Calcium Regulation of Exocytosis in PC12 Cells*

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The calcium (Ca$^{2+}$) regulation of neurotransmitter release is poorly understood. Here we investigated several aspects of this process in PC12 cells. We first showed that osmotic shock by 1 M sucrose stimulated rapid release of neurotransmitters from intact PC12 cells, indicating that most of the vesicles were docked at the plasma membrane. Second, we further investigated the mechanism of rescue of botulinum neurotoxin E inhibition of release by recombinant SNAP-25 COOH-terminal coil, which is known to be required in the triggering stage. We confirmed here that Ca$^{2+}$ was required simultaneously with the SNAP-25 peptide, with no significant increase in release if either the peptide or Ca$^{2+}$ was present during the priming stage as well as the triggering, suggesting that SNARE (soluble N-ethylmaleimidesensitive fusion protein attachment protein receptor) complex assembly was involved in the final Ca$^{2+}$-triggered event. Using this rescue system, we also identified a series of acidic surface SNAP-25 residues that rescued better than wild-type when mutated, due to broadened Ca$^{2+}$ sensitivity, suggesting that this charged patch may interact electrostatically with a negative regulator of membrane fusion. Finally, we showed that the previously demonstrated stimulation of exocytosis in this system by calmodulin required calcium binding, since calmodulin mutants defective in Ca$^{2+}$-binding were not able to enhance release.

The secretion of neurotransmitters from neurons and neuroendocrine cells is regulated by Ca$^{2+}$. Different populations of vesicles within cells exhibit distinct Ca$^{2+}$ concentration requirements for the stimulation of exocytosis and undergo fusion with varying kinetics (1). This is partly attributable to the spatial Ca$^{2+}$ concentration gradients originating from Ca$^{2+}$ channels (2, 3) and partly due to heterogeneity in the maturation states of the vesicles themselves (4). Ca$^{2+}$ not only triggers the final step of transmitter release, but is also involved in many other events important for vesicle recycling, for example, movement of vesicles from the reserve pool to the immediately releasable pool, which may involve Ca$^{2+}$-dependent cytoskeletal rearrangements (5). The norepinephrine-containing dense core vesicles in PC12 cells have been shown to be morphologically docked (6) and physically associated with the plasma membrane (7) and therefore do not require mobilization steps prior to exocytosis. Osmotic shock by applying high concentrations of sucrose has been used to estimate the size of the readily releasable pool in neurons (8) and is used in this study to confirm that PC12 cell dense core vesicles are docked. In the reconstituted exocytosis system using these cracked PC12 cells, Ca$^{2+}$ concentration is controlled by buffered solution, and only docked dense core vesicles are assayed, thus the system eliminates Ca$^{2+}$ concentration dynamics and provides a tool to analyze the late post-docking steps of vesicle fusion.

The role of Ca$^{2+}$ in the triggering of fusion with the plasma membrane of synaptic vesicles in neurons and large dense core vesicles in PC12 cells is likely in regulating the formation of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complexes (9). Syntaxin 1a is the t-(for target) or Q-SNARE of the plasma membrane, which together with the peripherally attached t- or Q-SNARE SNAP-25 binds to the vesicle-associated membrane protein VAMP2 (an R-SNARE) to form a trans-SNARE complex (10–12). Full formation of the SNARE complex bridging the two membranes is believed to force the membranes into close proximity, helping to overcome the energy barrier of membrane fusion (9, 13). Full models postulate that full SNARE complex formation is prevented by a Ca$^{2+}$-sensing protein, which binds to one or more of the SNAREs (or a partial SNARE complex) until it is displaced by the arrival of Ca$^{2+}$ ions that trigger exocytosis (14). However, the nature of the proposed Ca$^{2+}$ sensor and its precise binding site are still topics of debate (12, 15–19).

