We present evidence that venom from the Brazilian scorpion Tityus serrulatus and a purified fraction selectively cleave essential SNARE proteins within exocrine pancreatic tissue. Western blotting for vesicle-associated membrane protein type v-SNARE proteins (or synaptobrevins) reveals characteristic alterations to venom-treated excised pancreatic lobules in vitro. Immunocytochemistry by electron microscopy confirms both the SNARE identity as VAMP2 and the proteolysis of VAMP2 as a marked decrease in secondary antibody-conjugated colloidal gold particles that are predominantly associated with maturezymogen granules. Studies with recombinant SNARE proteins were used to determine the specific cleavage site in VAMP2 and the susceptibility of VAMP8 (endobrevin). The VAMP2 cleavage site is between the transmembrane anchor and the SNARE motif that assembles into the ternary SNARE complex. Inclusion of divalent chelating agents (EDTA) with fraction ν, an otherwise active purified component from venom, eliminates SNARE proteolysis, suggesting the active protein is a metalloprotease. The unique cleavages of VAMP2 and VAMP8 may be linked to pancreatitis that develops following scorpion envenomation as both of these v-SNARE proteins are associated withzymogen granule membranes in pancreatic acinar cells. We have isolated antarease, a metalloprotease from fraction ν that cleaves VAMP2, and report its amino acid sequence.

Eukaryotic intracompartmental transport and secretory processes require fusion of vesicles with cellular membranes (1–3). An essential step leading to fusion is assembly of a tetrameric coiled-coil structure formed from sets of membrane proteins known as soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)² (4). High resolution structures are available for the final, postfusion ternary SNARE complex that combines one vesicle protein (v-SNARE) with target membrane proteins (t-SNAREs); however, our understanding of the transient, intermediate states involved in assembly, disassembly, and catalysis of membrane fusion remains incomplete. The SNAREs responsible for neuronal secretion are among the best studied examples of this family of proteins. They include the v-SNARE vesicle-associated membrane protein (VAMP; also known as synaptobrevin) that is located on the secretory vesicle membrane and two t-SNAREs, syntaxin and SNAP25 (synaptosome-associated protein of 25 kDa), present on the target membrane (5, 6). SNAREs are critical for selective transport between cellular compartments (7, 8). Alterations or damage to proteins like SNAREs that perform integral transport functions often produce disabling or irreversible consequences. Failure of the normal vesicular traffic (unless transient) that is the basis for intracellular transport and secretion results in development of disease (9, 10). Toxins that attack this machinery thus have significant effects on normal cellular processes.

Regulated secretion requires coordinated vesicular trafficking for all processes, including biosynthesis, transport, storage, and discharge (11–13). Signaling in exocrine secretion produces characteristic phosphorylation patterns of intracellular proteins that represent control mechanisms, but the connections between receptor activation and secretory discharge remain incompletely understood.

Pancreatic exocrine secretory discharge is normally mediated by neurological (cholinergic) and hormonal (peptidergic) controls that operate in parallel (14, 15). Earlier work in our laboratory determined that scorpion venom also initiates pancreatic secretion in vitro (16, 17). Clinical studies report that scorpion venom induces significant pathology, including acute pancreatitis in humans following envenomation (18, 19). Experimental evaluation of cellular effects of scorpion venom and its bioactive components reveals signaling that differs from patterns produced by the natural cholinergic and peptidergic secretagogues.³ In previous work, we found that tissues treated with these venom preparations produce linear dose-response curves in comparison with cholinergic and peptidergic exo-

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2 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; TSV, T. serrulatus venom; Fx, fraction; ZG, zymogen granule(s); ZGM, zymogen granule membrane(s); WT, wild type; carbachol, carbamylcholine chloride; SELDI, surface-enhanced laser desorption ionization; TOF, time-of-flight; PBS, phosphate-buffered saline; KRB, Krebs-Ringer bicarbonate.
3 P. L. Fletcher, Jr. and M. D. Fletcher, unpublished observations.
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crine pancreatic secretagogues; however, discharge is diminished at levels in excess of optimum secretory doses (16, 17, 20). Information about the possible mechanism by which the scorpion venom decreases secretion at these dose levels has been absent. In the present study, we report evidence of specific proteolysis of SNARE proteins involved in pancreatic secretion by venom from the Brazilian scorpion Tityus serrulatus (TSV) and a fraction of the venom, fraction v (Fx v).

Initial discovery of unique scorpion venom-mediated activity on secretory mechanisms stemmed from changes in tyrosine phosphorylation immunoblot patterns of cellular proteins. Using Western blot analysis, we found scorpion venom-mediated cleavage of secretory SNARE proteins (VAMP2 (synaptobrevin), VAMP3 (cellubrevin), and VAMP8 (endobrevin)) in intact tissue in vitro that could disrupt cellular transport and secretion functions (21, 22). Studies with recombinant proteins and Fx v demonstrated that the cleavage site for VAMP2 is 7 residues from the membrane attachment segment, in the loop between the coiled-coil SNARE bundle binding region and the transmembrane segment adjacent to its C terminus. VAMP8 is cleaved at a homologous site.

An active protein in Fx v from TSV, named antarease, was isolated, and more than 90% of the sequence is presented here. The activity of this protease is dependent on divalent ions, in agreement with the zinc-binding motif present in the sequence. Before this report, proteolytic cleavage of SNARE family components was known to be associated only with large microbial systems. The activity of this protease is dependent on divalent ions, which is consistent with the activity of some simpler systems involved in cellular transport and secretion functions (21, 22). Studies with recombinant proteins and Fx v demonstrated that the cleavage site for VAMP2 is 7 residues from the membrane attachment segment, in the loop between the coiled-coil SNARE bundle binding region and the transmembrane segment adjacent to its C terminus. VAMP8 is cleaved at a homologous site.

