Pancreatic Acinar Cells Express Vesicle-associated Membrane Protein 2- and 8-Specific Populations of Zymogen Granules with Distinct and Overlapping Roles in Secretion*\textsuperscript{,} \textsuperscript{5}

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Previous studies have demonstrated roles for vesicle-associated membrane protein 2 (VAMP 2) and VAMP 8 in Ca\textsuperscript{2+}-regulated pancreatic acinar cell secretion, however, their coordinated function in the secretory pathway has not been addressed. Here we provide evidence using immunofluorescence microscopy, cell fractionation, and SNARE protein interaction studies that acinar cells contain two distinct populations of zymogen granules (ZGs) expressing either VAMP 2 or VAMP 8. Further, VAMP 8-positive granules also contain the synaptosome-associated protein 29, whereas VAMP 2-expressing granules do not. Analysis of acinar secretion by Texas red-dextran labeling indicated that VAMP 2-positive ZGs mediate the majority of exocytotic events during constitutive secretion and also participate in Ca\textsuperscript{2+}-regulated exocytosis, whereas VAMP 8-positive ZGs are more largely involved in Ca\textsuperscript{2+}-stimulated secretion. Previously undefined functional roles for VAMP and syntaxin isoforms in acinar secretion were established by introducing truncated constructs of these proteins into permeabilized acini. VAMP 2 and VAMP 8 constructs each attenuated Ca\textsuperscript{2+}-stimulated exocytosis by 50%, whereas the neuronal VAMP 1 had no effects. In comparison, the plasma membrane SNAREs syntaxin 2 and syntaxin 4 each inhibited basal exocytosis, but only syntaxin 4 significantly inhibited Ca\textsuperscript{2+}-stimulated secretion. Syntaxin 3, which is expressed on ZGs, had no effects. Collectively, these data demonstrate that individual acinar cells express VAMP 2- and VAMP 8-specific populations of ZGs that orchestrate the constitutive and Ca\textsuperscript{2+}-regulated secretory pathways.

The exocrine pancreas is responsible for synthesizing and secreting a variety of digestive enzymes that are essential for assimilation of the diet. Secretion occurs by exocytosis of large dense core zymogen granules (ZGs)\textsuperscript{3} localized in the apical cytoplasm of acinar cells. Similar to endocrine and neural cells, exocytosis is highly induced following acinar stimulation by secretagogues; however, acini are unique in that a significant proportion of exocytosis also occurs by a constitutive pathway under basal conditions. Secretagogue-stimulated exocytosis is mediated by G protein-coupled receptors, which signal through phospholipase C and/or adenylate cyclase, and numerous studies have established roles for Ca\textsuperscript{2+}, diacylglycerol, and cAMP in modulating secretion (reviewed in Ref. 1). For acinar cells of the pancreas, changes in cellular Ca\textsuperscript{2+} play a pivotal role to trigger exocytosis under normal conditions, and furthermore, during pathophysiological states, aberrant increases in Ca\textsuperscript{2+} may induce cell damage leading to the onset of pancreatitis (reviewed in Ref. 2).

The process of exocytosis and other membrane fusion events is widely held to be regulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) interactions in cells (3–5). SNARE proteins are classified as either Q-SNARE or R-SNARE based on conserved glutamine (Q) and arginine (R) residues positioned within their characteristic coiled-coil motifs. When brought in close opposition, Q- and R-SNAREs on each membrane form a heterotrimeric complex that provides the driving force for membrane fusion. In neurons, one R-containing coil of the complex is derived from vesicle-associated membrane protein (VAMP/synaptobrevin) present on synaptic vesicles, and one Q-containing coil is contributed by syntaxin 1 located on the pre-synaptic plasma membrane. The remaining two coils of the complex are contributed by synaptosome-associated protein (SNAP) 25, a Q-SNARE also located on the plasma membrane (6). VAMPs and syntaxins are integrated into phospholipids via C-terminal hydrophobic regions that orient their coiled-coil motifs into the cytoplasm. Uniquely, SNAP 25 is membrane inserted by palmitoylation of four central cysteine residues positioning separate amino and carboxyl coiled-coil motifs into the cytoplasm (7, 8).

Pancreatic acinar cells express SNAP 23, an isoform of SNAP 25, (9, 10), and also contain multiple syntaxin isoforms with discrete localizations, including syntaxin 2 on apical plasma membrane, syntaxin 3 on ZGs, and syntaxin 4 on both apical and basolateral membranes (10–12). ZGs also express VAMP 2, which modulates a portion of exocytosis, because cleavage of associated membrane protein; SNAP, synaptosome-associated protein; PFO, Perfringolysin O; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; MOPS, 4-morpholinepropanesulfonic acid.
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Although VAMP 2 and VAMP 8 are known to regulate acinar secretion, their concerted function in the overall secretory response has not been investigated. Likewise, despite having identified the major apical membrane Q-SNAREs syntaxin 2 and syntaxin 4, a definitive role for these molecules in the constitutive and regulated secretory pathways has not been established. In this study, we examined the SNARE complexes in pancreatic acinar cells that mediate the constitutive and Ca^{2+}-stimulated secretory pathways. Evidence demonstrates that individual acinar cells contain distinct populations of ZGs expressing either VAMP 2 or VAMP 8. Utilizing dextran labeling to identify sites of VAMP-specific ZG exocytosis indicated that VAMP 2-positive granules were highly localized at the apical plasma membrane under both basal and Ca^{2+}-stimulated conditions. In comparison, VAMP 8-positive granules were much less concentrated at the apical membrane and appeared to interact with the plasma membrane at regions that were distinct from VAMP-2/dextran staining. Utilization of truncated SNARE constructs in permeabilized acini revealed that syntaxin 2, which is exclusively present on the apical membrane, inhibited only the constitutive pathway. Taken together, these data indicate that VAMP 2 plays a major role in the constitutive secretory pathway and participates in Ca^{2+}-regulated secretion. In comparison, VAMP 8-positive granules appear to be more involved in Ca^{2+}-stimulated exocytosis.

