Communication

Novel Ca\(^{2+}\)-binding Protein (CAPS) Related to UNC-31 Required for Ca\(^{2+}\)-activated Exocytosis*

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Exocytotic secretion in neuroendocrine cells is activated by cytoplasmic Ca\(^{2+}\) increases. Late post-docking events in dense core vesicle exocytosis in permeable PC12 cells require cytosolic factors for sequential ATP-dependent priming and Ca\(^{2+}\)-dependent triggering steps. The cytosolic proteins phosphatidylinositol transfer protein and phosphatidylinositol (4)-phosphate 5-kinase, as well as membrane-bound N-ethylmaleimide-sensitive factor, are required for the ATP-dependent priming step. Following priming, the Ca\(^{2+}\)-dependent triggering of vesicle fusion requires an additional cytosolic factor, CAPS, which was purified as a 145-kDa protein. To clarify late Ca\(^{2+}\)-dependent events in vesicle fusion, the sequence of rat CAPS cDNA was determined and found to encode a novel protein that is the vertebrate homologue of the Caenorhabditis elegans UNC-31 protein shown genetically to be required for neurosecretion. Recombinant CAPS substituted for cytosol in the Ca\(^{2+}\) triggering step in permeable PC12 cells and exhibited moderate affinity (K\(_d\) = 270 \mu M) Ca\(^{2+}\) binding (2 mol Ca\(^{2+}\)/mol CAPS dimer), consistent with a role at a Ca\(^{2+}\)-regulated step in exocytosis.

Neurotransmitter and peptide hormone secretion are mediated by the fusion of secretory vesicles with the plasma membrane, an exocytotic process that requires ATP and is strongly dependent upon and activated by Ca\(^{2+}\). Insights into the molecular basis of regulated membrane fusion have been provided by the identification of several required synaptic proteins such as vesicle-associated membrane protein/synaptobrevin, syntaxin, and SNAP-25, which are substrates for clostridial neurotoxin proteases (1) and receptors for N-ethylmaleimide-sensitive factor/SNAP proteins (2), and synaptotagmin, a vesicle Ca\(^{2+}\)-binding protein (3). However, genetic screens in Caenorhabditis elegans indicate that a large number of other gene products are required for neurosecretion (4). Additional novel components required for regulated exocytosis in neuroendocrine cells have been identified by the reconstitution of Ca\(^{2+}\)-stimulated secretion in permeable cells (5–7).

Clostridial neurotoxin-sensitive secretion of norepinephrine (NE) via dense core granule exocytosis in permeable PC12 cells requires Ca\(^{2+}\), ATP, and cytosolic proteins (7, 8). The requirement for ATP precedes that for Ca\(^{2+}\) and both ATP-dependent and Ca\(^{2+}\)-activated steps require cytosolic factors (9). Preincubation of permeable PC12 cells with ATP and cytosolic proteins that catalyze phosphatidylinositol 4,5-bisphosphate synthesis (5, 6, 9) advances the exocytotic apparatus to a primed, post-docking state (10) from which vesicle fusion with the plasma membrane is rapidly triggered by Ca\(^{2+}\) and Ca\(^{2+}\)-activated exocytosis from the primed state is independent of MgATP but requires neural or neuroendocrine tissue cytosol (7, 9, 10). The required tissue-specific factor CAPS (for Ca\(^{2+}\)-dependent activator protein for secretion) was purified as a 145-kDa protein from rat brain cytosol (previously termed p145) based on its reconstituting activity (7). To further clarify the role and mechanism of CAPS action at a late post-priming step in the exocytotic pathway, cDNA characterization was undertaken. The sequence of rat CAPS revealed that it is the vertebrate homologue of the UNC-31 protein required for neurosecretion in C. elegans.

EXPERIMENTAL PROCEDURES

Secretion Assay—EGTA-extracted, permeable cells were prepared from [\(^{3}H\)]NE-labeled PC12 cells by a single pass through a ball homogenizer as described (7–9). Secretion assays were conducted in 0.05 mM HEPES, pH 7.2, 0.12 mM potassium glutamate, 0.02 mM potassium acetate, 0.002% EGTA, and 0.1% bovine serum albumin. Permeable cells were primed in 30-min incubations at 30 °C with 2 mM MgATP and 1.0 mg/ml rat brain cytosol, chilled, washed at 4 °C, and resuspended. Triggering incubations were conducted at 30 °C with 10 \mu M Ca\(^{2+}\) with rat brain cytosol, purified rat brain CAPS, or purified baculoviral recombinant CAPS. CAPS was purified from rat brain cytosol by modification of previous methods (7) omitting phenyl-TSK and Matrix Green chromatography. Immunoglobulins, when tested, were introduced after priming during a 60-min incubation at 4 °C prior to triggering. Immunoglobulins were purified by protein A-Sepharose chromatography of rabbit serum taken prior to or following immunization with purified rat CAPS. Release of [\(^{3}H\)]NE was determined as described (7–9).

