A Peptide That Mimics the C-terminal Sequence of SNAP-25 Inhibits Secretory Vesicle Docking in Chromaffin Cells*

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Excitation-secretion uncoupling peptides (ESUPs) are inhibitors of Ca2+-dependent exocytosis in neural and endocrine cells. Their mechanism of action, however, remains elusive. We report that ESUP-A, a 20-mer peptide patterned after the C terminus of SNAP-25 (synaptosomal associated protein of 25 kDa) and containing the cleavage sequence for botulinum neurotoxin A (BoNT A), abrogates the slow, ATP-dependent component of the exocytotic pathway, without affecting the fast, ATP-independent, Ca2+-mediated fusion event. Ultrastructural analysis indicates that ESUP-A induces a drastic accumulation of dense-core vesicles near the plasma membrane, mimicking the effect of BoNT A. Together, these findings argue in favor of the notion that ESUP-A inhibits ATP-primed exocytosis by blocking vesicle docking. Identification of blocking peptides which mimic sequences that bind to complementary partner domains on interacting proteins of the exocytotic machinery provides new pharmacological tools to dissect the molecular and mechanistic details of neurosecretion. Our findings may assist in developing ESUPs as substitute drugs to BoNTs for the treatment of spasmodic disorders.

Ca2+-dependent exocytosis is a highly regulated process in neural and endocrine cells. The molecular components and events involved in the exocytotic cascade are fast-emerging thanks to the convergence of genetic, biochemical, pharmacological, and biophysical approaches (1–7). The information accrued in recent years has led to the formulation of the SNAP1

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The abbreviations used are: SNAP, soluble NSF attachment protein; NSF, N-ethylmaleimide-sensitive fusion protein; ESUP, excitation-secretion uncoupling peptide; BoNT, botulinum neurotoxin; SNAP-25, synaptosomal associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; SNAP, SNAP receptor; v-SNARE, vesicle SNAP; t-SNARE, target SNAP; Fmoc, N-9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; NE, noradrenaline.
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sequence of segments from synaptotagmin (22, 23), SNAPs (24), synaptobrevin (25) and SNAP-25 (26, 27) were shown to be specific inhibitors of neurosecretion. These peptides, for which the term ESUP was coined to highlight their activity (26), are useful pharmacological tools to probe the functional role of distinct protein components in the secretory machinery, to dissect the contribution of specific domains in the protein-protein interactions that mediate the process and to identify steps in the exocytotic cascade. The use of this new set of reagents, however, remains limited because the mechanism underlying their inhibitory activity is unknown. Here, we characterize the molecular steps of the exocytotic process that are sensitive to the blocking activity of a 20-mer peptide that mimics the amino acid sequence of the C-terminal domain of SNAP-25 (SNAP-25-187–206): SNKTRIDEANQATKM-LGSG; denoted as ESUP-A. We find that ESUP-A arrests Ca\textsuperscript{2+}-dependent secretion from permeabilized chromaffin cells by inhibiting vesicle docking.

MATERIALS AND METHODS

Reagents—\textsuperscript{[3H]}Noradrenaline was from Du Pont. t-butoxycarbonyl and Fmoc amino acids, with standard side chain protecting groups, were obtained from Applied Biosystems (Foster City, CA). Solvents, reagents, and resins for peptide synthesis were obtained from Applied Biosystems (Foster City, CA). All other reagents were of analytical grade from Sigma. BoNT A was a gift of B. R. DasGupta (University of Wisconsin, Madison, WI).

Peptide Synthesis and Purification—ESUP-A (SNKTRIDEANQRATKLMGSG) and ESUP-A\textsuperscript{3-21} (TDSSGEMIKWKLQANTGR) were synthesized by t-butoxycarbonyl or Fmoc\textsuperscript{®} Fmoc chemistries in an Applied Biosystems 431A automated solid-phase peptide synthesizer, and cleaved as described (26). Cleaved peptides were purified by reverse-phase HPLC with a Vydac C-18 semi-preparative column. Samples of crude peptide (10–20 mg) were dissolved in 0.1% trifluoroacetic acid, 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 assessed by reverse-phase HPLC in a Vydac C-18 analytical column.