MATERIALS AND METHODS

Antibodies—Anti-SNAP 29 (catalog no. 111 302), anti-Syntaxin 2 (catalog no. 110 022), anti-syntaxin 4 (catalog no. 110 042), anti-VAMP 8 (catalog no. 104 302), rabbit polyclonal antibodies, and anti-VAMP 2 (catalog no. 104 211) mouse monoclonal antibody c169.1 were purchased from Synaptic Systems. Anti-syntaxin 4 (catalog no. 610439) mouse monoclonal antibody clone 49 was purchased from BD Transduction Laboratories. Anti-VAMP 2 (catalog no. VAS-SV006C) rabbit polyclonal antibody was purchased from StressGen. Anti-SNAP 23 (catalog no. ab 4114) polyclonal antibody was purchased from Abcam. Anti-VAMP 2 (cat AB5625) chicken polyclonal antibody was purchased from Chemicon. Anti-VAMP 8 rabbit polyclonal antibody was provided by Thomas Weimbs (17), and anti-SNAP 29 rabbit polyclonal antibody was from Mia Horowitz (18). Alexa-conjugated secondary antibodies (488, 546, 594, and 647), Alexa 488-conjugated anti-FITC, FITC, 3000K Texas red dextran, Rhodamine-Phalloidin, and Zenon secondary labeling kits were purchased from Molecular Probes. Peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from Amersham Biosciences.

Other Reagents—Soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride, Percoll, and Triton X-100 were purchased from Sigma, essential amino acid solution was from Invitrogen, and a protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, apro- tin, EDTA, leupeptin, and E64 was from Calbiochem. Protein A and protein G beads were from Pierce, Phadebas amylase assay kit was from Amersham Biosciences, and protein determination reagent was from Bio-Rad. The Perfringolysin O (PFO) bacterial expression plasmid was a kind gift from A. Johnson and R. Ramachandran of the University of Texas (19).

Isolation of Pancreatic Lobules and Dispersed Acini—The University of Wisconsin Committee on Use and Care of Animals approved all studies involving animals. Pancreatic lobules were prepared by microdissection of an adult male Sprague-Dawley rat pancreas that had been injected with incubation buffer consisting of (in mM) 10 HEPES, 137 NaCl, 4.7 KCl, 0.56 MgCl_2, 1.28 CaCl_2, 0.6 Na_3PO_4, 5.5 glucose, 2 l-glutamine, and an essential amino acid solution. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml bovine serum albumin, gassed with 100% O_2, and adjusted to pH 7.48. Dispersed cultures of pancreatic acinar cells were isolated from adult male Sprague-Dawley rats by collagenase digestion as described previously (20, 21). Acini were suspended in incubation buffer and cells were maintained at 37 °C for 1 h prior to performing assays.

Immunofluorescence Microscopy—The buffer used for all blocking, incubations, and washing steps contained: 1× phosphate-buffered saline, 3% bovine serum albumin, 2% goat serum, 0.7% cold water fish skin gelatin, and 0.2% Triton X-100. For cryostat sections of rat pancreas (Figs. 2 and 4A), lobules were fixed in 4% paraformaldehyde, and immunofluorescence microscopy was conducted on 9-μm-thick cryostat sections as previously described (22, 23). For images of acinar cell whole mounts (Fig. 5) isolated acini were allowed to settle on poly-L-lysine-coated microscope slides in wells created using a silicone slide cover. For dextran labeling, cells were incubated in HEPES buffer containing 1 mg/ml dextran for 5 min at room temperature before treatment with CCK-8 at room temperature. Following fixation in 4% paraformaldehyde, cells were blocked for 30 min at room temp, and primary antibodies were added simultaneously for 1.5 h at room temp. Secondary antibodies were added simultaneously for 1 h at room temp after washing (3 × 5 min). For VAMP 8, a secondary antibody amplification system (anti-rabbit-FITC followed by rabbit anti-FITC Alexa 488 conjugate) was used due to low signal intensity; however, initial studies conducted without amplification yielded identical results but required much higher laser settings. Due to the low expression levels of epithelial SNAREs, detection using secondary antibody amplification has previously been described (24). For ZG immunofluorescence (Fig. 4, B–D), Percoll-purified ZGs were fixed for 1 h in 4% paraformaldehyde, washed in phosphate-buffered saline, and embedded in embedding medium. Immunofluorescence microscopy was conducted on 9-μm-thick cryostat sections as described above. In all cases, specific-
ity of antibodies was determined by measuring fluorescence with secondary antibodies alone and in the case of VAMP 8, by antigen competition. Tissues were analyzed using a Radiance 2100 Bio–Rad Multiphoton/Confocal imaging system. For dual-immunofluorescence measurements, fluorophores were individually excited at the appropriate wavelength to ensure no overlapping excitation between channels. Captured images were overlaid and processed for presentation using ImageJ software. Movies of captured z-series and three-dimensional reconstruction of fixed acinar whole mounts, provided as supplemental movies for Fig. 5, were made using ImageJ and Windows Movie Maker software. For semiquantification of images in Fig. 4 (E and D), multiple z-series images from at least three separate tissue preparations were analyzed using ImageJ software with the colocalization threshold plug-in. All images used were raw data that had not been manipulated in any way prior to analysis. Threshold values for each image were automatically determined by the software and, therefore, were unbiased and provided conservative estimates. Analysis of VAMP 8-granule recruitment to the apical membrane (Fig. 5E) was conducted by counting the number of VAMP 8-positive puncta that colocalized with dextran alone or VAMP 2 and dextran in two optical fields obtained with a 100× objective for each treatment group. Data were quantified from three independent experiments utilizing different acinar preparations.