cDNA Characterization and Recombinant Protein Production—Rat brain (CLONTECH Laboratories RL1043b) and PC12 cell (kindly provided by Dr. J. Boulter) cDNA libraries were screened with CAPS antibodies (11) and with a polymerase chain reaction probe designed from degenerate primers (12) corresponding to amino acids 561–595. CAPS protein sequences were obtained from reverse phase high pressure liquid chromatography-purified peptides derived from cleavage of purified rat CAPS (7) with endoprotease Lys C. Two clones from the rat brain cDNA library and three clones from PC12 cDNA library were characterized. The coding region of 1289 codons (accession number U16802) began at an ATG (nucleotide 62) that conformed to consensus sequences for eukaryotic translation initiation (13) and ended at a termination codon (nucleotide 3929) followed by a polyadenylation signal (nucleotide 4901) and the beginning of a poly(A) tract (nucleotide 4924). Two cDNAs with slightly truncated coding regions representing alternative splicing at nucleotides 3505 or 3538 were also characterized. cDNAs were cloned into pBluescript vector and sequenced using Sequenase version 2.0 (U. S. Biochemical Corp.). Recombinant CAPS was produced by standard methods (14) employing the pBlueBacHis vector by expression in SF21 or High Five cells (Invitrogen Corp.). CAPS was purified from insect cell cytosol by anion exchange chromatography on a Poros HQ/M column (PerSeptive Biosystems Inc.). Northern blotting was conducted with a human multiple tissue blot (CLONTECH Laboratories) containing 2 \mu g of poly(A)

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1 The abbreviations used are: NE, norepinephrine; kb, kilobase(s); BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N,N-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

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Ca\(^{2+}\)-dependent NE secretion in ATP-primed permeable PC12 cells is stimulated by CAPS and inhibited by IgGs from CAPS immune serum but not preimmune serum. Incubations for the indicated times contained no additions (squelo), 10 \( \mu \text{M} \) Ca\(^{2+}\) without further additions (○), or 10 \( \mu \text{M} \) Ca\(^{2+}\) with 1 mg/ml CAPS immune IgGs (▲), 1 mg/ml preimmune IgGs (□), 0.5 mg/ml rat brain cytosol (●), or 5 \( \mu \text{g/ml} \) purified CAPS (□). CAPS immune IgGs completely block the reconstituting activity of rat brain cytosol or added CAPS (not shown). The inset shows initial 10 s of a similar experiment demonstrating that CAPS antibody blocked the exocytosis of all dense core vesicles at early times. Symbols show the mean of duplicate determinations that differed from the mean by less than 5%.

**Fig. 1.** CAPS is a necessary and sufficient cytosolic factor for Ca\(^{2+}\)-triggered vesicle exocytosis. Ca\(^{2+}\)-dependent NE secretion in ATP-primed permeable PC12 cells is stimulated by CAPS and inhibited by IgGs from CAPS immune serum but not preimmune serum. Incubations for the indicated times contained no additions (squelo), 10 \( \mu \text{M} \) Ca\(^{2+}\) without further additions (○), or 10 \( \mu \text{M} \) Ca\(^{2+}\) with 1 mg/ml CAPS immune IgGs (▲), 1 mg/ml preimmune IgGs (□), 0.5 mg/ml rat brain cytosol (●), or 5 \( \mu \text{g/ml} \) purified CAPS (□). CAPS immune IgGs completely block the reconstituting activity of rat brain cytosol or added CAPS (not shown). The inset shows initial 10 s of a similar experiment demonstrating that CAPS antibody blocked the exocytosis of all dense core vesicles at early times. Symbols show the mean of duplicate determinations that differed from the mean by less than 5%.