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 as described (26, 28). Cells were maintained in monolayer cultures at a density of 625,000 cells/cm\textsuperscript{2} and were used between the 3rd and 6th day after plating. All the experiments were performed at 37 °C.

Determination of Catecholamine Release from Detergent-permeabilized Chromaffin Cells—Secreted \textsuperscript{[3H]}noradrenaline was determined in digested permeabilized cells as described (26, 29). Briefly, cells were incubated with \textsuperscript{[3H]}noradrenaline (1 μCi/mL) in Dulbecco’s modified Eagle’s medium supplemented with 0.56 mM ascorbic acid during 4 h. Thereafter, monolayers were washed four 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\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgCl\textsubscript{2}, 2.5 mM CaCl\textsubscript{2}, 0.56 mM ascorbic acid, and 11 mM glucose. Cell permeabilization was accomplished with 20 μM digitonin in 20 mM Pipes, pH 6.8 with 140 mM monosodium glutamate, 2 mM MgCl\textsubscript{2}, 2 mM Mg-ATP, and 5 mM EGTA. This incubation was carried out in the absence or presence of 100 μM ESUP-A, ESUP-A\textsuperscript{3-21}, or 100 mM dithiothreitol (DTT)-reduced BoNT A as indicated. BoNT A was reduced with 10 mM DTT for 30 min at 37 °C. Following permeabilization, media were discarded, and cells were incubated for 10 additional min in digitonin-free medium in presence or absence of peptides. Basal secretion was measured in 5 mM EGTA, whereas stimulated secretion was measured in a medium containing 10 μM buffered Ca\textsuperscript{2+} solution. Media were collected, and released catecholamines as well as the total cell content were determined by liquid scintillation counting. Statistical significance was calculated using Student’s \textit{t} test with data from four or more independent experiments.

Electron Microscopy of Permeabilized Chromaffin Cells—Chromaffin cells (10\textsuperscript{5} cells/well) were permeabilized and peptide (100 μM) or DTT-reduced BoNT A (100 nM) incubated as described above. Cells were collected by low speed centrifugation (1,000 × g for 2 min) and fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.06 M cacodylate buffer at pH 7.3 for 2 h. The pellets were postfixed in 1.33% osmic acid during 1.5 h, in s-collidine buffer for ≥2 h, dehydrated, and embedded in Epon (30). Thin sections (~0.5 μm) were obtained using a Reichert Ultracut-E ultramicrotome (Vienna, Austria). Electron microscopy was performed using a transmission electron microscope (Zeiss EM-10, Oberkochen, Germany).

Vesicle density distribution was estimated in digitized micrographs using the NIH Image analysis program. For each chromaffin cell, the distance between the dense core granules and the plasma membrane was measured and binned using a bin width of 0.15 μm. To obtain a distance distribution histogram (Fig. 5), the number of granules occurring at a given point was plotted as a function of the average distance between the plasma membrane, defined at 0 μm, and the nuclear membrane, at ~3 μm. Data are given as mean with standard error of each group for each condition; the number of vesicles (n) counted per cell was 240 ≤ n ≤ 290, and the experimental error 10% ≤ e ≤ 15%.