Acinar Cell Permeabilization—Acini were suspended in a permeabilization buffer containing (in mM) 20 PIPES (pH 6.6), 139 K+-glutamate, 4 EGTA, 1.78 MgCl₂, 2 Mg-ATP, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 35 psi PFO. PFO is a cholesterol-dependent cytolysin that assembles to create large aqueous pores in cell membranes (19). PFO was allowed to bind to intact cells on ice for 10 min, and then centrifuged at 12,000 g for 1 min. The content of CaCl₂ added to the buffer was calculated based on dissociation constants using a computer program as previously described (21). To measure basal secretion, permeabilized cells were incubated at 37 °C with indicated amounts of recombinant proteins. The cell suspension was then diluted with an equal volume of the same buffer containing enough CaCl₂ to create the desired final concentration of free Ca²⁺. The quantity of CaCl₂ added to the buffer was determined by the software and, therefore, were unbiased and provided conservative estimates. Analysis of VAMP 8-granule recruitment to the apical membrane (Fig. 5E) was conducted by counting the number of VAMP 8-positive puncta that colocalized with dextran alone or VAMP 2 and dextran in two optical fields obtained with a 100× objective for each treatment group. Data were quantified from three independent experiments utilizing different acinar preparations.

Preparation of ZGs—Rat pancreases were minced in 5 vol of a buffer containing (in mM) 10 MOPS, pH 6.8, 250 sucrose, 0.1 MgCl₂, 0.1 phenylmethylsulfonyl fluoride, and 1 benzamidine. Tissue was homogenized by three strokes of a motor-driven homogenizer (5,000 rpm) using a Teflon pestle with 0.5- to 1-mm clearance. A postnuclear supernatant was prepared by centrifugation at 600 × g for 10 min and then further centrifuged at 1,300 × g for 10 min to produce a white pellet enriched in ZGs overlaid by a brown pellet enriched in mitochondria.

The remaining supernatant was centrifuged at 100,000 × g for 1 h to separate microsomal and cytosolic fractions. ZGs were further purified by Percoll gradient centrifugation (21) and then lysed by sonication in buffer consisting of (in mM) 50 Tris (pH 7.4), 100 NaCl, 5 EDTA, 25 NaF, 10 sodium pyrophosphate, and protease inhibitors. ZG membranes were separated from content by 100,000 × g centrifugation.

GST- and His-Protein Purification—Constructs containing the full-length cytoplasmic domains of human VAMP 2 and VAMP 8 were obtained from G. Reed (25). VAMP 1 constructs were prepared by PCR using full-length rat VAMP 1 as a template and the primers GGTTGATCCTATGTCGCTCAAGCTC (forward) and GATCTCAGTTAGCAGTTTTTCACCAATA (backward). GST fusion proteins incorporating cytoplasmic domains of VAMPs 1, 2, and 8 were purified by glutathione affinity chromatography and either eluted in buffer containing 10 mM glutathione or released by thrombin cleavage as previously described (26). PFO was purified as a His-tagged protein by Co²⁺-affinity chromatography in accordance with the manufacturer’s instructions.

GST Pull-down Assay and Immunoprecipitations—Membrane fractions prepared from isolated acini or purified ZG membranes were solubilized in lysis buffer containing 1% Triton X-100 and then incubated with 15 μg of GST alone, GST-VAMP 2 or GST-VAMP 8, together with 20 μl of glutathione-Sepharose beads at 4 °C. After 1-h incubation at 4 °C, the beads were washed extensively, and fusion proteins were eluted by incubating in cleavage buffer containing 0.03 unit/μl thrombin. Binding proteins were analyzed by immunoblotting. For immunoprecipitations, detergent-solubilized membrane fractions were incubated with indicated antibodies overnight at 4 °C. Antibodies were then precipitated with Protein A- or G-Sepharose beads for 1 h, and washed extensively in lysis buffer, and analyzed by SDS-PAGE and immunoblotting (27).

RESULTS

Subcellular Localization of SNAREs in Acini—Our initial studies using subcellular fractionation of pancreas confirmed previous reports that VAMP 2 and VAMP 8 are enriched in ZG membranes but absent in ZG content and cytosolic fractions (Fig. 1) (10, 13). Investigation of SNAP 25 homologs demonstrated that SNAP 23 and SNAP 29 also copurified with ZG membranes. Unlike SNAP 23, which is present exclusively in membrane, small amounts of SNAP 29 were evident in cytosolic fractions, likely reflecting that SNAP 29 does not contain the central cysteine residues that mediate palmitoylation and membrane insertion of SNAP 23 (7, 28). The presence of SNAP 29 in acini was recently reported by Chen et al. (29) who utilized a proteomic screen to identify novel ZG proteins. The distribution of syntaxin isoforms in pancreas confirmed a previous report (11) that syntaxin 3 is present on ZG membranes, whereas syntaxins 2 and 4 are localized to acinar membrane fractions.