EXPERIMENTAL PROCEDURES

Materials—Carbachol chloride (carbachol) was from Sigma, and EDTA was from Calbiochem. Caerulein was a gift from Dr. J. Jamieson (Yale University, New Haven, CT). Whole dried T. serrulatus (Lutz and Mello) scorpion venom was from the Instituto Butantan (Sao Paulo, Brazil). Toxin γ, fractions v and λ, were prepared as described previously (17). Centruroides sculpturatus (Ewing) scorpion venom was the gift of Dr. Dean Watt (Creighton University, Omaha, NE). H4 ProteinChips and the calibration standard molecules for the surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometer were purchased from Ciphergen Biosystems Inc. (Fremont, CA). α-Cyano-4-hydroxycinnamic acid, sinapinic acid, iodoacetic acid, CNBr, and angiotsensin were obtained from Sigma. N-Isopropylidioacetamide was a gift from Dr. J. Inman (National Institutes of Health, Bethesda, MD).

Animals—Dunkin-Hartley guinea pigs were used for collection of pancreatic lobules for in vitro experiments as described (16). Protocols for animal tissue studies were approved by the East Carolina University Institutional Animal Care and Use Committee.

Pancreatic Lobule Experiments—For radiolabeled secretion dose-response assays, previously published protocols were followed (16, 17). For Western blots, excised lobules were prepared as above. Incubations in KRB under various conditions and times in vitro were as described (16, 17) but without radioactive activity. Lobules were homogenized in 25 mM HEPES buffer, pH 6.8, with protease inhibitors (27) and then stored at −20 °C. Protein concentration was determined using the BCA assay (Pierce).

Electron Microscopic Immunocytochemistry—Lobules were diced into ~2-mm pieces and fixed in 0.25% glutaraldehyde (Polysciences, Warrington, PA), 4% paraformaldehyde (EMS, Hatfield, PA) in 0.1 M sodium cacodylate buffer, pH 7.4, at 4 °C overnight. Embedment was in LR White (Polysciences) with thermal cure. Thin sections of 90 nm were collected on Formvar-coated nickel grids. Nonspecific binding was blocked by pretreatment in 5% normal goat serum. Grids were then incubated overnight at 4 °C in polyclonal VAMP2 primary antibody (1:50) (Stressgen, Victoria, Canada). To confirm specific labeling, some grids were incubated as negative controls in PBS, 1% normal goat serum without primary antibody. After PBS rinse, grids were transferred to 10-nm gold-conjugated goat anti-rabbit IgG secondary antibody (1:100) (British Biocell International, Teg Pella, Inc., Redding, CA) for 1 h. Grids were stained in 4% aqueous uranyl acetate (EMS). Sections were examined in a JEOL 1200EX electron microscope equipped with iTEM digital image acquisition software (Soft Imaging System Gmbh, Munster, Germany). Morphometric image analysis was conducted with iTEM. Results represent two experiments and five immunogold incubations.

Isolation of Zymogen Granules and Membranes—Zymogen granules (ZG) and their membranes (ZGM) were prepared according to the method of Meldolesi et al. (28) and stored at −80 °C in protease inhibitor buffer (27). Briefly, ZG were purified from guinea pig pancreas homogenates by differential centrifugation. ZGM were prepared from lysed ZG. Protein concentration was measured with the BCA protein assay.

Electrophoresis and Blotting—Pancreatic homogenates, ZG, or ZGM (20–25 μg of protein/lane) or recombinant SNARE proteins (10–90 μM) were separated by PAGE on 14% SDS Laemmli gels and then electrophobled to polyvinylidene difluoride membranes. Visualization of bound conjugate on Western blots was by colorimetric staining. Primary antibodies were polyclonal VAMP2 (Stressgen), VAMP3 (Abcam, Cambridge, MA), VAMP8 (Synaptic Systems, Gottingen, Germany), and monoclonal PY20 (BD Transduction Laboratories, Lexington, KY). Secondary antibodies were anti-mouse IgG alkaline phosphatase conjugate and anti-rabbit IgG alkaline phosphatase conjugate (Promega Corp., Madison, WI). Transblot membranes were developed with 0.1% Coomassie Brilliant Blue R stain. Both Western blot and transblots were routinely repeated a minimum of three times per experiment. Images shown are representative of these.

Gel Filtration—Whole dried scorpion venom (500 mg) was solubilized and then applied to a Sephadex G-50 column...
(Superfine) (2.5 × 100 cm) as described (17). Fractions (5 ml) were collected, pooled, and freeze-dried.

**Reverse Phase Chromatography**—Reverse phase chromatography was carried out using a Vydac 245TP54 and Supelco wide pore C<sub>5</sub> or C<sub>18</sub> column. Elution utilized a linear gradient to 60% acetonitrile with 0.1% trifuluoroacetic acid and a uniform flow rate of 1.0 ml/min. Detection followed absorbance recording at both 280 and either 225 or 215 nm. Resolved components were collected accordingly.

**Ion Exchange Chromatography**—Similarly, ion exchange chromatography substituted a Waters Associates Shodex 5PW anion exchange column (7.5 mm × 7.5 cm) with a mobile phase of 20 mM Tris-HCl, pH 8.6, that incorporated a linear gradient to 200 mM NaCl at 1%/min and 1 ml/min flow rate.

**SELDI-TOF Mass Spectrometry**—The SELDI-TOF mass spectrometer was externally calibrated using the (M + H)<sup>+</sup> ion peaks of Arg8-vasopressin at 1084.24/ m/z, human ACTH 1–24 at 2933.50/ m/z, bovine insulin B chain at 3495.94/ m/z, human recombinant insulin at 5807.65/ m/z, and hirudin BKHV at 7033.61/ m/z. All mass spectra were recorded in the positive ion mode using a Ciphergen PBS II ProteinChip array reader, a linear laser desorption/ionization time-of-flight mass spectrometer with time lag focusing (29). Prior to SELDI-TOF mass spectrometry analysis, 1 μl of matrix (saturated α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid) was added to each feature of the ProteinChip surface for the analysis. Raw data were analyzed using the computer software provided by the manufacturer and are reported as average masses.