We previously established a system to study the role of SNAP-25 in large dense core vesicle exocytosis in cracked PC12 cells, in which the SNAP-25 COOH-terminal coil is inactivated by cleavage with botulinum neurotoxin E, and exocytosis of tritiated norepinephrine is rescued by addition of a recombinant SNAP-25 COOH-terminal coil (S25C; Ref. 9). To investigate whether, as in chromaffin cells for example (5, 20, 21), calcium facilitates earlier steps than the final triggering of exocytosis from PC12 cells, we took advantage of the fact that the norepinephrine release assay can be split into two stages: MgATP-dependent priming, followed by Ca$^{2+}$-dependent triggering (22). We examine whether the presence of low concentra-

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1 The abbreviations used are: SNAP, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; VAMP, vesicle-associated membrane protein; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SNAP, soluble N-ethylmaleimide attachment protein.
trations of Ca\(^{2+}\) and/or S25C during the priming step enhances the kinetics of either release or regular exocytosis during subsequent triggering. Prior exposure to calcium did not detectably enhance the rate of release, suggesting that early calcium-dependent maturation steps likely occur prior to priming and confirming that SNARE complex assembly appears to be involved in the final Ca\(^{2+}\)-triggered event. We further identify a series of charged surface residues in the COOH-terminal half of S25C, mutation of which results in enhanced rescue of exocytosis compared with wild-type S25C in our rescue assay. These mutants exhibit broadened tolerance to different Ca\(^{2+}\) concentrations, resulting in enhanced release at most of the Ca\(^{2+}\) concentrations tested. We speculate that these residues might be involved in the binding of the Ca\(^{2+}\)-sensor to the SNARE complexes, which normally suppresses exocytosis at suboptimal Ca\(^{2+}\) concentrations.

A candidate calcium sensor for membrane fusion events is calmodulin, which is a ubiquitous calcium mediator in eukaryotic cells. All vertebrates have one identical calmodulin protein, encoded by multiple genes (23). There has been substantial evidence implicating calmodulin in various membrane trafficking events (18, 24–26), and while its effector is still unknown, two recent reports proposed VAMP and Rab3 as possible candidates (27, 28). Calmodulin binds Ca\(^{2+}\) via a structural motif called the EF-hand. Upon binding Ca\(^{2+}\) at each of its four EF-hands, calmodulin exposes a hydrophobic surface that is thought to be critical for its interactions with its effector proteins (29). Mutation of one of the highly conserved Ca\(^{2+}\)-coordinating aspartates to alanine in the EF-hand motif of the essential yeast calmodulin protein dramatically reduces its affinity for Ca\(^{2+}\) (30), but surprisingly has little effect on yeast growth (30), implying that calmodulin has important functions that do not require Ca\(^{2+}\) binding. We have previously identified calmodulin as an active component of the membrane EGTA extract from brain that stimulated exocytosis from cracked PC12 cells (31). Here we show that mutations in the Ca\(^{2+}\)-binding domain of calmodulin prevent its ability to stimulate release, demonstrating that Ca\(^{2+}\) binding is required for calmodulin's function in exocytosis.

**EXPERIMENTAL PROCEDURES**

**Cracked PC12 Cell Assays—**PC12 cells were maintained, loaded with [\(^{3}H\)]norepinephrine, and cracked (mechanically permeabilized) as described previously (22, 31). After being incubated on ice in KGlu buffer (50 mM HEPES, pH 7.2, 150 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA) with 0.1% BSA and 9 mM additional EGTA at each of its four concentrations to achieve final total free concentrations ranging from 0 to 2 mM as indicated.

**Calmodulin Assay—**Cracked cells were primed in KGlu buffer containing 0.7–1 mg/ml rat brain cytosol and 2 mM MgATP at 30 °C for 30 min. The primed cells were centrifuged, washed once with KGlu buffer, and distributed into triggering reactions containing −1 μM free Ca\(^{2+}\) and 1.5 μM recombinant calmodulin (in the absence of cytosol and MgATP) and stimulated for 6 min at 30 °C.