SNAP 23 and SNAP 29 Are Present on Both ZG and Apical Membranes and Differentially Interact with VAMPs—Consistent with their purification in ZG membrane fractions, immunofluorescence microscopy indicated SNAP 23 and SNAP 29 were present in a granule-like pattern throughout the apical
cytoplasm (Fig. 2). Additionally, SNAP 23 was clearly present along the entire plasma membrane where it was abundant in basolateral regions but more dispersed along the apical membrane (Fig. 2A). In contrast, SNAP 29 was absent from basolateral regions and mainly confined in a punctate pattern in the apical cytoplasm (Fig. 2B). To identify if SNAP 29 is present on plasma membrane, acini were dual-labeled with Alexa 594 conjugated-wheat germ agglutinin, which binds with high affinity to plasma membrane, acini were dual-labeled with Alexa 594 conjugated-wheat germ agglutinin, which binds with high affinity to plasma membrane. Proteins from each fraction (60 µg) were analyzed by immunoblotting with specific SNARE antibodies. Data are a single representative experiment performed least three times on separate tissue preparations yielding identical results.

Identification of SNAP 23 and SNAP 29 on ZG membranes was unexpected, because the prototypical SNAP 25 is conventionally expressed on plasma membrane in secretory cells where it combines with syntaxin 1 during the initiation of SNARE complex formation (6, 7). To determine if SNAP 23 and SNAP 29 interact with resident ZG proteins, GST pull-down assays were performed with truncated constructs of VAMP 2 and VAMP 8 omitting the C-terminal membrane-spanning regions (Fig. 3A). Although GST-VAMP 8 clearly interacted with SNAP 23 and SNAP 29, GST-VAMP 2 bound only with SNAP 23 and did not interact with SNAP 29. These results were confirmed by coimmunoprecipitation, demonstrating that anti-VAMP 2 antibody coimmunoprecipitated SNAP 23 but not SNAP 29 (Fig. 3B). Densitometric analysis of the immunoblots from three independent tissue preparations indicated VAMP 2 coimmunoprecipitated 7.2 ± 3.7% (mean ± S.E., n = 3) of the total SNAP 23 present in the ZG-membrane starting material. Further supporting the selective VAMP 8/SNAP 29 interaction, anti-SNAP 29 antibody coimmunoprecipitated 4.5 ± 0.5% (mean ± S.E., n = 3) of the total VAMP 8 in the ZG lysate but did not coimmunoprecipitate VAMP 2 (Fig. 3C). The total VAMP 8/SNAP 29 interaction may be underestimated as the SNAP 29 antiseraum utilized would maximally immunoprecipitate only 60 ± 6.9% (mean ± S.E., n = 3) of the total SNAP 29 in the ZG lysate. Additional experiments conducted to determine a potential interaction between SNAP 23 or SNAP 29 and syntaxin 3, which is also expressed on ZGs, identified no association between these molecules (data not shown). Collectively, these results indicate that VAMP 2 readily forms a complex with SNAP 23, whereas VAMP 8 interacts with both SNAP 23 and SNAP 29 on ZG membranes.

VAMP 2 and VAMP 8 Are Expressed on Distinct Populations of Zymogen Granules—In agreement with tissue fractionation, immunostaining of VAMP 2 and VAMP 8 in pancreatic lobules confirmed that both molecules localized in a vesicular pattern within the apical cytoplasm and were largely absent from basal regions and nuclei (Fig. 4A). Significantly, dual excitation of fluorophores revealed only minimal colocalization of VAMP 2 and VAMP 8 within individual cells. Consistently, VAMP 2-positive ZGs were concentrated immediately below the apical membrane, whereas VAMP 8-positive granules were distributed deeper in the apical cytoplasm. Overlap of VAMP 2 and 8 occurred mainly along the apical membrane of cells. To better elucidate if the VAMPs occupied separate populations of granules, analysis was performed on intact ZGs that were purified by Percoll centrifugation to eliminate staining that may occur on endoplasmic reticulum, Golgi, and endosomal vesicles when analyzed in intact cells (Fig. 4B). Viewed by differential contrast microscopy Percoll-purified ZGs were highly uniform in size (1–2 µm in diameter) and largely devoid of additional membranous structures, consistent with a previous report utilizing electron microscopy to analyze the relative purity of ZGs isolated by this technique (30). Strikingly, only minimal overlap of VAMP 2 and VAMP 8 was detected in purified ZGs. Semiquantitative analysis was conducted on multiple z-series images obtained from three separate preparations of ZGs and confirmed a minimal 11% colocalization of the VAMP-specific fluorophores (Fig. 4E). Because protein interaction studies in ZG membranes identified a unique association of VAMP 8 with SNAP 29, dual immunofluorescence was also conducted to confirm this localization in purified organelles (Fig. 4, C and D). Whereas VAMP 2-positive granules showed only a 16% overlap with SNAP 29, a significantly greater 35% colocalization of VAMP 8-ZGs with this Q-SNARE was determined. Collectively, the SNARE interaction studies and immunofluorescence staining support the existence of two distinct populations of ZGs in acini expressing VAMP 2 or VAMP 8.