RESULTS AND DISCUSSION

ESUP-A Inhibits the Slow Component of the Ca\textsuperscript{2+}-evoked NE Release from Permeabilized Chromaffin Cells—Ca\textsuperscript{2+}-evoked exocytosis from permeabilized chromaffin cells can be kinetically dissected in two distinct components (31, 32) (Fig. 1): a fast component that may be detected few seconds after Ca\textsuperscript{2+} application, lasts ~1 min, and releases ~40% of the total \textsuperscript{[3H]}NE, followed by a slow component that proceeds for ~15 min. This biphasic behavior of catecholamine release has been associated with the presence of at least two distinct pools of secretory granules at different stages of the exocytotic cascade (3–6, 33). The fast component appears to represent the release of neurotransmitter from a population of primed, readily releasable vesicles, whereas the slow component corresponds to a pool of docked vesicles that must undergo priming prior to fusion and other releasable pools of nondocked granules (3–6, 31, 33). We investigated if ESUP-A targeted a specific step of the secretory pathway. As shown in Fig. 1, incubation of permeabilized chromaffin cells with 100 μM ESUP-A inhibited ~60% of catecholamine release by primarily altering the slow phase of secretion. These data suggest that the pool of primed vesicles is insensitive to the action of ESUP-A.

To gain further insights on the specific secretory step inhibited by ESUP-A, we attempted to dissociate the two distinct components of the exocytotic process by performing a double Ca\textsuperscript{2+} pulse secretion assay. The rationale considered that a short (2 min) Ca\textsuperscript{2+} pulse would primarily deplete the pool of primed vesicles, with minor effects on docked vesicles. A subsequent longer (10 min) Ca\textsuperscript{2+} pulse would induce docking, priming, and fusion of other vesicle pools. As illustrated in Fig. 2, a short Ca\textsuperscript{2+} pulse triggered significant exocytosis of \textsuperscript{[3H]}NE
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from chromaffin cells that is highly insensitive to 100 μM ESUP-A (−10% inhibition). In contrast, ESUP-A blocked ~80% of the Ca\textsuperscript{2+}-dependent exocytosis elicited by the second, longer Ca\textsuperscript{2+} pulse. A synthetic peptide with the same amino acid composition, yet random sequence (ESUP-A\textsuperscript{RDM}), blocked only ~10% of the Ca\textsuperscript{2+}-evoked neurosecretion, confirming that the ESUP-A inhibitory activity is sequence-specific. Taken together, these data suggest that ESUP-A inhibits Ca\textsuperscript{2+}-evoked catecholamine secretion by preventing vesicle docking or priming at the active zone, yet do not argue in favor of one or the other.

ESUP-A Blocks the Energy-dependent Steps of Ca\textsuperscript{2+}-mediated Exocytosis in Chromaffin Cells—Catecholamine release from chromaffin cells is an energy-dependent process requiring ATP hydrolysis to induce and stabilize the primed state of secretory vesicles before the Ca\textsuperscript{2+}-induced fusion event (1−7, 31, 32). Vesicle priming is composed primarily of an ATP-dependent component followed by a temperature-sensitive step that leads to the Ca\textsuperscript{2+}-induced exocytosis (32–34). As shown in Fig. 3, incubation of permeabilized chromaffin cells with 100 μM of ESUP-A attenuated ~60% of the Ca\textsuperscript{2+}-induced catecholamine secretion when 2 mM ATP was present in the incubation and stimulation media (Fig. 3). The inhibitory effect was sequence-specific as evidenced by the ineffectiveness of ESUP-A\textsuperscript{RDM}. Removal of ATP from the incubation and stimulation media diminished ~40% of the Ca\textsuperscript{2+}-evoked release of \textsuperscript{[3]H]NE (32, 34). Note that the ATP-independent secretion was not affected by ESUP-A. As expected, ESUP-A did not affect the temperature-sensitive step that follows the ATP-dependent transition (data not shown). These findings suggest that ESUP-A interferes with the steps that precede the ATP-dependent activation of secretory vesicles.

ESUP-A Induces Accumulation of Secretory Granules Near the Plasma Membrane of Chromaffin Cells—The morphology of secretory granules in chromaffin cells treated with peptides was examined by electron microscopy (Fig. 4). A main goal was to detect changes in secretory granule distribution induced by ESUP-A. For comparison purposes, the effect of BoNT A, known to prevent vesicle docking (35, 36), was investigated. Electron micrographs of permeabilized, unstimulated chromaffin cells display a random distribution of dense core secretory vesicles (Fig. 4A). Ca\textsuperscript{2+} stimulation induces a significant depletion of granules, as evidenced by the reduced number of vesicles in the cytosol (Fig. 4B), in accord with other reports (33). Notably, the presence of ESUP-A produced an accumulation of vesicles near the plasma membrane (Fig. 4C), similar to that produced by BoNT A (Fig. 4D).

