A peptide that mimics the carboxy-terminal domain of SNAP-25 blocks Ca^{2+}-dependent exocytosis in chromaffin cells

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Abstract SNAP-25, a synaptosomal associated membrane protein of 25 kDa, participates in the presynaptic process of vesicle–plasma membrane fusion that results in neurotransmitter release at central nervous system synapses. SNAP-25 occurs in neuroendocrine cells and, in analogy to its role in neurons, has been implicated in catecholamine secretion, yet the nature of the underlying mechanism remains obscure. Here we use an anti-SNAP-25 monoclonal antibody to show that SNAP-25 is localized at the cytosolic surface of the plasma membrane of chromaffin cells. This antibody inhibited the Ca^{2+}-evoked catecholamine release from digitonin-permeabilized chromaffin cells in a time- and dose-dependent manner. Remarkably, a 20-mer synthetic peptide representing the sequence of the C-terminal domain of SNAP-25 blocked Ca^{2+}-dependent catecholamine release with an IC_{50} = 20 μM. The inhibitory activity of the peptide was sequence-specific as evidenced by the inertness of a control peptide with the same amino acid composition but random order. The C-terminal segment of SNAP-25, therefore, plays a key role in regulating Ca^{2+}-dependent exocytosis, presumably mediated via interactions with other protein components of the fusion complex.

Key words: Vesicle fusion; Adrenomedullary cell; Catecholamine secretion; SNARE; SNAP-25

1. Introduction

Increasing evidence demonstrates the presence of common protein constituents on the exocytotic machinery in neuronal and neuroendocrine cells [1]. Membrane fusion is preceded by the docking and priming of neurotransmitter containing vesicles at active sites forming a multi-protein fusion complex [2–5]. This complex involves vesicle components such as synaptobrevin [6,7] and plasmalemma proteins as SNAP-25 and syntaxin [8]. SNAP-25, a neuronal-specific protein, is anchored to the plasma membrane via cysteine palmitoylation [9], and interacts with both synaptobrevin and syntaxin through its carboxy and amino-terminal domains, respectively [10,11].

Poignant evidence implicating these components in neurotransmision emerged from the action of clostridial tetanus and botulinum neurotoxins that block secretion by cleaving synaptobrevin, syntaxin and SNAP-25 [12–14]. The participation of SNAP-25 in synaptic vesicular trafficking and fusion was inferred from the structural similarity with the yeast SEC9 gene product, required for the fusion of Golgi-derived secretory vesicles with plasma membranes [15], and from the finding that antisense oligonucleotides inhibit axonal growth [16].

Significant expression of SNAP-25 is detected in neuroendocrine cells such as chromaffin cells [17,18], yet its role in Ca^{2+}-dependent secretion is not well understood. Here, we show that SNAP-25 and specifically its carboxy-terminal domain regulates Ca^{2+}-dependent exocytosis in chromaffin cells.

2. Materials and methods

2.1. Reagents

Monoclonal anti-SNAP-25 antibody was from Sternberger Monoclonals Inc. (Baltimore, MD); rhodamine-conjugated rabbit anti-mouse IgG and mouse IgG were from Sigma (St. Louis, MO); [^{3}H]Noradrenaline was from DuPont; t-Boc and Fmoc amino acids, with standard side chain protecting groups, were from Applied Biosystems (Foster City, CA), NovaBio-Chem (La Jolla, CA) or Peninsula Laboratories (Belmont, CA). Solvents, reagents and resins for peptide synthesis were from Applied Biosystems. All other reagents were of analytical grade from Sigma.

2.2. Chromaffin cell cultures

Chromaffin cells were prepared from bovine adrenal glands by collagenase digestion and further separated from debris and erythrocytes by centrifugation on Percoll gradients [19]. Cells were maintained in monolayer cultures at a density of 500,000 cells/cm^2 and used between the third and sixth day after plating. All the experiments were performed at 37°C.