**Amino Acid Sequence Analysis**—Chromatographic peaks were manually collected, and volume was reduced by evaporation in a Savant SpeedVac centrifuge followed by storage at −20 °C. The peptide cleavage products of VAMP2 and Fx ν isolated from incubation and separated by reverse phase chromatography were transferred to a Beckman peptide disk. These peptides and others isolated from the metalloprotease were sequenced on an ABI Procise model 492 sequencer using the computer software provided by the manufacturer and are reported as average masses.

**Bioinformatics and Molecular Modeling**—The authors used InsightII and Design Studio (Accelrys, Inc., San Diego, CA) and PyMOL (Delano Scientific LLC, Palo Alto, CA) to extend the molecular structures and homology of coiled-coil SNARE crystallography (Protein Data Bank code 1sfc) to provide a model for VAMP2.

**SNARE Cloning, Expression, Purification, and Characterization**—Plasmids, protein expression, and protein purification for full-length SNAP25A, truncated VAMP2 (amino acids 1–94 and 1–96; *Rattus norvegicus*), and syntaxin1A (1–263) have been described previously (30–34). The VAMP8 plasmid (amino acids 1–74; *R. norvegicus*) was a kind gift from Dr. Gottfried Mieskes (Max Planck Institute for Biophysical Chemistry). All proteins were expressed individually in *Escherichia coli* (BL21 (DE3)) in Terrific Broth medium as His<sub>6</sub> fusions in the pet28a vector (Novagen, Gibbstown, NJ) except VAMP2 (1–94), which was from the pGEX-4T vector (GE Healthcare), as described (35–37). All His<sub>6</sub>-tagged proteins were initially purified via nickel-nitritophytic acid-agarose (Qiagen, Germantown, MD) according to the manufacturer’s instructions using native conditions for the syntaxin and SNAP25 and denaturing protocols for VAMP2 and VAMP8. VAMP2 and VAMP8 were extensively dialyzed into native condition buffer (50 mm phosphate, pH 8.0, 300 mm NaCl, 1 mm dithiothreitol) before further use. VAMP2 (1–94) was purified by glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions. Syntaxin and SNAP25 were further purified by anion exchange chromatography on monoQ resin (GE Healthcare) in 20 mm Tris-HCl, pH 8.2, with elution in a NaCl gradient. The mutations in VAMP2 changing residues Lys<sup>85</sup> to Ala<sup>85</sup>, Arg<sup>86</sup> to Ser<sup>86</sup>, and Lys<sup>87</sup> to Ala<sup>87</sup> were introduced into the VAMP2 plasmid using the QuikChange method (Stratagene, Wilmington, DE) and were verified by sequencing the gene in the final plasmid. The triple mutant VAMP2 was purified by the same method as the wild type (WT).

**SNARE Proteolysis**—Recombinant SNARE proteins were incubated in PBS, pH 7.4, with 1.0 mM ZnCl<sub>2</sub> and 1.0 mM CaCl<sub>2</sub> for specified times at 37 °C in a water bath with agitation.
SNARE Complex Assembly—The ternary SNARE complex was assembled and purified as described previously (31). Briefly, SNARE complexes were formed by adding SNAP25 to syntaxin, followed by the addition of VAMP2 (35–37) in a ratio of 1:2:3 (syntaxin/SNAP25/VAMP2), generating SDS-resistant SNARE complexes (38). SNARE complex assembly was carried out in 20 mM Tris–HCl, pH 8.2, 200 mM NaCl (TBS) by first incubating at 42 °C for 1.5 h and then 4 °C for 12 h. For the purpose of additional purification of the assembled complex, the histidine tag was not cleaved from VAMP2 but was removed by thrombin treatment from syntaxin and SNAP25 before assembly. The assembly reaction was rebound to Ni\(^{2+}\)-nitrilotriacetic acid-agarose after the 12-h incubation, extensively washed with TBS to remove excess syntaxin and SNAP25, and eluted in TBS containing 200 mM imidazole. The assembled complex was then rebound to monoQ resin. VAMP2-(1–96) does not bind monoQ under loading conditions, so excess VAMP2 flows through. The purified ternary SNARE complex was then eluted in a NaCl gradient.

RESULTS

Pancreatic Exocrine-regulated Secretion and SNARE Proteins—The exocrine pancreas has great protein biosynthetic capacity as well as the ability to store large quantities of proteins for regulated secretory discharge upon presentation of the proper secretory agonist. The basic unit of this tissue, the pancreatic acinar cell, produces and stores exocrine proteins in vesicular elements in the trans-Golgi network that become mature zymogen or secretory granules positioned near or at the apical plasmalemma. Others have shown that docking and fusion events utilize the SNARE family of proteins to vectorially transport secretory proteins from endoplasmic reticulum through Golgi processing to apical discharge stages of these relatively large granules (≥0.5 μm) (39).

Experimental protocols were designed to differentially characterize stimulation from scorpion venom secretagogues in comparison with classical cholinergic (carbachol) and peptidergic (caerulein) secretagogues. Tissues treated with these venom preparations in comparison with carbachol and caerulein similarly produce linear dose–response curves only as far as an optimal level of venom (Fig. 1A). Protein discharge is diminished at higher levels of venom in excess of optimum secretory doses (16, 20). The TSV plot shown is similar to one we previously published (16) and is included only for comparison and reference.

TSV Stimulates Pancreatic Exocrine Secretion and Cleaves VAMP SNAREs—Experiments were carried out at the optimum dose–response level (1 μg/ml) to produce maximal secretion and at hyperstimulatory amounts of TSV (5 μg/ml and 50 μg/ml). Both positive stimulated (carbachol and caerulein) and unstimulated controls were included (Fig. 1, B and D).