**Time Course Analysis—**The percentage of [\(^{3}H\)]norepinephrine release achieved during S25C rescue or regular release, was calculated and the averages of the indicated number of independent experiments plotted versus time using DeltaGraph® 4.0.5 software. The curves were fitted with exponential curves given by $y = a + (1 - e^{-rt}) + c(\text{II} + c(\text{II})$ typically 0.99–0.999). The initial rate of release (initial slope of each time course curve), $V_0$, is the product of $a$ and $b$. For the toxin-treated cells, the $V_0$ value in the absence of S25C (0 μM) represents the background leakage and so was subtracted from the $V_0$ values of the appropriate S25C curves.

**Intact PC12 Cell Succrose Assay—**PC12 cells were labeled with [\(^{3}H\)]norepinephrine as described previously (22), then washed three times in MBB (124 mM NaCl, 5 mM KCl, 1.5 mM Na\(_2\)HPO\(_4\), 2 mM MgCl\(_2\), 6 mM glucose, 25 mM HEPES, pH 7.4). All cells, except for those stimulated with 5 mM Ca\(^{2+}\) and ionomycin, were treated with 50 μM EDTA (a membrane-permeable methyl ester of EGTA) for 20 min at 37 °C to allow chelation of cytosolic Ca\(^{2+}\) to ensure there was no free Ca\(^{2+}\) in the cells. Release was initiated by incubation at 30 °C for various amounts of time by either adding 5 mM calcium and 1 μM ionomycin (in the absence of EDTA) or succrose to the cells in MBB + 2 mM MgATP and terminated by chilling on ice. [\(^{3}H\)]Norepinephrine release was quantitated as for the cracked cells (above).

**Recombinant Proteins—**S25C (amino acids 142–206 of mouse SNAP-25) wild-type and mutant proteins, S25N (amino acids 1–82 of mouse SNAP-25), rat syntaxin 1a H3 domain (amino acids 191–266), and rat VAMP2 coil domain (amino acids 25–94) were expressed in bacteria with a His-terminus, a thrombin cleavage tag that was removed by thrombin cleavage after purification on glutathione-agarose beads, as described previously (32). The purity of the proteins was confirmed by SDS-PAGE (>95% pure), and the protein concentration was assayed using the BCA kit (Pierce). Proteins used in the cracked cell assay were all dialyzed into KGlu buffer and, if necessary, concentrated on the day of the experiment to prevent precipitation over time, and no aggregation was observed.

**S25C Mutants** were constructed by polymerase chain reaction using the QuikChange site-directed mutagenesis protocol (Stratagene) and verified by sequencing. CD analysis of fast protein liquid chromatography gel filtration-purified S25N/S25C/VAMP2/Syntaxin 1a H3 complex was performed as described previously (9).

**Intact PC12 Cell assay** was used for measurement of S25C rescue or regular release, was calculated and the averages of the indicated number of independent experiments plotted versus time using DeltaGraph® 4.0.5 software. The curves were fitted with exponential curves given by $y = a + (1 - e^{-rt}) + c(\text{II} + c(\text{II})$ typically 0.99–0.999). The initial rate of release (initial slope of each time course curve), $V_0$, is the product of $a$ and $b$. For the toxin-treated cells, the $V_0$ value in the absence of S25C (0 μM) represents the background leakage and so was subtracted from the $V_0$ values of the appropriate S25C curves.