FIG. 2. ESUP-A blocks catecholamine secretion in permeabilized chromaffin cells evoked by the second of two consecutive Ca\textsuperscript{2+} pulses. Secretion was elicited by 10 μM of free Ca\textsuperscript{2+} or in basal media (5 mM EGTA) for 2 min (1st pulse). Media were collected, and cells were incubated for 5 min in Ca\textsuperscript{2+}-free buffer. Thereafter, a 10 min Ca\textsuperscript{2+} stimulation was applied (2nd pulse). Three conditions were assayed: Control, cells incubated with 100 μM ESUP-A (SNKTRIDEANQBATKMLGSG); and cells exposed to 100 μM ESUP-A\textsuperscript{RDM}, a 20-mer peptide with the same amino acid composition of ESUP-A but random sequence (TDSSGREMIKQLANGTR). The counts/min released from control cells under basal conditions were ~4,000 and increased to ~13,000 after stimulation with 10 μM Ca\textsuperscript{2+}. The total number of counts obtained from detergent-permeabilized cells was ~130,000. Thus, the normalized basal and the Ca\textsuperscript{2+}-evoked release represent the 3.5% and ~10% of the total secretion, respectively. Data are given as mean ± S.E. with n (number of experiments performed in triplicate) = 4. Other conditions are the same as described in the legend to Fig. 1.

FIG. 3. ESUP-A affects the ATP-dependent step of the exocytotic process. Ca\textsuperscript{2+}-evoked catecholamine secretion was elicited in presence (left) or absence (right) of 2 mM Mg-ATP at 37°C. Three conditions were assayed: Control, 100 μM ESUP-A; and 100 μM ESUP-A\textsuperscript{RDM}. Catecholamine secretion was triggered with 10 μM Ca\textsuperscript{2+} for 10 min. Basal secretion was evoked with 5 mM EGTA. Data are given as mean ± S.E. with n = 4 (experiments performed in triplicate). Other conditions were the same as described in the legend to Fig. 1.

FIG. 4. ESUP-A induces changes in chromaffin granule distribution after Ca\textsuperscript{2+} stimulation of digitonin-permeabilized cells. Cells were permeabilized as described in presence or absence of peptide. Secretion was triggered with 10 μM Ca\textsuperscript{2+} for 10 min. Thereafter, cells were collected by low speed centrifugation (1,000 × g, 2 min), fixed, and processed for electron microscopy. A, photomicrographs of cells incubated in a 5 mM EGTA medium without added Ca\textsuperscript{2+} (Unstimulated); B, cells stimulated with 10 μM of free Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-stimulated). C, Ca\textsuperscript{2+}-stimulated cells in the presence of 10 μM of ESUP-A. D, Ca\textsuperscript{2+}-stimulated cells treated with BoNT A. BoNT A (100 nM) inhibited ~60% of Ca\textsuperscript{2+}-induced neurotransmitter release. N denotes the cell nucleus. Magnification is 4,000-fold for A and 6,000-fold for B–D.
Frequency distribution histograms show that unstimulated (control) chromaffin cells are characterized by the occurrence of two populations of cargo vesicles (Fig. 5A, left): a predominant population with a mean distance to the plasma membrane of $-$0.5 $\mu$m and a second most frequent with a mean distance of $-$2.3 $\mu$m. The relative frequency of occurrence of these two vesicle populations was not significantly altered by treating the cells with peptides or BoNT A (Fig. 5, B–D, left). Ca$^{2+}$-stimulated exocytosis in control cells (Fig. 5A, right) or cells incubated with 100 $\mu$m ESUP-A$^{\text{RD M}}$ (Fig. 5B, right) decreased the total number of vesicles, as evidenced by the reduction in the area of both granule populations (Figs. 4B and 5A, right). By contrast, treatment with 100 $\mu$m ESUP-A or 100 nM BoNT A notably skewed the distribution of vesicles toward the plasma membrane, resulting in the virtual disappearance of the population centered at $-$2.3 $\mu$m and the ensuing increase of the granule population centered at $-$0.5 $\mu$m (Fig. 5, C and D, right). The majority of vesicles gathered at the active zone, however, are not in tight contact with the membrane, lending support to the notion that ESUP-A prevents vesicle docking.