**Calcium Binding—** Recombinant CAPS at 50–70 \( \mu \text{M} \) with 0.05 mg/ml α2 macroglobulin to inhibit proteolysis was dialyzed in small (30 \( \mu \text{l} \)) floating dialysis chambers at 4 °C for 24 h with 10 \( \mu \text{Cl/ml} \) Ca\(^{2+}\) (NEN Life Science Products) in 0.02 M HEPES, pH 7.2, 0.1 \( \mu \text{M} \) KCl, 0.0001 \( \mu \text{M} \) EGTA adjusted to indicated free Ca\(^{2+}\) concentrations as calculated with the program CHELATOR. Ca\(^{2+}\) binding at equilibrium was determined in triplicate. CAPS concentration following dialysis was determined by dye binding (Bio-Rad) and by Coomassie staining following gel electrophoresis. Data are representative of two complete and seven partial binding curve determinations. Negative (bovine serum albumin and H\textsubscript{2}O\textsubscript{2}-SNAP) and positive (troponin C and calmodulin) controls for Ca\(^{2+}\) binding were determined in parallel with CAPS. Binding data were fit to a two-site model with cooperative and hyperbolic components using the equation \( B = [B_{\text{max}} \times x \text{Ca}^{2+} \times I] / [K_{\text{ICa}^{2+}} + (\text{Ca}^{2+})^n] + [B_{\text{max}} - x \text{Ca}^{2+} \times I] / [K_{\text{ICa}^{2+}} + (\text{Ca}^{2+})^n] \). Proteolytic digestions of CAPS were conducted at room temperature for 60 min in 0.002 \( \mu \text{M} \) Tris, pH 8.0, 0.001 \( \mu \text{M} \) ATP, 0.05 \( \mu \text{M} \) KCl, 0.001 \( \mu \text{M} \) BAPTA, 0.001 \( \mu \text{M} \) dithiothreitol at the indicated free Ca\(^{2+}\) concentrations with a mass ratio of proteinase K to CAPS of 0.01–1. Digestions were terminated by the addition of 0.02 \( \mu \text{l} \) phenylmethylsulfonyl fluoride plus 0.001 \( \mu \text{M} \) aminoethylbenzenesulfonyl fluoride, and the samples were analyzed by SDS-PAGE for Coomassie staining and quantitation by densitometry. Control experiments with several proteins established that proteinase K activity toward other substrates was not affected by Ca\(^{2+}\) over this range of concentrations.

**RESULTS AND DISCUSSION**

Following completion of an ATP-dependent priming step, Ca\(^{2+}\) elicits rapid NE secretion from permeable PC12 cells, which is stimulated by a brain cytosol fraction (Fig. 1). Purified CAPS is entirely sufficient for replacing crude brain cytosol in Ca\(^{2+}\)-triggered secretion. Without added CAPS, Ca\(^{2+}\) triggered some NE secretion from the primed state, but this was completely eliminated by preincubation with CAPS-specific antibody (Fig. 1), indicating that it is mediated by the membrane-associated CAPS present in the permeable cells. The results show that Ca\(^{2+}\)-triggered exocytosis exhibits an absolute requirement for CAPS, implying a site of action at a late stage in the exocytotic pathway.

To further clarify the mechanism of CAPS action at a late post-docking, post-priming step, the amino acid sequence of CAPS was inferred from its cDNA. cDNAs from PC12 cell and rat brain libraries encoded a protein of 1289 amino acids with a predicted molecular mass of 146 kDa (Fig. 2A) corresponding closely to the estimated 145 kDa for CAPS by SDS-PAGE (7). Sequences of four endoprotease Lys C-derived CAPS peptides were present in the encoded protein (Fig. 2A), indicating that the cDNA corresponded to CAPS mRNA. cDNAs representing variants alternatively spliced in the 3′ coding region that encode 135- and 145-kDa bands, whereas CAPS (1282–1289)-specific antibody recognized only the 145-kDa band (not shown), consistent with the existence of isoforms of CAPS with C-terminal truncations.

The sequence of CAPS was overall hydrophilic without extended hydrophobic stretches despite the membrane association of a significant fraction of CAPS in brain homogenates. Two regions (residues 94–129 and 869–898) show significant potential for forming a coiled-coil (16), which could mediate the dimerization of CAPS (7) or its interaction with other proteins. A full-length CAPS protein expressed in insect cells was of predicted size and was reactive with native rat brain CAPS antibodies (Fig. 2B). Moreover, the recombinant CAPS supported Ca\(^{2+}\)-dependent secretion in permeable PC12 cells (Fig. 2B), confirming the authenticity of the CAPS cDNA. Northern blot analysis detected a 5.6-kb CAPS mRNA in brain, pancreas (Fig. 2C), hypothalamus, pituitary, and adrenal (not shown) but not in heart, placenta, lung, liver, skeletal muscle, or kidney (Fig. 2C). This tissue distribution for CAPS mRNA is similar to that of the CAPS protein with expression detected only in neural and endocrine secretory tissues (7).

Fasta (17) and Blast (18) searches indicated that the CAPS amino acid sequence was novel, exhibiting only limited similarity to data base entries in regions of low sequence complexity. A comparison of the rat CAPS sequence with that of the C. elegans unc-31 protein (19) (Fig. 2A) revealed that these proteins are homologues (75% similarity, 54% identity). Loss-of-function unc-31 mutants exhibit pleiotropic nervous system defects (19, 20) and enhanced resistance to acetylcholinesterase inhibitors (4). These properties and the distribution of the UNC-31 protein throughout the nervous system in C. elegans (19) suggest an essential role for CAPS in neurotransmitter and/or neuropeptide secretion.