VAMP 2-positive ZGs Mediate a Major Proportion of the Initial Phase of Stimulated Secretion—To examine a role for VAMP-specific granules in secretion, acini were incubated with Texas red-conjugated dextran under basal or CCK-8-stimu-
lated conditions (100 pm) and then immediately fixed in paraformaldehyde to specifically label sites of ZG exocytosis (Fig. 5). Previously, incubation of acini with lysine-fixable dextran was shown to identify omega-shaped structures exclusively at the apical membrane representing individual sites of ZG fusion (31). In our experiments, dextran label was confined to the acinar lumen allowing easy identification of sites of secretion along the apical membrane; however, individual omega structures were often difficult to discern. Under basal conditions, dextran accumulated primarily in VAMP 2-positive regions along the apical membrane, whereas colocalization with VAMP 8 was comparatively limited (Fig. 5A, also supplemental movies S1–S3 showing z-series and three dimensional projection). Unlike the expanded VAMP 2 staining along the apical membrane, VAMP 8-positive ZGs typically retained a spherical appearance. Stimulation with CCK-8 (100 pm) for 1 min (Fig. 5B), 5 min (Fig. 5C), and 15 min (not shown) dilated the acinar lumen with corresponding increases in dextran and VAMP 2 colocalization, but again VAMP-8/dextran colocalization was more limited. Semiquantitative analysis of multiple z-series images from three separate tissue preparations supported the expanded colocalization of VAMP 2 and dextran under basal and CCK-8-stimulated conditions, which ranged from 32 to 46% (Fig. 5D). Likewise, relatively constant but considerably less colocalization of ~12% between VAMP 8 and dextran was detected under basal and stimulated conditions. Collectively, the pronounced overlap of VAMP 2 and dextran under basal and stimulated conditions suggests that VAMP 2-positive granules mediate the majority of the constitutive pathway as well as the initial phase of stimulated secretion. Analysis of the VAMP/dextran overlap does not quantify the overall increase in ZG accumulation at the cell apex during CCK stimulation. Because VAMP 8 staining retained a spherical or punctate appearance when colocalized with dextran, VAMP 8 granule recruitment to sites of exocytosis was quantified by counting the number of VAMP 8-positive puncta that colocalized with VAMP 2 and dextran or with dextran alone (Fig. 5E). Clearly, the majority of VAMP 8 granules colocalized with dextran independent of VAMP 2 staining. When analyzed as a percentage of total VAMP-8 and dextran-positive ZGs colocalized VAMP 2, <20% overlap was detected under control and CCK-stimulated conditions. These results suggest that VAMP 2- and VAMP 8-expressing granules dock at distinct sites along the apical membrane. A similar analysis of VAMP 2 granules was precluded, because the majority of staining did not retain a punctate or spherical appearance but, rather, appeared to coalesce with the apical membrane.

**Syntaxins 2 and 4 Are Present on the Acinar Cell Apical Plasma Membrane—Immunofluorescence analysis of syntaxin isoforms in acini demonstrated that syntaxin 2 was present exclusively along the apical membrane and absent from basolateral regions (Fig. 6). In contrast syntaxin 4 was seen along the entire plasma membrane with prominent staining in basolateral regions and a more dispersed punctate pattern noted along the apical membrane. Additionally, faint syn-

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**VAMPs and Acinar Secretion**

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**FIGURE 2. SNAP 23 and SNAP 29 are present on ZG and apical plasma membranes.** Cryostat sections of paraformaldehyde-fixed pancreatic lobules were (A) stained with anti-SNAP 23 antibody (1:50) and detected using Alexa 488-conjugated secondary antibody, or (B and C) labeled with SNAP 29-specific antibody (1:100) (18). Preincubating sections with Alexa 594-wheat germ agglutinin (C) identified acinar plasma membrane (shown in “red”). SNAP 29 antibody was detected using an FITC-conjugated secondary antibody and amplified using an Alexa 488-conjugated anti-FITC antibody. A single optical section (0.6 mm) is shown. Note that SNAP 23 and SNAP 29 are localized in ZG regions along the apical membrane, whereas SNAP 29 is sparsely localized in the acinar membrane. Asterisks and arrows indicate positions of nuclei and acinar lumen, respectively. All images are single representative experiments performed on multiple sections from at least three separate tissue preparations yielding identical results.

**FIGURE 3. VAMP 8 specifically interacts with SNAP 29 on ZG membranes.** Zymogen granule membranes (ZGM) were solubilized in lysis buffer containing 1% Triton X-100 and incubated with GST-VAMP constructs (A), anti-VAMP 2 antibodies (B), or anti-SNAP 29 antibodies (C). Precipitated proteins were analyzed by immunoblotting (IB) with indicated antibodies. Note that VAMP 8 binds to SNAP 23 and SNAP 29, whereas VAMP 2 binds only to SNAP 23. Data are single representative experiments performed at least three times on separate tissue preparations.
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FIGURE 4. VAMP 2 and VAMP 8 are present on separate populations of ZGs. A, VAMP 2 and 8 VAMP 8 were localized in 4% paraformaldehyde-fixed sections of pancreatic lobules. VAMP 8 was detected with a rabbit polyclonal antibody (AB5625, 1:100). An anti-rabbit FITC Alexa 488-conjugated rabbit IgG (1:500) secondary amplification system and an Alexa 546-conjugated anti-chicken IgG (1:5000), were used for immunolocalization. B–D, microscopy was conducted on cryostat sections of Percoll-purified ZGs. VAMP 8 was detected with a rabbit polyclonal antibody (104 302, 1:50), and VAMP 2 with a mouse monoclonal antibody (104 211, 1:100) or a chicken polyclonal antibody (AB5625, 1:100, in C). SNAP 29 was detected with a rabbit polyclonal antibody (1:100) (18). Immunoreactivity was localized using an Alexa 488-conjugated anti-rabbit IgG, Alexa 546-conjugated anti-mouse IgG, Alexa 546-conjugated anti-chicken IgG, or Alexa 488 conjugated anti-rabbit IgG, respectively. In D, VAMP 8 was detected with a rabbit polyclonal antibody (104 302, 1:50) and SNAP 29 with a rabbit polyclonal antibody (1:100) using an Alexa 488-conjugated and Alexa 546-conjugated Zenon secondary detection kit, respectively. Arrows indicate isolated areas of VAMP 8 staining. Arrowheads indicate isolated areas of VAMP 2 staining. Note in A that VAMP 2 is localized below the acinar lumen, whereas VAMP 8 occupies deeper areas of the apical cytoplasm. Also note the pronounced overlap of VAMP 8 and SNAP 29 (dotted-line arrowheads in D) but minimal overlap of VAMP 2 and SNAP 29 in ZG fractions (C). E, semiquantitative analysis of SNARE protein colocalization acquired from multiple z-series images from three separate ZG preparations. Data are mean and S.E. (n = 30, 12, and 12 for VAMP 2/VAMP 8, VAMP 2/SNAP 29, and VAMP 8/SNAP 29, respectively). All images are a single representative experiment performed on multiple sections from at least three separate tissue preparations.