2.3. Immunofluorescence labelling of SNAP-25 in intact and permeabilized chromaffin cells

Cultured chromaffin cells were incubated for 90 min with the primary anti-SNAP-25 monoclonal antibody (mAb) (1:250 dilution) in Krebs/HEPES solution containing 1% BSA and then washed 4 times with antibody-free solution. Thereafter, cells were fixed for 5 min with 4% paraformaldehyde in TBS buffer (50 mM Tris, pH 7.5 with 500 mM NaCl 1% BSA). After washing 3 times with TBS, cells were incubated for 1 h with the secondary antibody, rhodamine-conjugated rabbit anti-mouse IgG (1:200 dilution) in 1% BSA in TBS. Cells were washed 3 times with TBS, mounted with 80% glycerol in TBS, and photographed using a Zeiss Axiophot fluorescence microscope. For permeabilized cells immunostaining, cultures were fixed and permeabilized for 15 min in pure methanol at −20°C and then incubated with the primary and secondary antibodies, as described.

2.4. Determination of catecholamine release from detergent-permeabilized chromaffin cells

Secreted [^{3}H]noradrenaline was determined in digitonin-permeabilized cells as described [20]. Briefly, cells were incubated with [^{3}H]noradrenaline (1 μCi/ml) in DMEM during 4 h. Thereafter, monolayers were washed 4 times with a Krebs/HEPES basal solution: 15 mM HEPES, pH 7.4, with 134 mM NaCl 4.7 mM KCl 1.2 mM KH_2PO_4 1.2 mM $MgCl_2$ 2.5 mM $CaCl_2$ 0.56 mM ascorbic acid 11 mM Glucose. Cells

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Abbreviations: SNAP-25, synaptosomal associated protein of 25 kDa; BoTx, botulinum neurotoxin; SNAP, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein; SNARE, SNAP receptor.

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were permeabilized using 20 μM digitonin in 20 mM PIPES, pH 6.8 with 140 mM monosodium glutamate 2 mM $MgCl_2$ 2 mM Mg-ATP 5 mM EGTA, for 5 min, unless otherwise indicated. Permeabilization was carried out in the absence or presence of the different immunoglobulins or peptides. Thereafter, media were discarded and cells were incubated for 10 additional min in digitonin-free medium in presence or absence of additives. Basal or stimulated secretion were measured in media containing 5 mM EGTA or 10 μM Ca^{2+}, respectively. Media were collected and released catecholamines as well as the total cell content were determined by liquid scintillation counting.

2.5. Peptide synthesis and purification

Peptides were synthesized by t-boc or Fastmoc Fmoc chemistries using an Applied Biosystems 431A automated solid-phase peptide synthesizer, and cleaved as described [21]. Cleaved peptides were purified by RP-HPLC on a VydaC C-18 semipreparative column. Samples of crude peptide (10–20 mg) dissolved in 0.1% trifluoroacetic acid were applied to the column and eluted with a linear gradient of 90% acetonitrile in 0.1% trifluoroacetic acid. Eluted peaks were monitored by absorbance measurements at 214 nm, pooled and lyophilized. Peptide purity was assesed by RP-HPLC on a VydaC C-18 analytical column.

3. Results and discussion

3.1. SNAP-25 is located at the cytosolic surface of the chromaffin cell plasma membrane

An anti-SNAP-25 mAb was used to localize SNAP-25 in both intact and permeabilized chromaffin cells in culture. Only permeabilized cells were immunostained by the antibody, as shown by the annular distribution of the fluorescence tag (Fig. 1A). In contrast, insignificant fluorescence was detected on cells labelled prior to fixation and permeabilization (Fig. 1B,C). Thus, the epitope recognized by the anti-SNAP-25 antibody, which corresponds to a single band of 26 kDa band present in adrenomedullary microsomal and plasma membrane fractions (data not shown), appears to be located at the plasma membrane and oriented to the cytosolic side.

3.2. The anti-SNAP-25 mAb inhibits Ca^{2+}-evoked catecholamine release from chromaffin cells

Digitonin-permeabilized chromaffin cells, which are exocytosis competent [22], were used to test the activity of the anti-SNAP-25 mAb on catecholamine secretion. Cells were permeabilized with 20 μM digitonin for different time periods before a 10 μM Ca^{2+} solution was added to trigger catecholamine release (Fig. 2A). The antibody produced both time- and dose-dependent inhibition of Ca^{2+}-evoked noradrenaline release (Fig. 2A,B). When cells were permeabilized in presence of Ca^{2+} ($t = 0$), the mAb present in the medium caused no effect on the stimulated secretion. In contrast, it inhibited Ca^{2+}-dependent [^{3}H]noradrenaline release when cells were permeabilized for longer periods prior to Ca^{2+} triggering (Fig. 2A). A dose–response curve using 5 min permeabilization time is shown in Fig. 2B. Half-maximal effect was obtained 5 μg IgG/ml of mAb (Fig. 2B), with a maximal inhibition of 60% at 25 μg/ml IgG/ml of mAb. Specificity was assessed using mouse IgG which had no effect. SNAP-25 is, therefore, involved in Ca^{2+}-dependent exocytosis in chromaffin cells.