**Calmodulin Mutants—**Yeast shuttle vector-based plasmids pJ61, pJ62, pJ65, and pJ66, carrying the EF-hand mutant vertebrate calmodulin genes D20A/D93A (CaM-D1,3A), D56A/D129A (CaM-D2,4A), D20A/D56A/D93A/D129A (CaM-D1,2,3,4A), and D56A (CaM-D2A), respectively (30), were restriction-digested to generate a unique 367-base pair HindIII/HindIII fragment that then replaced the equivalent wild-type fragment of calmodulin in the pCR2 vector (a pET23d-based construct from Novagen; Ref. 33). The expression constructs thus obtained were confirmed by sequencing and wild-type vertebrate calmodulins were expressed in E. coli (DE3) Escherichia coli cells using the pET system (Novagen). Bacteria were resuspended in cold lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA) and disrupted by French pressing, followed by DNase I (Sigma) treatment in 10 mM MgCl\(_2\) at 25 °C until no longer viscous. All further steps were performed at room temperature. The lysate was cleared by low speed centrifugation, and calmodulin was added to

rotixin E light chain in the presence of 2 mM MgATP and −3.5 mg/ml rat brain cytosol for 6–10 min at 30 °C. Cells were then washed three times in KGlu/BSA buffer prior to rescue by addition to tubes containing S25C in addition to cytosol, MgATP, and Ca\(^{2+}\) as in the typical release reactions above. For the dose response curves, the purity and concentrations of the S25C mutants (measured by S25C assay (Pierce)) were verified by SDS-PAGE to ensure equivalent amounts were used in the assays. To determine the Ca\(^{2+}\) sensitivity of the S25C proteins, the cells were stimulated for 30 min at 30 °C in the presence of MgATP, cytosol, 40 μM each S25C protein, and Ca\(^{2+}\) of different concentrations to achieve final total free concentrations ranging from 0 to 1.5 mM as indicated.
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RESULTS

Osmotic Shock Induces Fusion of Most of the Dense Core Vesicles—In neurons, the application of 0.5 osm sucrose induces the fusion of docked synaptic vesicles with the presynaptic membrane and has been used as a tool to estimate the size of the readily releasable pool of norepinephrine containing dense core vesicles, as suggested previously (6, 7). The higher apparent release in the cracked cell system (e.g. Fig. 4 and Ref. 35) is likely due to the removal of the cytosolic pool of norepinephrine that is not taken up into vesicles during loading (the results are expressed as a percentage of the total [3H]norepinephrine).

The Effect of Ca\(^{2+}\) in Priming—In adrenal chromaffin cells, Ca\(^{2+}\) concentrations lower than those required to trigger fusion can assist cytoskeletal rearrangements and other upstream events in the maturation of vesicles into the readily releasable pool (5, 20, 21). We therefore took advantage of the fact that exocytosis can be resolved into a MgATP-dependent, Ca\(^{2+}\)-independent priming stage and a subsequent Ca\(^{2+}\)-dependent (MgATP-independent) triggering of release (22) to examine whether calcium is required at early, as well as late, steps in PC12 cell exocytosis. We investigated whether the rate of S25C-dependent rescue or regular norepinephrine release during triggering would increase if low concentrations of Ca\(^{2+}\) (<100 nm, i.e. less than the –1 μM normally added in triggering) were present during the priming step. Triggering was then initiated by addition of the remaining complement of Ca\(^{2+}\) (to –1 μM final) and S25C in the case of the toxin-treated cells, and the time course of release was followed. We were unable to resolve any differences in the initial rates of fusion following the different priming protocols (data not shown), suggesting that unlike in chromaffin cells, PC12 cell dense core vesicles have probably bypassed early Ca\(^{2+}\)-dependent steps of maturation.

We reported previously that S25C is required in the triggering but not in the priming step of rescue (9), leading us to conclude that Ca\(^{2+}\) triggers the final step of SNARE complex formation. This means that both Ca\(^{2+}\) and S25C are required to form fully zippered SNARE complexes and drive fusion. We wondered, however, whether S25C’s presence in the priming reaction might affect the triggering reaction, for example its release kinetics, that we did not closely examine in the previous study. Thus, we conducted experiments to compare the time course of rescue after priming the cells with two different concentrations (one low and one high) of S25C in the absence of Ca\(^{2+}\) to that of cells primed with Ca\(^{2+}\) in the absence of S25C (Fig. 2). The initial rate of release was calculated based on curve-fitting data and found not to differ significantly between any of the priming protocols (Fig. 2). Thus, the presence of S25C in the early stage of exocytosis does not affect release kinetics in any way that we can detect in our system.