Tissue homogenates produced intriguing results in PY20 Western blots. Fig. 1B reveals part of the pattern of phosphorylation of Tyr residues (PY20) in proteins of homogenates of guinea pig pancreatic lobules incubated for 3 and 4 h at various concentrations of TSV. Significant bands indicating Tyr phosphorylation in pancreatic homogenates were prominent at 17 kDa in control tissue. Samples that included the lowest concentration (1 μg/ml) of TSV appeared the same as control samples. At the highest venom levels (5 and 50 μg/ml) a different pattern emerged. The appearance of bands at 14 and 12 kDa and the concomitant disappearance of the 17 kDa band coinciding with higher concentrations of TSV stimulation at 3 and 4 h suggested the possibility of proteolysis. Bands from venom-treated tissue showed marked decreases as quickly as 5 min, even at 4 °C (Fig. 1C). Crude membrane preparations showed the same patterns, but postmicrosomal supernates did not (not shown).

We tentatively identified the PY20-positive band of 17 kDa as VAMP because of the apparent molecular weight similarities and membrane association. Samples were probed with anti-VAMP antisera (Fig. 1B) that provided putative identity as VAMP2 (2 Tyr residues) or VAMP3 (1 Tyr residue). Fig. 1D shows that none of the classical secretagogues (carbachol and caerulein) or the venom secretagogue toxin γ cause tissue alterations during 3 h of incubation, whereas TSV cleaves the native v-SNAREs, VAMP2 and VAMP8.

Identification of a Proteolytic Fraction in TSV—Isolation of a potential venom protease activity involves systematic purification stages. Our publication (17) showed that the majority of secretagogue activity is contained in lower molecular weight Sephadex G-50 fractions. We identified these bioactive components as agonists and antagonists of voltage-gated ion channels (17). Revisiting these materials, we found the proteolytic activity in Fx ν (see Fig. 9A), the highest molecular weight fraction, using in vitro incubations with recombinant VAMP2. Assays of Fx ν for stimulation of radiolabeled (newly synthesized) proteins revealed weak secretory activity that is diminished in comparison with TSV (Fig. 1A). This fraction is known to have limited or absent animal toxicity. Fx A that follows chromatographically is the next lower molecular weight fraction and has stronger secretagogue activity (Fig. 1A); however, no proteolytic activity was detectable.

Electron Microscopy Shows VAMP2 Destruction—Excised guinea pig pancreatic lobules were incubated in vitro with a hyperstimulatory dose of TSV (50 μg/ml) for 1 h at 37 °C, rinsed in KRB, and then prepared for electron microscopy. Untreated (control) lobules were incubated in KRB alone. Thin sections were probed with primary antisera against VAMP2 and then labeled with secondary antibodies conjugated with gold nanoparticles (10 nm) to detect bound immunoglobulins. Examples of these sections are seen in Fig. 2, A and B. Immunocytochemistry of tissues treated in vitro shows that VAMP2 demonstrated decreased colloidal gold labeling mainly associated with mature ZG as a result of apparent venom proteolytic activity.

The essential conclusion is that because the epitope for this antigen in the TSV-treated tissue in Fig. 2B has significantly fewer gold nanoparticles than the control cells (Fig. 2A), cleavage of VAMP2 has occurred. These electron microscopy immunogold studies provide structural verification of the biochemical findings seen in Fig. 1C. A separate experiment extending incubation to 3 h with 100 μg/ml TSV documented the same dramatic reduction of VAMP2 gold particles (not shown).

TSV and Fx ν Degrade VAMP2 and VAMP8 in Isolated Zymogen Granules and Zymogen Granule Membranes—We carried out subcellular fractionation to distinguish organelles
and membrane systems from cytoplasmic proteins. Zymogen granules and their isolated ZGM provided significantly less protein diversity; however, the alterations produced by exposure to venom in vitro remained constant. As indicated in Fig. 3, isolated ZG developed cleavages similar to those in pancreatic lobules. TSV produced cleavages in VAMP2 in both ZG (Fig. 3A) and ZGM (Fig. 3B). As seen in Fig. 3C, VAMP8 is also a proteolytic target in ZG during incubation with Fx ν. Fig. 3D shows that whole TSV or Fx ν rapidly cleave VAMP2 (top) even in short incubation periods (5 and 30 min). The PY20 immunoblots (bottom) parallel this result and are probably reflective of VAMP2 because VAMP8 has no tyrosine residues (see Fig. 6). Carbachol, caerulein, and toxin γ do not cause cleavage of VAMP2 in ZG, and patterns are the same as the control in Western blots (not shown). Although immunoblots showed cleavage, chemical substrate confirmation remained elusive. By limiting the source of cellular proteins to the ZG and ZGM, the range of potential cleavage targets had been further limited. ZGM include small amounts of VAMP2, known to have a blocked (acetyl) N terminus, as well as VAMP8 and other similar sized proteins (40). The cleavages produced on these materials were not sufficient to provide a definition for the site of attack. Interference from integral ZGM proteins and retained cytoplasmic and zymogen proteins prevented assignment of a precise cleavage site with in vitro experiments using tissue subfractions. Based upon apparent localization and molecular weight, we decided to investigate a source of pure cytoplasmic protein substrate, recombinant VAMP2.

Recombinant VAMP2 Provides Proteolytic Target Identification—To confirm substrate identity and reveal cleavage sites, we employed both whole venom and chromatographically purified venom proteins with a bacterially expressed, recombinant VAMP2.
Novel Metalloprotease Cleavage of VAMP

A modified version of the truncated soluble VAMP2-(1–94) protein as substrate. VAMP2 was incubated with TSV, Fx ν, or Fx λ at 37 °C for 30 min. Fig. 4A (left) shows that VAMP2 is cleaved by TSV. The middle and right panels of Fig. 4A show that Fx ν produces VAMP2 cleavage under these conditions, but Fx λ causes no proteolysis. Fig. 4D reveals a potential cleavage product band differing from the substrate protein by −2 kDa that appears at 5 min of incubation with Fx ν and then becomes more prominent until 120 min, evident only faintly at 180 min. Substrate bands diminish at all time points with the exception of the undigested control samples that retain original density. Results in Fig. 4D are both transblots and Western blots probed for VAMP2. Bands shown in each reveal similar outcomes, and the immunobLOTS visualize another band at 6 kDa. Similar results were observed with the 1–96 fragment of VAMP2. Venom from another New World scorpion, C. sculpturatus, was tested, and its proteolytic activity with VAMP2 substrate is shown in Fig. 4E.

