35). Amperometric recordings were carried out from enhanced green fluorescent protein-positive cells that were depolarized with KCl. As shown in Fig. 5A, the

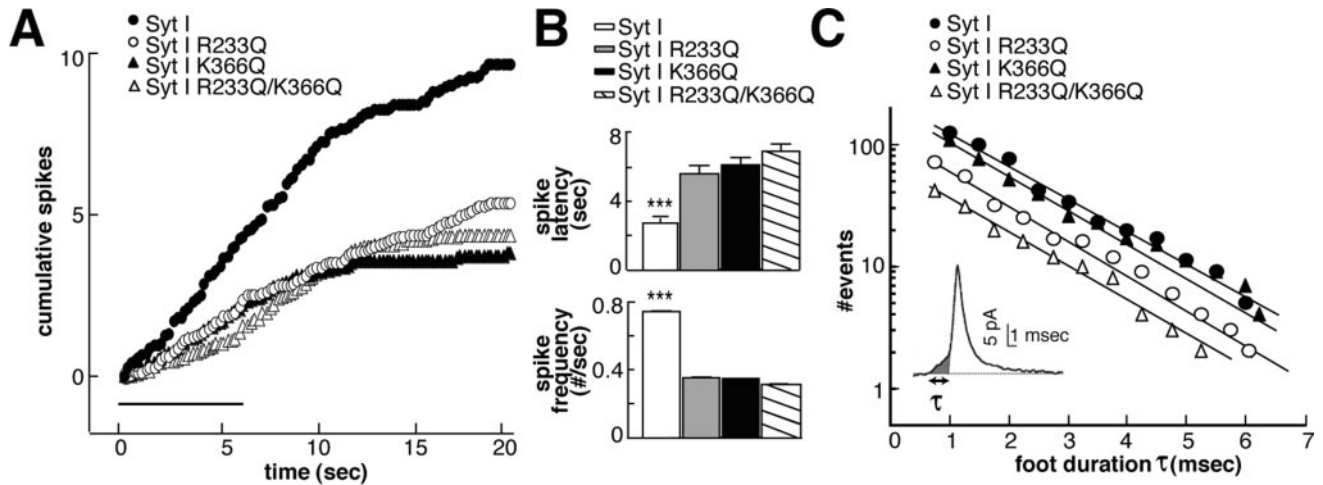


FIG. 5. R233Q and K366Q mutations in syt reduce the rate of exocytosis in PC12 cells but do not affect fusion pore dilation kinetics. A, amperometry was used to measure secretion from PC12 cells transiently transfected with R233Q, K366Q, or R233Q/K366Q mutant syt. The bar below indicates depolarization with 105 mM KCl for 6.5 s. The number of fusion events (spikes) were measured from 13–28 cells in at least four independent transfections. For each condition, the cumulative average number of spikes per cell is plotted *versus* time. B, spike latency was obtained by averaging the first spike latency from all recordings in A. Mean frequency was calculated as the maximum slope of the curves in A. $***, p < 0.001$. C, the log of the cumulative number of prespike feet was plotted *versus* the foot duration. Data were then fitted with single exponential functions to yield the mean open time (τ). These values were: WT syt, $\tau = 1.61 \pm 0.09$ ms; R233Q, $\tau = 1.52 \pm 0.06$ ms; K366Q, $\tau = 1.54 \pm 0.06$ ms; R233Q/K366Q, $\tau = 1.58 \pm 0.06$ ms. The *inset* shows a single release spike; the prespike foot signal is shaded, and its duration is defined as τ .

rate of secretion was reduced by $\sim 50\%$ by expression of R233Q, K366Q, or the R233Q/K366Q mutant. Consistent with the biochemical results above, the R233Q and K366Q mutations again were not additive. The decrease in the kinetics of secretion was quantified in Fig. 5B; in the *upper panel*, the latencies to the first release event are increased to similar extents in the mutants, and in the *lower panel*, the spike frequency was quantified and is decreased to similar extents in all of the mutants. These data demonstrate that Arg²³³ and Lys³⁶⁶ play critical roles in excitation-secretion coupling.