taxin 4 punctate staining was present in the cytoplasm, likely reflecting its presence on vesicular carriers distinct from ZGs, because syntaxin 4 did not copurify with these organelles in tissue fractionation studies (see Fig. 1). As previously reported (11), syntaxin 3 staining was detected in a granular pattern in the apical cytoplasm confirming its localization on ZGs (data not shown).

Soluble Forms of Recombinant SNAREs Selectively Inhibit Digestive Enzyme Secretion—As previously reported (10), coimmunoprecipitation analysis of SNARE protein interactions in acini using a variety of antibodies revealed that syntaxin 4 interacts with VAMP 8 and SNAP 23. Moreover, we were unable to detect any reproducible interaction of VAMP 2 with syntaxin isoforms (data not shown). SNARE coimmunoprecipitations in acini are notoriously difficult to conduct because of the low expression of SNARE proteins in comparison to other secretory cells and the small fraction of total granules that undergo fusion (32).

As an alternative, we utilized soluble constructs of SNARE proteins in PFO-permeabilized acini to more directly evaluate their roles in secretion. Recombinant SNARE proteins were made soluble by removing their respective C-terminal membrane spanning domains. These truncation mutants are known to act as selective inhibitors of exocytosis, presumably by competing with endogenous proteins in forming SNARE complexes (25, 33). Preliminary experiments measuring the Ca\(^{2+}\) sensitivity of amylase secretion from PFO permeabilized cells indicated maximum secretion was achieved at 3 \(\mu\)M free Ca\(^{2+}\) (data not shown). Therefore, permeabilized cells were incubated with various concentrations of VAMP 1, 2, or 8 and concurrently stimulated with 3 \(\mu\)M Ca\(^{2+}\) for 25 min (Fig. 7, A–D). VAMP 2 and VAMP 8 each inhibited Ca\(^{2+}\)-stimulated secretion in a concentration-dependent manner with a maximum 50% inhibition achieved at 200–300 \(\mu\)g/ml of recombinant protein. No further inhibition was detected with higher concentrations of VAMP proteins or at later times. Combined incubation of cells with maximal concentrations of both VAMPS showed no additive effect (data not shown). No effects
of exogenous VAMPs were detected during the first 10–15 min of incubation and then became evident between 15 and 30 min (data not shown). As a control, acini were incubated with the neuron-specific VAMP 1, which is not expressed in acini, and it had no effects on secretion at concentrations as high as 300 μg/ml. Because VAMP 1 and VAMP 2 are essentially identical within their coiled-coil domains, an additional construct of VAMP 1 containing only the coiled-coil region was tested, and it too had no significant effect on secretion (Fig. 7E). Neither VAMP isoform had any effect during the first 30 min but strongly inhibited constitutive secretion by 70% when measured between 30 and 60 min.

The parallel effects of VAMP 2 and VAMP 8 constructs on exocytosis suggested the proteins were acting through a common SNARE protein (potentially SNAP 23) to inhibit secretion. As an alternative, soluble constructs of syntaxin 2, 3, and 4 omitting their C-terminal membrane-spanning regions were utilized (Fig. 8, A–D). Analysis of the effects of recombinant syntaxins on Ca2+ -stimulated secretion revealed that only syntaxin 4 significantly inhibited exocytosis. Measured at 30 min, a maximal 50% inhibition was achieved at 200 μg/ml of syntaxin 4. In comparison, syntaxin 2 and syntaxin 3 had a minor non-significant effect at concentrations as high as 300 μg/ml. As seen with exogenously added VAMPs, no effect of syntaxin 4 was detected during the first 10–15 min of Ca2+ stimulation and then became evident between 15 and 30 min (data not shown). When tested at high concentrations under basal conditions (≤10 nM Ca2+) the syntaxin isoforms did not inhibit secretion during the first 30 min of incubation (Fig. 8E). However, syntaxin 2 and syntaxin 4 each caused a significant 50% reduction in total secretion by 90 min. Moreover, when analyzed between 30 and 90 min, syntaxin 2 and syntaxin 4 fully inhibited basal secretion. Syntaxin 3, which is expressed on ZGs, had no significant effects at early or late times. These data suggest that, whereas syntaxin 2 and 4 modulate basal secretion, only syntaxin 4 significantly inhibited the Ca2+ -stimulated response.