Fx ν Proteolysis of VAMP2 Requires Divalent Cations—Initial structure studies of the protease revealed a putative divalent cation binding site. The sequence (HESVHLLGSPHD) that we identified is a recognized motif for zinc binding (41). With this suggestive information, we designed experiments to examine the effects of divalent cation chelation.

Fx ν was preincubated with 10 mM EDTA for 60 min prior to the addition of 10 μM VAMP2-(1–94) substrate. This chelation step was sufficient to prevent detectable VAMP2 cleavage by Fx ν after 60 min at 37 °C, as shown in Fig. 4B. The metalloprotease characterization of Fx ν was further strengthened with this combined information.

Amino Acid Substitutions at the VAMP2 Cleavage Site Prevent Proteolysis—N-terminal sequencing revealed that the major bands are the majority of this protein (residues 1–87) and that the smaller cleaved C terminus was not recovered due to its brief sequence (amino acids 88–94) of 7 residues. Reverse phase chromatography of aliquots of the digestion mixtures provided cleaved peptides of both N and C termini, as determined by protein sequencing (Fig. 5A). Cleavage peptides that were recovered and sequenced indicated enzymatic hydrolysis with new N termini at residues Lys85, Arg86, and Lys87. These peptides revealed a novel cleavage site within the VAMP2 cytoplasmic portion. Proteolysis on the N-terminal side of Lys85, Arg86, and Lys87 leaves cytoplasmic portions of no more than 10 residues or as short as 7 residues from the transmembrane segment (Fig. 6). It is clear from the chromatogram in Fig. 5B that VAMP2 incubated as a control in the same experiment remains intact.

A modified version of the cytoplasmic VAMP2 sequence was expressed that substituted an altered amino acid sequence composition replacing the cleavage site. Alanine and serine were substituted for lysine and arginine, respectively, so that the wild type Lys85, Arg86, Lys87 that was previously determined...
to be a cleavage site for the Fx \(v\) metalloprotease activity was altered to Ala\(^{85}\)-Ser\(^{86}\)-Ala\(^{87}\). Based upon results of both PAGE and reverse phase chromatography (C18), these substitutions prevented the previously observed cleavage (Fig. 4C). Primary amino acid determinations using the \(^{85}\)ASA\(^{87}\)-altered cytoplasmic VAMP2-(1–96) following incubation with Fx \(v\) at 37 °C yielded no new \(N\) termini. From these data, we conclude that this protein contains only one site for enzymatic cleavage, Lys\(^{85}\)-Arg\(^{86}\)-Lys\(^{87}\), and substituting ASA for this sequence eliminates Fx \(v\) hydrolysis of cytoplasmic VAMP2.

Fx \(v\) Also Cleaves VAMP8 and SNAP25—Bacterially expressed, recombinant VAMP8-(1–74) is cleaved upon exposure to Fx \(v\) (Fig. 7). The cleavage site is homologous with that determined for VAMP2. The two cleavage products are at the \(N\) termini of Arg\(^{67}\) and Lys\(^{68}\). This cleavage produces 8 (1135.38 Da) and 9 (1291.57 Da) \(C\) terminus residues remaining before the transmembrane segment. These products are confirmed by mass spectrometry. BLASTP-aligned sequences are shown in Fig. 6 (top). Similar results are demonstrated in Western blots of treated guinea pig pancreas \textit{in vitro} (Fig. 1D) and ZG (Fig. 3C). Recombinant SNAP25-(1–206) is a \(t\)-SNARE that is also cleaved by Fx \(v\) (Fig. 7). However, it does not include transmembrane segments because it is attached via nearly centrally located (palmitoylated) cysteine residues (Cys\(^{85}\), Cys\(^{88}\), Cys\(^{90}\), and Cys\(^{92}\)).

**Assembled SNARE Complex and Fx \(v\)**—A cleavage site is identified in the recombinant monomeric SNARE component VAMP2 molecules (Fig. 6). The cleavage site is located at the end of the coiled-coil SNARE motif in VAMP2. We examined the potential for proteolysis of assembled coiled-coil SNAREs. The SNARE component proteins, even those without attached transmembrane segments, can be assembled into aligned coiled-coil SNARE structures that share extraordinary characteristics with natural SNARE complexes. These characteristics include stability in 2% SDS, 8 M urea solutions and concurrent heating in excess of 80 °C. Fig. 8 (Laemmli denaturing gel system; 14% acrylamide) demonstrates this unusual stability. Assembled (resistant) SNARE complex (not boiled) as well as disassembled SNARE complex pro-
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FIGURE 6. Molecular model of VAMP2 and description of cleavage sites by clostridial toxins and Fx ν. Top, VAMP2 and VAMP8 FASTA homology alignment with cleavage sites (red) and transmembrane segments (violet). Center, VAMP2 amino acid sequence coordinated with a molecular model, where font color reflects sequence features: Fx ν cleavage (red) at broken ribbon, BoNT and TeNT cleavages (yellow and orange) at arrows, and SNARE motifs, X1 and X2 (blue). The intervening sequence ribbon is green. The model and FASTA homology were generated by InsightII (Accelrys).