In PC12 cells, most of the fusion events are preceded by prespike foot signals that correspond to the initial open state of the fusion pore (Fig. 5C, *inset*) (39). Subsequent dilation of the pore results in the rapid upstroke of the spike. Lifetime distributions of prespike foot signals were generated as described (35) and are plotted in Fig. 5C; the data are well fitted by single exponential functions. The foot durations were as follows: WT syt, $\tau = 1.61 \pm 0.09$ ms; R233Q, $\tau = 1.52 \pm 0.06$ ms; K366Q, $\tau = 1.54 \pm 0.06$ ms; and R233Q/K366Q, $\tau = 1.58 \pm 0.06$ ms. The mutations in syt had no apparent effect on the time constants of prespike foot signals.

DISCUSSION

In previous studies, the effects of mutations in each C2 domain of syt have been investigated. These studies indicate that C2B plays an essential role in synaptic transmission (5, 40), but a consensus as to the function of the C2A domain has not been reached (7, 41). In one study, the R233Q mutation was reported to reduce synaptic transmission (4). However, in another study, mutations that virtually abolish the ability of C2A to bind PS/PC in response to Ca^{2+} did not reduce synaptic transmission (8).

Here, we have carried out a detailed analysis of the R233Q mutation and have extended these studies to the analogous residue in C2B, K366Q. If C2A and C2B function as independent modules, we would expect that the effects of these mutations would be additive. To test this, we also analyzed a version of syt that harbored both mutations. Either mutation significantly decreased the affinity of syt for liposomes composed of PS/PC (Fig. 3B) and resulted in a 2-fold increase in the Ca^{2+} requirements for binding to PS/PC (Fig. 1B). The

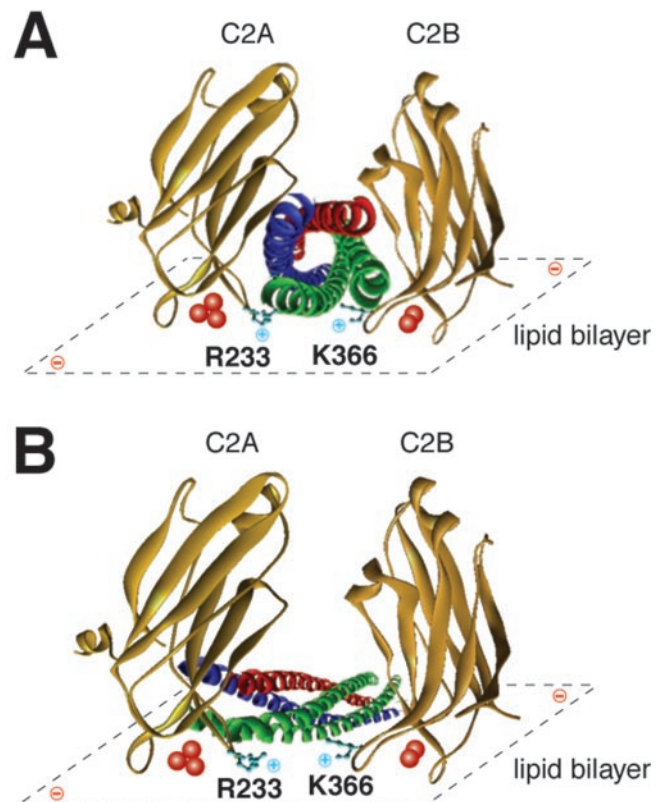


FIG. 6. Models depicting the interaction between the C2 domains of syt (46, 47), the core of the SNARE complex (16), and anionic membranes. Residues Arg²³³ and Lys³⁶⁶ are indicated (these residues are adjacent to residues Phe²³⁴ and Ile³⁶⁷, shown previously to penetrate PS/PC bilayers (13)). Putative electrostatic interactions between these charged residues and anionic lipid head groups are indicated by the *plus* and *minus* signs. Two models are shown; in A, the C2 domains are shown straddling the core of the SNARE complex, and in B the C2 domains bind, side by side, to the side of the SNARE complex. In both models, Arg²³³ and Lys³⁶⁶ simultaneously interact with SNAP-25 and membranes; neither residue makes direct contact with syntaxin. The model was generated using WebLab Viewer Lite; in the SNARE complex, SNAP-25 is rendered in green, syntaxin in red, and synaptobrevin in blue. The red spheres represent Ca^{2+} ions.

decrease in membrane-binding affinity may account, at least in part, for the increase in the $[Ca^{2+}]_{1/2}$ for binding PS/PC, since syt binds Ca^{2+} only weakly in the absence of anionic lipids (42).