To characterize potential mechanisms of CAPS function, the properties of a recombinant protein were further studied. The Ca\(^{2+}\)-dependent retention of CAPS in hydrophobic interaction chromatography (7) suggested that CAPS may be a Ca\(^{2+}\)-binding protein. Direct studies by equilibrium dialysis confirmed this suggestion (Fig. 3A), revealing two classes of Ca\(^{2+}\)-binding sites. The higher affinity of these sites \( (K_a = 270 \mu \text{M}) \), corresponding to 2 mol bound Ca\(^{2+}\)/mol CAPS dimer, exhibited cooperative \( (n = 2.7) \) binding over the range of 10–1000 \( \mu \text{M} \) Ca\(^{2+}\) (Fig. 3A, inset). The lower affinity sites \( (K_a = 4.3 \text{ mM}) \), corresponding to 10 mol bound Ca\(^{2+}\)/mol CAPS dimer, are unlikely to be of physiological significance and may correspond to clusters of acidic residues in the CAPS sequence. Ca\(^{2+}\)-binding to higher affinity sites promoted an increased susceptibility

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2 B. Berwin, personal communication.
FIG. 2. CAPS is a novel neural/endocrine-specific protein homologous to the C. elegans UNC-31 protein. A, amino acid sequence of rat CAPS (upper) and UNC-31 protein (lower). Sequences of Lys C-derived peptides from rat CAPS are in bold. The UNC-31 protein sequence of 1360 amino acids is from D. Livingstone (19). Inserts in the UNC-31 sequence of indicated length are indicated by carets. Amino acid sequences are shown in single-letter codes with identities and similarities indicated by lines and dots, respectively.

B, recombinant CAPS supports Ca\(^{2+}\)-triggered NE secretion. Left, baculovirus-encoded CAPS expressed as a 153-kDa fusion protein (arrowhead) in SF21 cell cytosol was analyzed by SDS-PAGE and Coomassie stain (left lane) or immunoblotting with CAPS antibody (right lane). Uninfected SF21 cells did not express the 153-kDa protein (not shown). Middle, stimulation of NE secretion by purified baculoviral CAPS (rCAPS) was Ca\(^{2+}\)-dependent with no activity observed in the absence of Ca\(^{2+}\). Incubations with ATP-primed permeable PC12 cells contained 10 μM Ca\(^{2+}\) and/or 0.01 μg/ml rCAPS as indicated. Right, purified baculoviral CAPS (●) supported Ca\(^{2+}\)-activated NE secretion from ATP-primed permeable PC12 cells to the same extent as purified rat brain CAPS (○). Incubations contained 10 μM Ca\(^{2+}\) and indicated amounts of CAPS in 0.1-ml reactions. C, Northern blot analysis of CAPS mRNA. A 5.6-kb RNA was detected in poly(A)\(^{+}\) RNA from brain and pancreas. Similar studies (not shown) detected the 5.6-kb RNA in pituitary, adrenal, hypothalamus, and pancreatic islets. A human β-actin cDNA probe was used to evaluate RNA loading.
of CAPS to proteolytic cleavage (Fig. 3B), consistent with a possible Ca\(^{2+}\)-dependent conformational change in the protein. Ca\(^{2+}\) regulates the exocytotic pathway at multiple sites that include actin cytoskeleton rearrangements (21), recruitment of vesicles to a release-ready pool (22), fusion of docked vesicles (23, 24), and expansion of the fusion pore (25), indicating that several stage-specific Ca\(^{2+}\) sensors participate in the regulation of exocytosis. There is evidence that synaptotagmin functions as a Ca\(^{2+}\)-dependent regulator of synaptic vesicle exocytosis (26, 27), although residual Ca\(^{2+}\)-dependent secretion in synaptotagmin null mutants suggests there may be additional Ca\(^{2+}\) sensors (26–28). The Ca\(^{2+}\) binding affinity exhibited by CAPS (K\(_d\) = 270 μM) is close to the half-maximal Ca\(^{2+}\) concentration (EC\(_{50}\) = ~200 μM) effective for triggering synaptic vesicle exocytosis (23). A role for CAPS in synaptic vesicle exocytosis may be indicated by the increased sensitivity of loss-of-function unc-31 mutants in C. elegans to acetylcholinesterase inhibitors (4). Direct studies demonstrate a role for CAPS in the exocytosis of dense core vesicles in neuroendocrine cells, but this process is triggered by lower levels (EC\(_{50}\) = −3–30 μM) of Ca\(^{2+}\) (24, 29). Additional studies are required to determine whether the function of CAPS at a late step in dense core vesicle exocytosis is Ca\(^{2+}\)-dependent. The availability of a functional recombinant CAPS should facilitate determination of its mechanism in exocytosis.

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