Identification of S25C Residues That Broaden the Ca\(^{2+}\) Sensitivity of Release—Using a recombinant SNAP-25 COOH-terminal coil (S25C) to rescue norepinephrine exocytosis in botulinum neurotoxin E-treated PC12 cells, we found previously that several mutations yielded increased rescue compared with
the wild-type S25C coil (9). In particular, several aspartic acid or glutamic acid residues at “b” or “f” positions on the surface of the four-helix bundle of the SNARE complex (for example mutants D186A, E183A, D193A in Ref. 9; see also Fig. 3) resulted in enhanced rescue when mutated to alanine. In a three-dimensional helical structure, these residues are clustered together on the surface of the SNARE complex (36), which may create a binding site for a regulatory protein, so we constructed additional mutations to alanine in this area (Fig. 3) and tested their effect on botulinum neurotoxin E rescue.

With the exception of R176A, all other mutants, including surface-charged mutants D172A, D179A, D186A, and D193A, and a hydrophobic “d” layer asparagine mutant (N188A), consistently resulted in increased rescue relative to wild-type S25C at most concentrations (Figs. 3 and 4A). To further test the effect of the putative surface acidic patch, we constructed a double mutant containing two contiguous “b” mutations (D193A/D186A) and found that this was slightly more efficient at rescue than either single mutant alone (Figs. 3 and 4B). Similarly, a triple contiguous “b” position mutant (D193A/D186A/D179A) was more efficient still. Addition of a fourth contiguous “b” mutation (D193A/D186A/D179A/D172A) reaching back past the ionic layer also further enhanced rescue.

The increase in rescue by these S25C surface mutants could either be due to increased complex stability, resulting in greater efficiency of release, or due to weaker interaction with a negative regulator of fusion. To distinguish between these possibilities, we purified core complexes containing several of the above S25C mutants by gel filtration and monitored their thermal unfolding profiles by circular dichroism spectroscopy. While mutant D186A produced a marginally more thermostable complex than wild-type S25C (94.8 °C versus 94 °C, respectively), complexes containing the other surface mutants were of similar or lower stability, particularly the triple mutant, which appeared to unfold less cooperatively than the others (Fig. 5A). The complex containing N188A (at a “d” position) did not fully denature even at 99 °C, thus increased complex stability could perhaps account for the higher rescue by this mutant. These data confirm our previous findings that thermostability only strictly correlates with rescue for the hydrophobic layers (9, 32).

To try to explain the increased rescue by these mutants, we examined the sensitivity of their rescue to Ca2+ . As shown in
alane might weaken the interaction with such a fusion clamp, resulting in the greater level of exocytosis observed over a wider concentration range than is accommodated by wild-type S25C. Interestingly, the N188A mutant also displayed the same shift in Ca\textsuperscript{2+} sensitivity, even though Asn188 is involved in inter-SNARE core interactions. While it remains possible that this asparagine to alanine mutation on the other side of the S25C helix to the acidic patch indirectly causes the same weakened interaction with the regulator, we also consider it possible that a general enhancement of SNARE complex stability, indicating that their enhancement of rescue is not due to greater complex stability. B, broadened Ca\textsuperscript{2+} sensitivity in the S25C surface mutants. 30-min rescue assays were carried out in the presence of a 40 \mu M concentration of the displayed S25C mutants at the indicated concentrations of Ca\textsuperscript{2+}, and the percentage of norepinephrine release was plotted versus free Ca\textsuperscript{2+} concentration. The data shown are from a single representative experiment that was repeated several times.

normal Ca\textsuperscript{2+}-dependent exocytosis (39), to examine whether the S25C mutants would no longer rescue better than wild-type. For unknown reasons, these cells leak [\textsuperscript{3}H]norepinephrine during the cracking and 30 °C incubation (data not shown), resulting in a very low final \textsuperscript{3}H-signal (only 14% of that with wild-type cells), thus there is some uncertainty in the results obtained using this cell line. Nevertheless, the calcium sensitivity of norepinephrine release was unaltered, peaking at −1 \mu M free calcium as in the wild-type cells (data not shown), and the double (D186A/D193A) and triple (D186A/D193A/D179A) S25C mutants did appear to afford enhanced rescue relative to wild-type (Fig. 6), suggesting that synaptotagmins I or II may not be the hypothesized regulator.