3.3. A peptide with the sequence of the C-terminal domain of SNAP-25 specifically blocks Ca^{2+}-dependent catecholamine release from chromaffin cells

We used synthetic peptides to probe the role of specific protein domains of SNAP-25 in excitation-secretion coupling. Specifically, we focused on the carboxy-terminus of SNAP-25 because it is a target of clostridial neurotoxins A and E (BoTx A and E), known potent inhibitors of exocytosis [2,23,24], and it has been shown to interact with synaptobrevin and syntaxin [2,3,10,11,25]. Notably, a 20-mer peptide encompassing the C-terminal region of SNAP-25 (aa 187–206: SNKTRIDEANQRATKMLGSG), hereinafter denoted as SNAP-25(187–206), blocked the Ca^{2+}-evoked catecholamine release from digitonin-

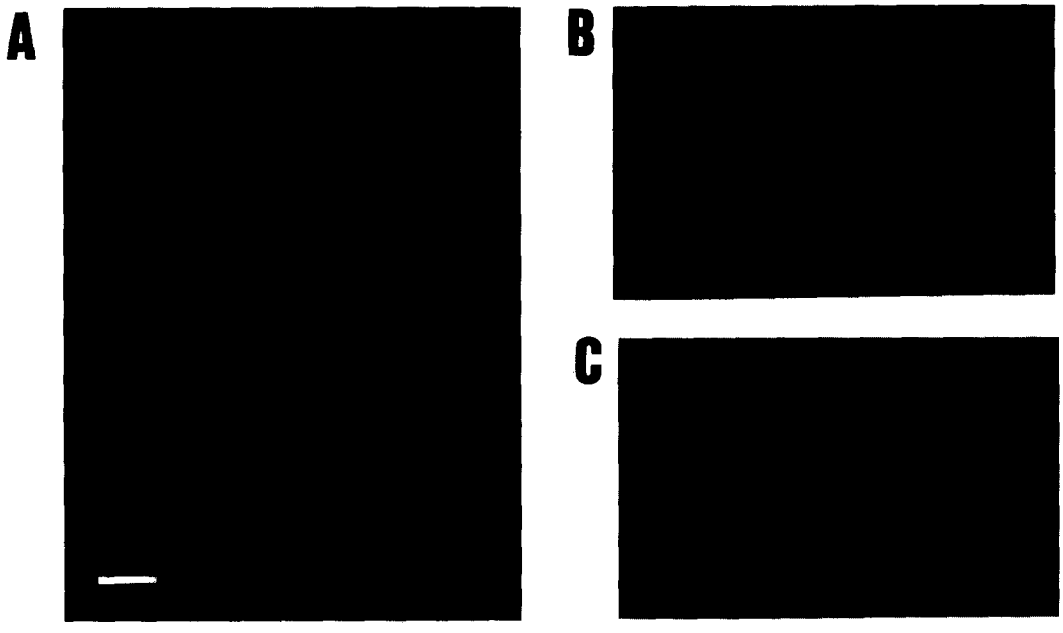

Fig. 1. Immunostaining of permeabilized and intact cultured chromaffin cells. (A) Permeabilized cells. Cells were fixed and permeabilized before incubation with the anti-SNAP-25 mAb. Scale bar = 20 μm. (B) Non-permeabilized cells. Intact cells were incubated with anti-SNAP-25 mAb before fixation in 4% paraformaldehyde. Immunolocalization was performed using a rhodamine-conjugated anti-mouse IgG (A and B). (C) Phase contrast microscopy of the same field shown in (B).