**DISCUSSION**

Results demonstrating separate VAMP 2 and VAMP 8 localization in intact acini and purified ZGs strongly support that distinct populations of ZGs exist in individual cells. These findings are further substantiated by the codistribution of SNAP 29 with VAMP 8-positive granules. The possibility that acinar cells express a heterogeneous population of ZGs that may change in accordance with diet has been postulated for some time.
From a mechanistic standpoint, Castle et al. (35) provided evidence in parotid acini supporting the existence of three separate secretory pathways that mediate the exocytosis of both common and unique secretory products. It was proposed that the sequential activation of a "minor regulated pathway" mediates the insertion of syntaxin 3 into the apical membrane and thereby provides fusion sites for a separate population of secretory granules. Further, Nemoto et al. (36), utilizing multiphoton imaging of acini provided compelling evidence that secretion proceeds through an initial rapidly activated phase that could not be resolved as single fusion events followed by a second phase involving compound exocytosis. The present study is clearly concordant with Chen et al. (37), and suggests that the initial rapidly activated phase of exocytosis may be mediated by VAMP 2-positive ZGs poised along the apical membrane. Moreover, our observation that VAMP 8-positive granules retained a spherical or punctate appearance when colocalized with dextran supports that they represent individual fusion events. However, demonstration that VAMP 8-positive granules most commonly associated with dextran-positive regions along the acinar lumen independent of VAMP 2, suggest that VAMP-specific populations of granules undergo exocytosis at distinct apical membrane sites.

Identification of SNARE protein interactions on purified ZGs indicated the presence of binary complexes involving VAMP 8 with SNAP 23 or SNAP 29 and VAMP 2 with SNAP 23. Biophysical studies using prototypical SNAREs demonstrated that SNARE-pin formation begins most efficiently with a syntaxin-SNAP 25 intermediate present on the plasma membrane that then strongly interacts with VAMP (38). Thus, whether the abundant SNAP 23 and SNAP 29 expression on ZGs participates in fusion reactions with the apical membrane is uncertain. Because SNAP 23 and SNAP 29 are also expressed along the apical membrane, it is likely that the ZG-associated isoforms have alternative functions. Possibilities include ZG to ZG fusion during compound exocytosis, and/or ZG fusion to endosomal vesicles involved in the recycling of regulatory proteins (including VAMPs) from the apical membrane. Although in preliminary experiments we were unable to detect an interaction between syntaxin 3 and

**FIGURE 6. Syntaxins 2 and 4 are present on the apical membrane.** Syntaxins 2 and 4 were localized in cryostat sections of 4% paraformaldehyde-fixed lobules. Syntaxin 2 was identified with a rabbit polyclonal antibody (110 022, 1:50) and syntaxin 4 with a mouse monoclonal antibody (610439, 1:50); corresponding immunoreactivity was detected using an Alexa 546-conjugated anti-rabbit IgG (1:500) and Alexa 488-conjugated anti-mouse IgG (1:500), respectively. Arrows indicate apical membrane. All images are single representative experiments performed on multiple sections from at least three separate tissue preparations with identical results.

**FIGURE 7. Truncated VAMP proteins inhibit amylase secretion.** Isolated acini were permeabilized with PFO and incubated with the indicated concentrations of recombinant VAMP proteins or the coiled-coil domain of VAMP 1. A–C, amylase secretion was measured under basal (≤10 nM free Ca²⁺) or stimulated conditions by clamping the free Ca²⁺ concentration at 3 μM for 25 min. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. D, data are replotted as % of Ca²⁺-stimulated secretion following subtraction of basal amylase secretion, which was not altered by addition of recombinant proteins at 25 min. Black bars, VAMP 1; gray bars, VAMP 2; open bars, VAMP 8; hatched bar, VAMP 1 coiled-coil. E, acini were incubated with maximal inhibitory concentrations of VAMP 2 (200 μg/ml) or VAMP 8 (300 μg/ml) and basal amylase secretion in response to ≤10 nM Ca²⁺ was measured at 30 and 60 min. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. All data are the mean and S.E. of at least three independent experiments each performed in triplicate. Statistical significance (*, p < 0.05; **, p < 0.01) from control was determined using Student’s t test.
SNAP 23 or 29 on ZG membranes, recent evidence indicates that ZGs also contain syntaxins 7 and 8 (10), clearly supporting SNARE interactions involving VAMP 8 and syntaxin 4.

SNAP 29 is homologous to SNAP 23 and SNAP 25 and contains the two characteristic coiled-coils that mediate SNARE protein interactions in vitro (7); however, demonstration of a direct involvement of SNAP 29 in membrane fusion has been lacking. SNAP 29 is distinguishable by the absence of central cysteine residues that mediate membrane insertion of SNAP 23 and SNAP 25 (7). In neurons, overexpression of SNAP 29 inhibited synaptic transmission by competing with preformed complexes.