Ca\textsuperscript{2+} Sensing by Calmodulin Is Required for Its Stimulatory Effect in Exocytosis—A potential positively acting calcium sensor for exocytosis is calmodulin, which we previously identified as a stimulator of norepinephrine release, acting in the triggering stage of the cracked PC12 cell assay (31). Calmodulin contains four EF-hand motifs in which an aspartic acid residue is known to be important for Ca\textsuperscript{2+} binding (30, 40). Yeast calmodulin EF-hand mutants grow normally, while calmodulin
knockouts are lethal (30), suggesting that Ca\(^{2+}\) binding to calmodulin is not always required. Therefore we investigated whether Ca\(^{2+}\) binding to calmodulin was necessary for the latter’s ability to stimulate the Ca\(^{2+}\)-dependent triggering of exocytosis. We generated bacterial expression constructs for four mutant vertebrate calmodulins with varying Ca\(^{2+}\) binding affinities, CaM (D\(_{2}\)A), CaM (D\(_{1,3}\)A), CaM (D\(_{2,4}\)A), and CaM (D\(_{1,2,3,4}\)A), with the subscript number indicating which of the four EF-hands are mutated. We examined the ability of the recombinant proteins to bind Ca\(^{2+}\), using an established gel-shift assay (41). Wild-type calmodulin, when electrophoresed in sample buffer containing Ca\(^{2+}\), migrates faster on SDS-PAGE gels than in the presence of EGTA (Fig. 7A, lanes 1 and 2). Calmodulin mutated in the second EF-hand (D\(_{2}\)A, lanes 3 and 4 of Fig. 7A), or in the first and third EF-hands (D\(_{1,3}\)A, lanes 5 and 6), displayed a less pronounced increase in migration in the presence of Ca\(^{2+}\), indicating reduced Ca\(^{2+}\)-binding. Mutations in the second and fourth EF-hands together (D\(_{2,4}\)A, lanes 7 and 8) decreased the mobility shift still further, while removal of all four aspartic acid residues (D\(_{1,2,3,4}\)A, lanes 9 and 10) completely abolished Ca\(^{2+}\) binding.

We assayed the ability of these reduced Ca\(^{2+}\)-binding calmodulin mutants to stimulate exocytosis in the triggering stage of the cracked PC12 cell assay. The D\(_{2}\)A mutant displayed similar Ca\(^{2+}\) sensitivity to wild-type calmodulin, peaking at ~1.5 \(\mu\)M free Ca\(^{2+}\), but total norepinephrine release at this peak level was suppressed compared with wild-type calmodulin (although not at other Ca\(^{2+}\) concentrations). The D\(_{1,3}\)A resulted in lower overall stimulation compared with wild-type calmodulin at every Ca\(^{2+}\) concentration. The low and non-Ca\(^{2+}\) binding mutants (D\(_{2}\)A and D\(_{1,2,3,4}\)A, respectively) did not stimulate release at all, and even slightly inhibited release below background levels at some Ca\(^{2+}\) concentrations (data not shown). Thus, the ability of calmodulin to bind Ca\(^{2+}\) is essential for its stimulation of exocytosis.

Attempts to reveal the stimulatory effect of calmodulin in the S25C rescue assay were unsuccessful, perhaps due to the presence of high concentrations of S25C (tens of \(\mu\)M) in the triggering reaction. Thus, we were unable to assay the effect of acidic mutations in S25C on calmodulin’s ability to stimulate exocytosis.