Unexpectedly, the R233Q and K366Q mutations were not additive; the double mutant exhibited a $[Ca^{2+}]_{1/2}$ (Fig. 1B) and affinity for PS/PC that was indistinguishable from C2A-C2B that harbored the single point mutations (Fig. 3B). This observation indicates that mutations in one C2 domain can be "dominant" over an adjacent wild type C2 domain. Consistent with this interpretation, overexpression of the single mutants or the double mutant had virtually identical dominant negative effects on large dense core vesicle fusion in PC12 cells; in all cases, the rate of release was reduced by ~50% (Fig. 5). We interpret the dominant-negative effect to be due to the displacement of WT syt (isoforms I and IX) (23, 43, 44) at sites of fusion. These functional data, in conjunction with biochemical data, provide additional support for the idea that the tandem C2 domains of syt interact and cooperate with one another, at least in the presence of effectors (13, 37). The mechanism that mediates cooperation/interactions between C2A and C2B is not yet understood.

A goal of this study was to selectively diminish syt-membrane interactions in order to discern their function during exocytosis. However, we discovered that the R233Q, K366Q, and double mutants all exhibited ~50% reductions in the maximal extent of SNAP-25 binding activity. Interactions with syntaxin were unaffected by these mutations. These data suggest that when syt engages partially or fully assembled SNARE complexes via direct contacts with SNAP-25 and syntaxin (5, 11, 19, 44), position 233 in C2A and position 366 in C2B lie at an interface between membranes and SNAP-25. We propose two possible models for the structure of the ternary complex; in one model, the C2 domains straddle the SNARE complex during assembly (Fig. 6A); alternatively, the C2 domains bind side-by-side to the complex (Fig. 6B). In either model, syntaxin lies along a distinct surface of the four-helix bundle and would interact with distinct determinants on syt (11, 16). This model is further supported by the finding that syt can penetrate membranes and bind to SNAREs at the same time (11). To confirm this, we assayed whether PS/PC liposomes can compete with SNAP-25 for binding to C2A-C2B; no apparent competition was observed (data not shown; see Ref. 45 for a different view).

Syt binds to t-SNAREs in the absence of Ca^{2+} , but binding is enhanced by relatively high concentrations of Ca^{2+} (~100 μM ; Fig. 4). Because the affinity of syt for Ca^{2+} is increased by anionic lipids, syt is likely to bind t-SNAREs at much lower $[Ca^{2+}]$ *in situ*. So far, we have been unable to measure the Ca^{2+} requirements for the interaction of recombinant syt with t-SNAREs in the presence of anionic membranes, because these components assemble into large aggregates that precipitate out of solution. However, this idea was borne out by cross-linking experiments in PC12 cells where 10 μM Ca^{2+} drove nearly complete complex formation between syt and SNAP-25 on native membranes (23). According to this view, interactions with membranes would precede interactions with t-SNAREs. If correct, this model predicts that the loss in excitation-secretion coupling seen with the R233Q and K366Q mutants is due predominantly to loss of membrane binding activity. This idea is supported by the finding that the loop mutations decrease the overall rate of fusion but do not alter fusion pore lifetimes, indicating that rates out of the open fusion pore are not affected. Thus, Arg²³³ and Lys³⁶⁶ probably influence an earlier step in Ca^{2+} -triggered exocytosis, which may precede or include fusion pore opening

(i.e. syt-membrane interactions). In contrast, mutations that selectively disrupt t-SNARE binding activity, without affecting membrane binding activity, alter fusion pore kinetics.²

In summary, our data implicate the Ca^{2+} -binding loops in both C2 domains of syt in mediating syt-membrane and SNAP-25 interactions; perturbation of these interactions disrupts excitation-secretion coupling. Thus, each C2 domain of syt plays an important role in Ca^{2+} -triggered exocytosis. The fact that the R233Q and K366Q mutations are not additive in both biochemical and functional assays suggests that the tandem C2 domains of syt cooperate to execute the function of this protein during secretion.

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**Mutations in the Effector Binding Loops in the C2A and C2B Domains of
Synaptotagmin I Disrupt Exocytosis in a Nonadditive Manner**
Ping Wang, Chih-Tien Wang, Jihong Bai, Meyer B. Jackson and Edwin R. Chapman

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