FIGURE 7. TSV and Fx ν cleave other recombinant SNARE proteins. Transblots are shown. 90 μM WT endobrevin (VAMP8) cytoplasmic portion 1–74 (left) or 20 μM WT SNAP25 full-length 1–206 (right) was incubated with either 10 μg/ml TSV or 10 μg/ml Fx ν in vitro at 37 °C for 30 min. The number of experiments was as follows: VAMP8, n = 3; SNAP25, n = 7.

FIGURE 8. The assembled SNARE complex and Fx ν. A transblot is shown. SNARE complex (CPX) was incubated with 10 μg/ml Fx ν in vitro at 37 °C for 60 min. After mixing with Laemmli buffer, one sample was gel-loaded as intact complex (not boiled), and another sample was boiled for 5 min for disassembly prior to loading (boiled). The image is representative of 14 blots from three experiments using three sequentially purified SNARE complexes.
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Amino Acid Sequence Determination—Purified Fx ν protease isolated from whole T. serrulatus venom after successive chromatography on Sephadex G-50 and then either Waters Shodex DEAE anion exchange or Supelco wide pore C18 reverse phase chromatography was reduced and alkylated and then loaded on a polyvinylidene difluoride disk. N-terminal sequencing by automated Edman degradation provided the initial 29 residue identities (Fig. 11). Additional amino acid sequence was determined from a combination of chemical (CNBr) and proteolytic enzyme cleavages (trypsin, Lys-C, Arg-C, Asp-N, and Glu-C) and C5 and C18 reverse phase peptide separations for recovery of the proteolytic fragments. Overlapping amino acid sequences provided the assembled peptide map that is consistent with a single protein sequence (Uniprot accession number P86392) for antarease of 245 amino acid residues and 25,500 Da, as diagrammed in Fig. 11. The assembled peptides as illustrated and the intact protein have the same mass as determined by mass spectrometry of 25,500 ± 100 Da. Additional residue identities remain undetermined at two locations: residues 38–47 (10 residues) and 193–201 (9 residues), thus totaling 19 or 92% complete.

DISCUSSION

SNARE complex formation is integral to membrane fusion in vesicular trafficking and exocytosis in both neuronal and nonneuronal cells. Effects of clostridial toxins that cleave SNARE proteins in neuronal cells include inhibition of neurotransmitter release (25). In exocrine cells, VAMP2 and VAMP8, described in separate ZG populations, may have different roles but share unique participation in transport and exocytotic events (42). VAMP2 is thought to modulate constitutive secretion, whereas VAMP8 has a primary function during regulated secretion (42, 43). The intricate nature of these actions, whether distinct, redundant, or integrative, is not defined, yet understanding of these functions is fundamental to extending knowledge of transport in normal and diseased cells. Simultaneous cleavage of multiple SNAREs, such as VAMP2, VAMP3, and VAMP8, would presumably have major physiological consequences because membrane fusion in exocytosis is SNARE-dependent in acinar cells (7). Our earlier results concerning changes in exocrine pancreatic secretory discharge in hyperstimulation may point to these specific effects. Current studies are examining whether venom-induced VAMP cleavage interferes with pancreatic intracellular transport, exocytosis, and response to secretory signaling. Cleavage of the v-SNAREs VAMP2 and VAMP8 should result in significant alterations to vesicular transport and similarly would impact secretory mechanisms. Others found that tetanus neurotoxin cleavage of VAMP2 in pancreatic acini would impact secretory mechanisms (44). VAMP8 null mice show a major inhibition of CCK-stimulated regulated pancreatic secretion of amylase but maintain resting secretion (43).

Electron microscopic immunocytochemistry showed that VAMP2 demonstrated decreased colloidal gold labeling associated with mature ZG as a result of apparent venom proteolytic activity (Fig. 2). Results define VAMP2 localization to multiple points on the ZGM. Also, we found gold particles on
Microvesicle-like structures within the ZG content. Following exposure to TSV, few gold particles remain whether confined to the ZGM or granule content. Enrichment protocols with both isolated ZG and ZGM produced similar cleavage results.

In vitro experiments with ZGM provided limited amounts of substrate protein VAMP2, which has a blocked N terminus (45), as well as interference from similar sized intracellular proteins. VAMP2 was expressed in E. coli to provide substrate confirmation in experiments with chromatographically purified scorpion venom metalloprotease preparations. Amino acid sequencing of cleaved peptides recovered by chromatography revealed novel cleavage within the cytoplasmic portion of VAMP2. Three consecutive cleavage sites were determined to be 7 residues from the transmembrane segment, closer than botulinum A (18 residues), G (13 residues), and tetanus (18 residues) (Fig. 6).

Scorpion venom protease activity with potential pathogenic mechanisms has not been described previously. The primary structure of the isolated venom protease is not found in existing amino acid sequence data bases. While our biochemical characterization is in development, the scorpion venom metalloprotease, antarease, is composed of 245 residues and is therefore distinguished from snake venom metalloproteinasesthat comprise more than 400 residues (46). The antarease sequence has been included in the SwissProtein data base Uniprot with accession number P86392. This unique venom protease appears to be penetrating intact tissue, targeting SNARE proteins that are cleaved within loop segments outside coiled-coil tetrameric SNARE assemblies.

Understanding of the functional significance of SNARE assemblies remains incomplete and is a subject of investigational focus in vesicular transport and secretion research (39). Current knowledge of intracellular SNARE cleavage is based upon...
metalloprotease toxins from Clostridium botulinum and Clostridium tetani (23, 26). The principal clostridial toxins produce VAMP cleavages within the region of tetrameric assembly (Fig. 6) (47). Clostridial protease cleavage is also restricted to single microbial serotypes that define single cleavage sites (47). The cleavage site presented here for the venom metalloprotease is within the loop region immediately following the sequence directly involved in the coiled-coil tetramer and precedes the transmembrane segment that is near the C terminus (Fig. 6). Depending upon the nature of binding and exposure of the cleavage site, antarease may allow for cleavage of VAMP2 regardless of its presence in an assembled SNARE complex. Findings by others (25) revealed that SNARE tetrameric complex assembly is resistant to proteolysis by clostridial toxins; thus, this venom metalloprotease is further distinguished as a unique proteolytic activity.