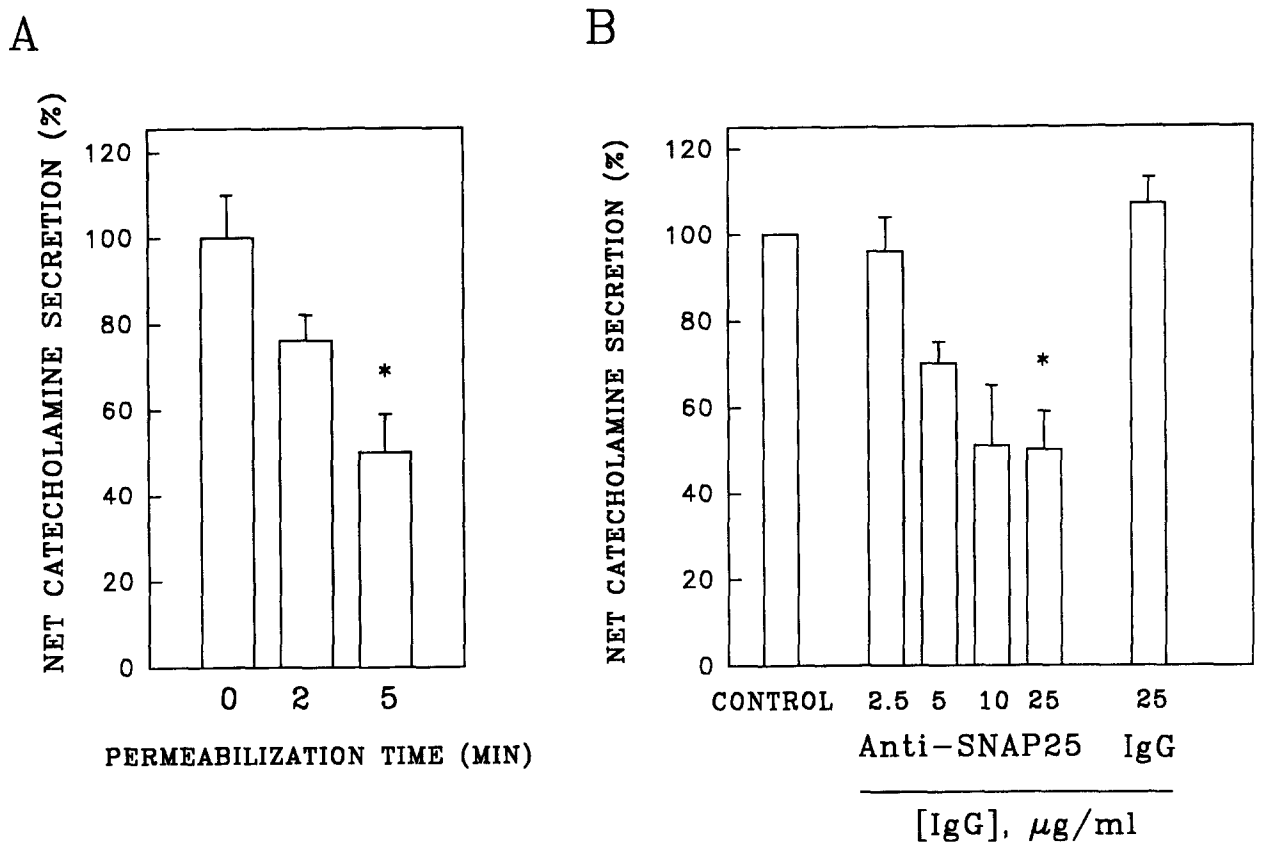

Fig. 2. Anti-SNAP-25 monoclonal antibody blocks Ca^{2+}-dependent catecholamine release from permeabilized chromaffin cells. (A) Time-dependence of inhibition. Cultured cells were incubated with [^{3}H]noradrenaline before digitonin-permeabilization for the indicated time periods in the presence or absence of anti-SNAP-25 mAb (25 μg IgG/ml). Thereafter, secretion was induced for 10 min in media containing either 5 mM EGTA or 10 μM Ca^{2+} both in the absence or presence of the antibody. (B) Dose–response curve. Increasing amounts of the anti-SNAP-25 mAb were incubated during detergent permeabilization for 5 min. Control denotes the absence of the antibody or the presence of mouse IgG at the indicated concentration. Secretion is expressed as net release (difference between stimulated and basal). Data are mean ± S.E.M. from 4 independent experiments. Statistical significance was assessed using the Student's *t*-test. $*P < 0.01$ with respect to control.