**DISCUSSION**

In this study, we have investigated several aspects of Ca\(^{2+}\) regulation of exocytosis of norepinephrine in PC12 cells.

We showed that osmotic shock induced rapid exocytosis of almost all dense core vesicles from intact PC12 cells, confirming previous ultrastructural evidence that vesicles are docked at the plasma membrane in cracked PC12 cells (Fig. 1; Refs. 6 and 7). Therefore, any cytoskeletal arrangements influenced by Ca\(^{2+}\) must have occurred prior to the cracking, and the vesicles are poised to be released. This is consistent with the fact that we could not detect any early Ca\(^{2+}\)-requiring step in regular release, and in rescue assays we could not detect any early Ca\(^{2+}\)-requiring step independent of S25C. Using either S25C or Ca\(^{2+}\) in the priming reaction prior to triggering of exocytosis, we observed no significant increase in the rate of release com-
pared with cells primed only with MgATP and cytosol, suggesting that the Ca\textsuperscript{2+}-triggered steps in membrane fusion. However, the time resolution of this assay is low and the curve-fitting program takes all data points equally into account, so any differences limited to the beginning of the time course may be obscured, as evidenced by only a small increase in fusion rate in primed cells compared with unprimed cells (Fig. 2). While we cannot exclude the possibility that Ca\textsuperscript{2+} may regulate multiple late events in the exocytic process in PC12 cells, SNAREs appear to be at the heart of the Ca\textsuperscript{2+} regulation of vesicle fusion.

We have identified a contiguous series of acidic S25C residues that rescue botulinum neurotoxin E inhibition more potently than wild-type S25C when mutated. Mutation of these residues results in a broader concentration range of Ca\textsuperscript{2+} for stimulation of release. These negatively charged residues thus identify a potential binding site for a Ca\textsuperscript{2+}-sensitive negative regulator of fusion on the surface of the SNAP-25-C coil of the SNARE complex. However, the molecular nature of such a Ca\textsuperscript{2+} sensor remains to be determined. Since the acidic patch mutants also rescue better than wild-type in SNAPtagmin I/II-deficient PC12 cells, these two SNAREtagmins are unlikely to be the SNAP-25-binding calcium sensor hypothesized here. However, we cannot exclude that other SNAPtagmin family members may regulate calcium-sensitive release in this SNAPtagmin I/II-deficient cell line.

The complexin (or synaphin) proteins, which bind to SNARE complexes through syntaxin (42), have been recently implicated in the Ca\textsuperscript{2+} dependence of neurotransmitter secretion, since complexin-deficient neurons exhibit decreased Ca\textsuperscript{2+} sensitivity that correlates with defective release (19). Addition of recombinant complexin to cracked PC12 cells did not alter the Ca\textsuperscript{2+} sensitivity of regular norepinephrine release (data not shown), perhaps because it only affects fast synaptic release (19, 44). However, adding calcineurin or its inhibitor FK506 did not affect calmodulin-stimulated release either (data not shown), implying that it does not mediate calmodulin’s actions in PC12 cells. This might agree with more recent data suggesting that the downstream target of calmodulin in yeast is the V\textsubscript{S} subunit of the vacuolar H\textsuperscript{+}-ATPase (47). Other downstream targets of calmodulin in eukaryotes are the L- and P/Q-type Ca\textsuperscript{2+} channels, whose activities have been shown to be modulated by distinct domains of calmodulin in a Ca\textsuperscript{2+}-dependent fashion (48, 49). More recently, Ca\textsuperscript{2+}/calmodulin was shown to bind to the membrane-proximal domain of VAMP2 in vitro (27), but the physiological relevance of this interaction remains to be determined, since it hinders SNARE complex formation and so is not easily reconciled with the stimulatory role of calmodulin. Further study is required to identify the effect(s) of calmodulin in a mammalian exocytosis system.

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