Our studies of the effects of scorpion venom and its bioactive protein components presumed that direct effects were limited to extracellular or plasma membrane components, primarily ion channels (17, 20). Enzymes in scorpion venom have not been implicated as primary pathology mediators. The relatively abundant scorpion venom hyaluronidase was considered the most important enzyme (48). The role for this hyaluronidase activity was thought significant only in facilitating tissue penetration and distribution of bioactive venom components in vivo (i.e. as in capture of prey or in human envenomation) (49). In morphological studies, we documented the in vitro and in vivo effects of TSV and some component protein toxins that produce secretory discharge and tissue alterations that are similar to clinical appearances of acute pancreatitis (16, 50). Secretagogues of non-scorpion venom origin used by others can also produce similar effects but require excessive levels of administration in vivo in order to achieve those results (51).

Pathology has been attributed to the majority presence in scorpion venom of ion channel mediator activities (52, 53). These neurotoxins are modulators of voltage-gated sodium and potassium channels, chloride channels, and calcium channels. There is little correlation between toxin action and physiological effects from stings except that the autonomic nervous system is the primary target (53). No scorpion toxins have been associated with intracellular targets until this report. A definitive function in pathogenesis for the metalloprotease activity of scorpion venom remains to be determined beyond a theoretical role. Current studies are aimed at further characterizations of cleavage of other SNARE proteins, including SNAP23, SNAP25, and syntaxin1A. Initial information is included here with SNAP25 cleavage. It is not likely that there is uniform cleavage of all SNARE components, so the asymmetrical function of remaining cellular SNAREs would reasonably produce unbalanced vesicular traffic. Previously, we noted the appearance of intracellular vacuoles, endoplasmic reticulum Golgi intermediate compartment (ERGIC) aggregates, effacement of the apical plasmalemma with loss of microvilli, and distension of endoplasmic reticulum cisternae as consequences of scorpion venom exposure in vitro and in vivo (16, 50). These structural alterations are possible effects, but they have not been linked with specific SNARE cleavage by antarease. Because SNAREs are likely cleaved, the apical exocytotic functions would be abrogated. In this case, secretory discharge may become shifted to basolateral membrane sites. If these conditions are confirmed, then the possibility for basolateral exocrine pancreatic discharge would suggest an obvious mechanism for onset of acute pancreatitis upon exposure to scorpion venom in concert with clinical reports (54). Research by others (9, 55) implicates SNARE alterations and redirected exocytosis as the root for pancreatitis pathology. At this time, we have similarly connected SNARE cleavage with our earlier descriptions of scorpion venom-mediated changes to pancreatic morphogenesis and secretory biochemical characteristics.

In summary, we report intracellular cleavage of the vesicular SNAREs VAMP2 and VAMP8 in exocrine pancreatic acinar cells from excised tissue incubated in vitro with venom from the Brazilian scorpion T. serrulatus. Immunocytochemistry by electron microscopy located major changes in VAMP2 content in mature ZG following incubation. Studies with isolated ZG and ZGM provided reinforcement for these findings. Definitive results were obtained using bacterially expressed recombinant, cytoplasmic VAMP2. Amino acid sequencing established that the cleavage sites were residues lysine 85, arginine 86, and lysine 87. Replacing these VAMP2 residues with alanine 85, serine 86, and alanine 87 prevented cleavage. The cleavage sites are not shared with the sites for the only known proteases that target SNARE proteins, the microbial toxins from C. botulinum and C. tetani.

These results have important implications for potential effects on secretory discharge as well as vesicular transport mechanisms in the exocrine pancreas. The novel effects on SNARE components suggest that antarease can produce important information concerning vesicular docking and fusion mechanisms. The presence of a metalloprotease in scorpion venom may provide insight into mechanisms of scorpion envenomation clinical pathogenesis. This mode of action for a venom toxin represents a new and exciting dimension for a non-microbial SNARE targeting enzyme, also a cell biological tool especially for the study of SNARE structure and function.