Results showing that VAMP 2 and VAMP 8 constructs equally inhibited secretion suggest they were acting through a common mechanism. Because both VAMPs interact with SNAP 23, it is possible that each protein commonly neutralized SNAP 23 in acini. Interestingly, VAMP 1, which is not expressed in acini, but highly homologous to VAMP 2, had no discernable effects on secretion. VAMP 1 and VAMP 2 differ only in their N-terminal 26 amino acids. Unlike VAMP 1, VAMP 2 contains an N-terminal proline-rich motif (PAAPXGx₃PP) that was shown to inhibit exocytosis when injected into presynaptic neurons of *Aplysia* (40). Similarly, in cultured adipocytes, both VAMP 2 and a VAMP 2 N-terminal peptide inhibited GLUT4 trafficking, whereas VAMP 1 had no effect (41). As an alternative, we utilized a truncated VAMP 1 construct, omitting the 26-amino acid N terminus and composed of only the coiled-coil motif, that is identical to VAMP 2, and it too was without effect on secretion. These data indicate that the N-terminal region of VAMP 2 is essential to direct normal SNARE formation in acini. Interestingly, when analyzed following an overnight incubation in acinar lysates, protein constructs on exocytosis may be due to the presence of a pool of primed ZGs present during cell permeabilization that limit the ability of exogenous SNAREs to interfere with preformed complexes. These results are strikingly similar to the effects of clostridial neurotoxins to inhibit acinar secretion in permeabilized acini. Giasano et al. (13) demonstrated that introduction of tetanus toxin into permeabilized acini cleaved VAMP 2 but inhibited stimulated secretion by only 30%. In contrast, Padfield (14) demonstrated a 75% inhibition of Ca²⁺-stimulated secretion by tetanus toxin in permeabilized acini and reasoned that the greater inhibition was due to prolonged incubation of the toxin in the presence of cytosol and ATP to promote dissociation of existing SNARE complexes and thereby enhancing cleavage. Immunofluorescence evidence that VAMP 2-positive ZGs mediate the constitutive pathway as well as the initial round of exocytosis following stimulation is compatible with VAMP 2-positive granules representing the pool of docked granules along the apical membrane. The presence of a docked pool of ZGs would explain why exogenous SNAREs failed to inhibit secretion during the 15 min of incubation. Presumably, exogenous proteins would only be available to inhibit the secondary phase of exocytosis prior to SNARE-pin formation and/or to neutralize those SNAREs involved in the initial exocytotic phase after their disassembly from existing complexes.

FIGURE 8. Truncation constructs of syntaxin proteins selectively inhibit amylase secretion. Isolated acini were permeabilized with PFO and incubated with the indicated concentrations of recombinant soluble forms of syntaxins 2, 3, or 4. A–C, amylase secretion was measured under basal (≤10 nm free Ca²⁺) or stimulated conditions by clamping the free Ca²⁺ concentration at 3 µM for 30 min. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. D, data are replotted as % of Ca²⁺-stimulated secretion following subtraction of basal amylase secretion, which was not altered by addition of recombinant proteins at 30 min. Black bars, syntaxin 2; gray bars, syntaxin 3; open bars, syntaxin 4. E, permeabilized acini were incubated with maximal inhibitory concentrations of syntaxins (300 µg/ml) and basal amylase secretion in response to 10 nM free Ca²⁺ was measured at 30, 60, and 90 min. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. Data in A–D are the mean ± S.E., and E the mean ± S.D. of at least three independent experiments each performed in triplicate. Statistical significance (*, p < 0.05; **, p < 0.01) from control was determined using Student’s t test.
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VAMP 1 interacted with SNAP 23 (data not shown). Thus, whether or not VAMP 2 and VAMP 8 inhibited secretion by neutralizing SNAP 23, syntaxin isoforms, or some additional regulatory molecule is uncertain. Nevertheless, this selectivity of acinar-specific SNARE proteins to inhibit secretion underscores the specificity of these molecules in regulating various forms of membrane fusion in diverse cell types.

Previous studies (10, 11, 14) have characterized the localization of syntaxin isoforms in pancreatic acini, however, there is insufficient functional evidence implicating these molecules in secretion. Hansen et al. (12), using an in vitro fusion assay, reported that treatment of plasma membrane fractions with botulinum toxin C cleaved syntaxin 2 but not syntaxin 4 and fully inhibited ZG fusion, suggesting that syntaxin 2 is the major isoform regulating secretion. In the present study, exogenous syntaxin 2 inhibited basal secretion and produced a minor non-significant effect on stimulated secretion. Rather, syntaxin 4 significantly inhibited both basal and Ca$^{2+}$-stimulated exocytosis suggesting that this isoform plays a primary role in the Ca$^{2+}$-regulated pathway. Consistent with our findings, an association between VAMP 8 and syntaxin 4 was previously shown in mouse acini (10). In contrast, other studies utilizing GST-VAMP 2 pull-down assays (10) and syntaxin coimmunoprecipitations (11) reported an interaction between VAMP 2 and syntaxin 4.

The differences among these reports are no doubt related to the experimental approach and overall difficulty of analyzing SNARE interactions in acini. That being the case, we concede that interactions between VAMP 2 and syntaxin 4 may occur; moreover, this SNARE pairing is not inconsistent with our functional data implicating syntaxin 4 in the constitutive pathway.

Evidence that syntaxin 4 preferentially associates with VAMP 8 is consistent with a model where VAMP 2-positive granules primarily mediate constitutive exocytosis and VAMP 8-positive granules stimulated exocytosis. This was recently proposed by Wang et al. (10) based on the finding that acini from VAMP 8 null mice had a 4.5-fold increase in total cellular membrane was not reported, the basic principle of an initial round of ZG fusion directing a sustained response is supported. Although some VAMP 8 colocalization with VAMP 2 and dextran was detected in the present study, it appeared that VAMP 8 ZGs preferentially undergo homotypic fusion during compound exocytosis. The nature of the VAMP 2 staining made it difficult to identify if these granules undergo compound exocytosis. Clearly, the present data demonstrating separate VAMP 2- and 8-specific pools of ZGs in acini raises many possibilities not only related to exocytosis but also the molecular nature of ZG formation, maturation, composition of cargo, trafficking, and retrieval from the apical membrane.

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