**A Molecular Mechanism Underlying the Inhibitory Activity of ESUP-A**—The main result of our study is that a 20-mer peptide that mimics the C terminus of SNAP-25, effectively inhibits Ca$^{2+}$-dependent exocytosis in endocrine cells by preventing the docking of cargo vesicles at the plasma membrane. Catecholamine release in permeabilized chromaffin cells exhibits a biphasic time course characterized by an early ATP-independent component, and a slow, ATP-dependent phase (31–35). The ATP-independent phase presumably represents the Ca$^{2+}$-mediated steps that ultimately lead to fusion, while the ATP-dependent phase may be associated with vesicle priming events (1–7, 33). The final stages of neurosecretion in endocrine cells, therefore, may be described according to the following model (32, 34),

$$[\text{ATP}] \xrightarrow{\Delta T} \text{A} \xrightarrow{\text{Docking}} \text{B}_{\text{docked}} \xrightarrow{\text{Priming}} \text{C}' \xrightarrow{\text{Fusion}} \text{D}_{\text{primed}}$$

**Scheme 1**

where A denotes the state that leads to the docked state B, and C and C' represent the energy-dependent intermediates in the process of vesicle activation that lead to the primed state D; Ca$^{2+}$ triggers the process of fusion. Several lines of evidence support this mechanism: (i) the identification of an ATP-dependent step ([ATP]) before the actual Ca$^{2+}$-triggered fusion event (B$\Rightarrow$C) (32); (ii) the involvement of a temperature-sensitive process ($\Delta T$) between the ATP-dependent step and the Ca$^{2+}$-mediated fusion event, which appears to be the overall rate-limiting factor during priming (C$\Rightarrow$C') (34); (iii) the abrogation of vesicle docking by *Clostridial* neurotoxins (A$\Rightarrow$B), arresting the ATP-dependent steps of the cascade (3–6, 35–37).

Although this model is an oversimplification of the complex exocytic cascade, it provides a basic framework to understand the molecular mechanism underlying the inhibitory activity of excitation-secretion uncouplers such as ESUP-A.

Given the mechanism depicted in Scheme 1, our results with ESUP-A are compatible with the concept that the peptide pre-

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**Fig. 5.** ESUP-A induces accumulation of secretory vesicles in the active zone of chromaffin cells. The figure depicts frequency distribution histograms of number of granules as a function of distance to the plasma membrane for unstimulated cells (left panels) and Ca$^{2+}$-stimulated cells (right panel). Vesicle distance to plasma membrane was measured in digitized micrographs using the NIH Image program. Distance was binned using a bin width of 0.15 $\mu$m within the boundaries of 0–3 $\mu$m, defined as the average distance between the plasma membrane (0 $\mu$m) and the nucleus ($-$3 $\mu$m). Distance distribution histograms were obtained for unstimulated cells (Unstimulated) and cells stimulated with 10 $\mu$m Ca$^{2+}$ (Ca$^{2+}$-stimulated). Four conditions were analyzed: Control, untreated cells; ESUP-A$^{\text{RD M}}$, cells treated with 100 $\mu$m ESUP-A$^{\text{RD M}}$; ESUP-A, cells treated with 100 $\mu$m ESUP-A; and BoNT A, cells treated with 100 nM DTT-reduced BoNT A. Data are given as mean with number of cells analyzed (n) $\geq$ 5.