permeabilized chromaffin cells (Fig. 3A). At 100 μM, SNAP-25(187–206) inhibited 65% of the Ca^{2+}-dependent [^{3}H]noradrenaline release, but had minor effects on constitutive exocytosis. A control peptide, with the same amino acid composition as SNAP-25(187–206) but in random order (Fig. 3A, SNAP-25(187–206)RD: TDSSGREMIKANKQLANGTR), had no effect on Ca^{2+}-dependent secretion. Therefore, the inhibitory effect of SNAP-25(187–206) is sequence-specific. Shorter peptides were inactive (Fig. 3A, SNAP-25(195–201): ANQRATK), suggesting that a minimum peptide length may be neccesary to support an active conformation. Furthermore, a peptide mimicking an N-terminal motif presumably involved in the interaction with the light chain of BoTx A and E [26] (SNAP-25(49–59) (aa 49–59: MLDEQGEQLER)) did not affect Ca^{2+}-triggered secretion (Fig. 3A). A dose–response curve for the inhibition of [^{3}H]noradrenaline release by SNAP-25(187–206) is shown in Fig. 3B. The concentration of peptide required to inhibit half of the maximal secretion was 20 μM, with maximal inhibition of 65% at 100 μM of peptide.

Taken together, these data indicate that the carboxy-terminal domain of SNAP-25 is involved in Ca^{2+}-evoked catecholamine release. The inhibition potency of SNAP-25(187–206) is 10-fold higher than that reported for peptides mimicking regions of the soluble *N*-ethylmaleimide sensitive fusion protein attachment (SNAP) proteins injected in the giant synapse of the squid axon [27], suggesting that SNAP-25 is critical for Ca^{2+}-regulated membrane fusion. The partial blockade of Ca^{2+}-evoked secretion exerted by the SNAP-25(187–206) and the anti-SNAP-25 mAb resembles that produced by BoTx A in chromaffin and pancreatic β cells [28–30]. Incomplete block of Ca^{2+}-regulated exocytosis appears compatible with the occurrence of at least two releasable vesicle pools, the docked and primed vesicles [2,30,31]. Our observations suggest that SNAP-25(187–206) might interfere with vesicle priming at the plasma membrane. Accordingly, the pool of primed vesicles, which would be insensitive to SNAP-25(187–206), the mAb or of BoTx A, may account for the remaining unblocked secretion.

3.4. The C-terminal domain of SNAP-25 regulates excitation–secretion coupling in endocrine cells

Our results provide functional evidence for the presence of the SNAP receptor (SNARE) proteins in neuroendocrine cells [17,18,32], which together with action of clostridial neurotoxins on secretion [2,12–14], strongly support the hypothesis of a common secretory machinery present in neural and neuroendocrine cells consisting of the described SNARE elements of the fusion complex [33].