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REFERENCES
1. Ferro-Novick, S., and Jahn, R. (1994) Nature 370, 191–193
2. Rothman, J. E. (1994) Nature 372, 55–63
3. Südhof, T. C. (1995) Nature 375, 645–653
4. Jahn, R., and Scheller, R. H. (2006) Nat. Rev. 7, 631–643
5. Breidenbach, M. A., and Brunger, A. T. (2005) Trends Mol. Med. 11, 377–381
6. Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T. R. (2002) Nat. Struct. Biol. 9, 107–111
7. Hansen, N. J., Antonin, W., and Edvardsson, J. M. (1999) J. Biol. Chem. 274, 22871–22876
8. Jahn, R., and Südhof, T. C. (1999) Annu. Rev. Biochem. 68, 863–911
9. Gaisano, H. Y. (2000) Pancreas 20, 217–226
10. Antonin, W., Wagner, M., Riedel, D., Brose, N., and Jahn, R. (2002) Mol. Cell. Biol. 22, 1545–1554
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11. Jena, B. P., Gumkowski, F. D., Konieczko, E. M., von Mollard, G. F., Jahn, R., and Jamieson, J. D. (1994) *J. Cell Biol.* **124**, 43–53
12. Mayer, A. (2002) *Annu. Rev. Cell Dev. Biol.* **18**, 289–314
13. Xu, T., Rammer, B., Margittai, M., Artalejo, A. R., Neher, E., and Jahn, R. (1999) *Cell* **99**, 713–722
14. Palade, G. E. (1975) *Science* **189**, 347–358
15. Scheele, G. A., and Palade, G. E. (1975) *J. Biol. Chem.* **250**, 2660–2670
16. Fletcher, P. L., Jr., Fletcher, M. D., and Possani, L. D. (1992) *Eur. J. Cell Biol.* **58**, 259–270
17. Possani, L. D., Martin, B. M., Fletcher, M. D., and Fletcher, P. L., Jr. (1991) *J. Biol. Chem.* **266**, 3178–3185
18. Waterman, J. A. (1938) *Trans. R. Soc. Trop. Med. Hyg.* **31**, 607–624
19. Bartholomew, C. (1999) *Arch. Biochem. Biophys.* **359**, 832–835
20. Fletcher, P. L., Fletcher, M., Fainter, L. K., and Terrian, D. M. (1996) *Biophys. J.* **70**, 717–766
21. Fletcher, P. L., Fletcher, M. D., Weninger, K., Anderson, T. E., and Martin, B. M. (2007) *Mol. Biol. Cell* **18**, (suppl.), 272a
22. Fletcher, P. L., Fletcher, M. D., Weninger, K., Anderson, T. E., and Martin, B. M. (2008) *Mol. Biol. Cell* **19**, (suppl.), 1727a
23. Pellizzari, R., Rossetto, O., Schiavo, G., and Montecucco, C. (1999) *Phil. Trans. R. Soc. Lond. B* **354**, 259–268
24. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Poverino de laureto, P., DasGupta, B. R., and Montecucco, C. (1992) *Nature* **359**, 832–835
25. Rossetto, O., Seveso, M., Caccin, P., Schiavo, G., and Montecucco, C. (2001) *Toxicon* **39**, 27–41
26. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) *Physiol. Rev.* **80**, 717–766
27. Valentijn, J. A., Sengupta, D., Gumkowski, F. D., Tang, L. H., Konieczko, E. M., and Jamieson, J. D. (1996) *Eur. J. Cell Biol.* **70**, 33–41
28. Meldolesi, J., Jamieson, J. D., and Palade, G. E. (1971) *J. Cell Biol.* **49**, 109–129
29. Merchant, M., and Weinberger, S. R. (2000) *Electrophoresis* **21**, 1164–1177
30. Li, Y., Augustine, G. J., and Weninger, K. (2007) *Biophys. J.* **93**, 2178–2187
31. Weninger, K., Bowen, M. E., Chu, S., and Brunger, A. T. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14800–14805
32. Weninger, K., Bowen, M. E., Choi, U. B., Chu, S., and Brunger, A. T. (2008) *Structure* **16**, 308–320
33. Bowen, M. E., Weninger, K., Brunger, A. T., and Chu, S. (2004) *Biophys. J.* **87**, 3569–3584
34. Bowen, M. E., Weninger, K., Ernst, J., Chu, S., and Brunger, A. T. (2005) *Biophys. J.* **89**, 690–702
35. Fasshauer, D., Elaison, W. K., Brünger, A. T., and Jahn, R. (1998) *Biochemistry* **37**, 10354–10362
36. Fasshauer, D., Otto, H., Elaison, W. K., Jahn, R., and Brünger, A. T. (1997) *J. Biol. Chem.* **272**, 28036–28041
37. Fasshauer, D., Bruns, D., Shen, B., Jahn, R., and Brünger, A. T. (1997) *J. Biol. Chem.* **272**, 4582–4590
38. Hu, K., Carroll, I., Fedorovich, S., Rickman, C., Sukhodub, A., and Davletov, B. (2002) *Nature* **415**, 646–650
39. Wäsle, B., and Edwardson, J. M. (2002) *Cell. Signal.* **14**, 191–197
40. Pickett, J. A., Campos-Toimil, M., Thomas, P., and Edwardson, J. M. (2007) *Biochem. Biophys. Res. Commun.* **359**, 599–603
41. Alberts, I. L., Nadassy, K., and Wodak, S. J. (1998) *Protein Sci.* **7**, 1700–1716
42. Weng, N., Thomas, D. D., and Groblewski, G. E. (2007) *J. Biol. Chem.* **282**, 9635–9645
43. Wang, C. C., Ng, C. P., Lu, L., Atlashkin, V., Zhang, W., Seet, L. F., and Hong, W. (2004) *Dev. Cell* **7**, 359–371
44. Rosado, J. A., Redondo, P. C., Salido, G. M., Sage, S. O., and Pariente, J. A. (2005) *Am. J. Physiol. Cell Physiol.* **288**, C214–C221
45. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* **362**, 318–324
46. Fox, J. W., and Serrano, S. M. (2008) *FEBS J.* **275**, 3016–3030
47. Brunger, A. T., Jin, R., Breidenbach, M. A. (2008) *Cell. Mol. Life Sci.* **65**, 2296–2306
48. Possani, L. D., Alagón, A. C., Fletcher, P. L., Jr., and Erickson, B. W. (1977) *Arch. Biochem. Biophys.* **180**, 394–403
49. Pessini, A. C., Takao, T. T., Cavalheiro, E. C., Vichnewski, W., Sampaio, S. V., Giglio, J. R., and Arantes, E. C. (2001) *Toxicon* **39**, 1495–1504
50. Fletcher, M. D., Possani, L. D., and Fletcher, P. L., Jr. (1994) *Cell Tissue Res.* **278**, 255–264
51. Klippel, G., Dreyer, T., Willemer, S., Kern, H. F., and Adler, G. (1986) *Virchows Arch. A Pathol. Anat. Histopathol.* **409**, 791–803
52. Gwee, M. C., Narthanhan, S., Khoo, H. E., Gopalakrishnakone, P., Kini, R. M., and Cheah, L. S. (2002) *Clin. Exp. Pharmacol. Physiol.* **29**, 795–801
53. Ismail, M. (1995) *Toxicon* **33**, 825–858
54. George Angus, L. D., Salzman, S., Fritz, K., Ramirez, J., Yaman, M., and Gintautas, J. (1995) *Ann. Trop. Paediatr.* **15**, 285–289
55. Cosen-Binker, L. I., Binker, M. G., Wang, C. C., Hong, W., and Gaisano, H. Y. (2008) *J. Clin. Invest.* **118**, 2535–2551