Vesicles measured per cell was 240 $\leq \mu$ 290. The experimental error is 10% $\leq \epsilon$ 15%. Solid lines depict the best fit to a Lorentzian distribution with two components. For control cells these components were characterized by the following values: $\mu_1$ = 0.53 and $\mu_2$ = 2.3 $\mu$m, where $\mu$ is the mean distance to the plasma membrane. Different treatments and Ca$^{2+}$ stimulation did not affect the mean distance; the modifications were in the relative frequency of the two vesicle populations.
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Fast and slow components denote the net catecholamine release in a double Ca\(^{2+}\)-pulse secretion assay (Fig. 2). ATP-dependent and independent exocytosis refer to Ca\(^{2+}\)-evoked release in presence or absence of 2 mM ATP at 37 °C, respectively (Fig. 3). Number of vesicles in active zone are defined as vesicles near the plasma membrane (Figs. 4 and 5). The concentration of BoNT A was 100 nm. ESUP-A concentration was 100 μM. ND, not determined.

|                      | BoNT A       | ESUP-A       |
|----------------------|--------------|--------------|
| Ca\(^{2+}\)-dependent exocytosis | -60% inhibition | -60% inhibition |
| Fast component       | Insensitive  | Insensitive  |
| Slow component       | ND           | ~80% inhibition |
| ATP-dependent exocytosis | -70% inhibition | -60% inhibition |
| ATP-independent exocytosis | Insensitive  | Insensitive  |
| Number of vesicles in active zone | Increase | Increase |

* BoNT A values were taken from Lawrence et al. (35).

vent the formation of the docked state B and the ensuing intermediates in the exocytic pathway. The finding that ESUP-A does not affect the fast, ATP-independent, Ca\(^{2+}\)-mediated component (Figs. 1 and 2) suggests that its site of action is upstream of the fusion event. That ESUP-A abrogates the slow, ATP-dependent (B \(\rightarrow\) C) component (Fig. 3) is consistent with an inhibition of the formation of the primed state C, either by blocking the transition from the docked state B to the first step of the priming process or by inhibiting vesicle docking. The observation that ESUP-A promotes a massive accumulation of secretory vesicles near the plasma membrane of chromaffin cells (Figs. 4 and 5), but not in tight contact, implies that the peptide blocks the formation of the docked state. This mechanism of action of ESUP-A is similar to that proposed for BoNT A (Table I) (35–37).

How does ESUP-A prevent vesicle docking? During docking, a ternary complex involving VAMP, SNAP-25, and syntaxin is formed, the so-called SDS-resistant complex (3–6, 38). A sequence of protein-protein interactions starts with binding of SNAP proteins to the ternary aggregate and recruitment of NSF. The next step involves ATP hydrolysis that energizes and rearranges the core complex, making it competent for Ca\(^{2+}\)-induced fusion (3–6). Since the C-terminal domain of SNAP-25 binds tightly to VAMP during docking (5, 38), it is conceivable that VAMP is a complementary binding partner for the peptide. How does ESUP-A binding to VAMP block exocytosis? A plausible mechanism considers that ESUP-A competes with SNAP-25 for binding to VAMP and, thereby, prevents the formation of the critical SDS-resistant complex, comprising SNAP-25-VAMP-syntaxin (3–6, 38, 39). Absence of this ternary complex would hinder the association of SNAP and NSF proteins and the subsequent ATP-hydrolysis, therefore, preventing vesicle docking, priming, and fusion. Two findings suggest that the peptide binds directly to VAMP and interrupts the subsequent chain of protein-protein interaction events that lead to vesicle fusion. First, ESUP-A promotes the accumulation of secretory vesicles near the active zone (Figs. 4 and 5). Second, the peptide arrests the ATP-dependent maturation of the secretory granules (Fig. 3). Direct binding measurements of ESUP-A to VAMP in vitro have produced inconclusive results, presumably arising from low affinity (micromolar) of the interaction. Indeed, given a micromolar affinity constant, and as-

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