A main result of this study is that a 20-mer peptide that

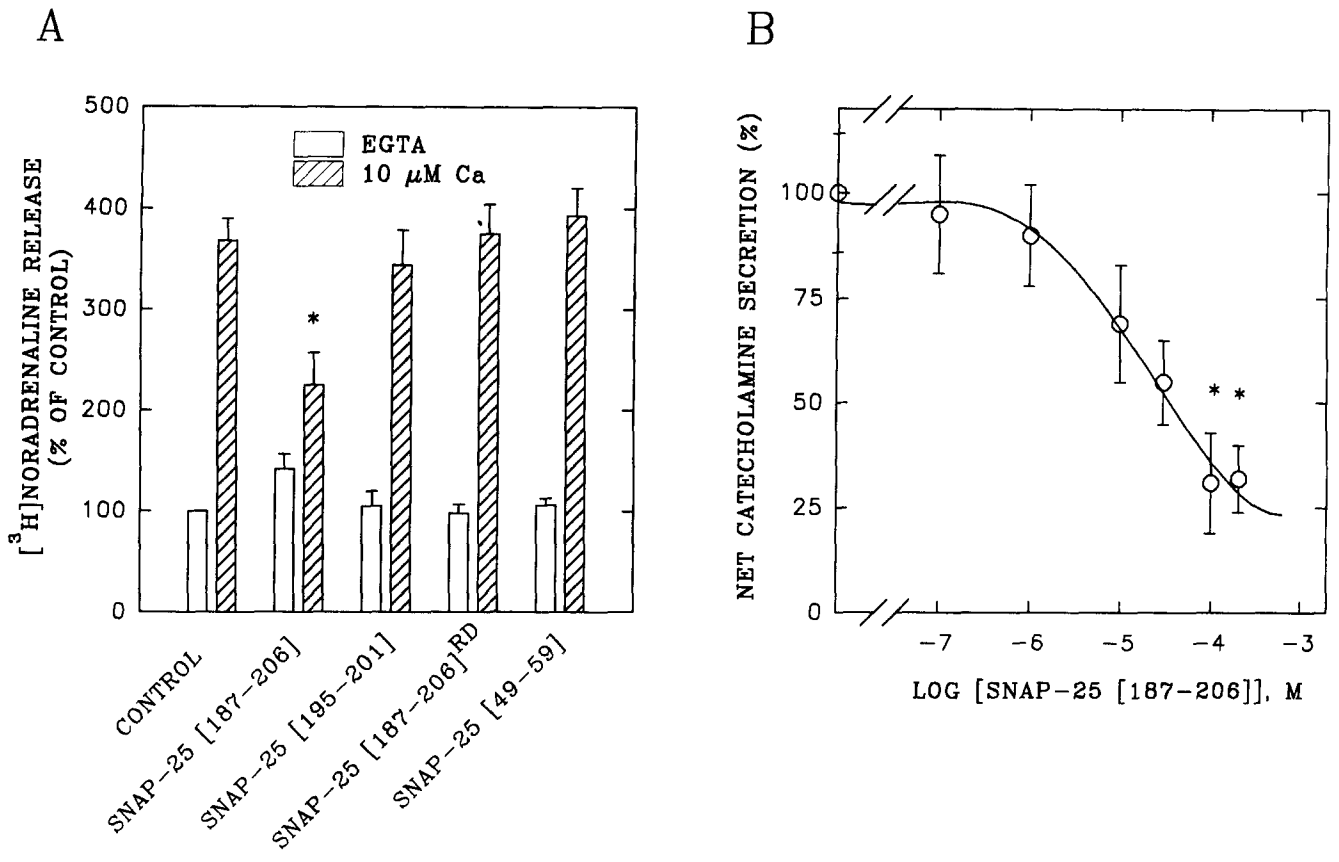

Fig. 3. A synthetic peptide representing the C-terminal sequence of SNAP-25 inhibited Ca^{2+}-regulated exocytosis from permeabilized chromaffin cells. Digitonin-permeabilization lasted 5 min and [^{3}H]noradrenaline secretion was evoked in the absence (5 mM EGTA) or presence of Ca^{2+}. (A) Inhibition of exocytosis is sequence specific. Shown is the effect on the basal and Ca^{2+}-stimulated release of 100 μM of SNAP-25(187–206) (SNKTRIDEANQRATKMLGSG), SNAP-25(195–201) (ANQRATK), SNAP-25(187–206)RD (TDSSGREMIKANKLANGTR) and SNAP-25(49–59) (MLDEQGEQLER). (B) Dose-dependent inhibition of Ca^{2+}-evoked secretion by SNAP-25(187–206). [^{3}H]Noradrenaline is expressed as net release. Data are mean ± S.E.M. from 4 different experiments performed in triplicate. Statistical significance was assessed using the Student's *t*-test. $^*P < 0.01$ as compared with control in the absence of peptide.

mimics the carboxy-terminal domain of SNAP-25 effectively blocks the Ca^{2+}-dependent release of catecholamines from chromaffin cells without affecting the constitutive exocytotic pathway. The blocking effect mimics that exerted by the catalytically-active light chains of BoTx A and E [12–14], that cleave SNAP-25 single sites in the C-terminal domain [23–24]. This cleavage is sufficient to disable the fusion process that leads to secretion by inhibiting vesicle priming [2,30]. Presumably, the C-terminal domain of SNAP-25 is required for the competent attachment of the fusion complex to the plasma membrane that precedes the fusion event [2,11]. The SNAP-25 C-terminal peptide effectively produces similar effects to BoTx A and E and conceivably may inhibit vesicle priming. In other words, the peptide appears to uncouple excitation from secretion. We therefore propose the term ESUP for the 'excitation–secretion uncoupling peptide'. It is anticipated that ESUP would block the calcium regulated fusion events that lead to neurotransmitter release in neuronal synapses. The notion embodied in the ESUP activity suggests alternative pathways to regulate synaptic vesicle exocytosis and points to previously unrecognized means of intervention.

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