Expression, localization, and functional role for synaptotagmins in pancreatic acinar cells

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Falkowski MA, Thomas DD, Messenger SW, Martin TF, Groblewski GE. Expression, localization, and functional role for synaptotagmins in pancreatic acinar cells. Am J Physiol Gastrointest Liver Physiol 301: G306–G316, 2011. First published June 2, 2011; doi:10.1152/ajpgi.00108.2011.—Secretagogue-induced changes in intracellular Ca2+ play a pivotal role in secretion in pancreatic acini yet the molecules that respond to Ca2+ are uncertain. Zymogen granule (ZG) exocytosis is regulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. In nerve and endocrine cells, Ca2+-stimulated exocytosis is regulated by the SNARE-associated family of proteins termed synaptotagmins. This study examined a potential role for synaptotagmins in acinar secretion. RT-PCR revealed that synaptotagmin isoforms 1, 3, 6, and 7 are present in isolated acini. Immunoblotting and immunofluorescence using three different antibodies demonstrated synaptotagmin 1 immunoreactivity in apical cytoplasm and ZG fractions of acini, where it colocalized with vesicle-associated membrane protein 2. Synaptotagmin 3 immunoreactivity was detected in membrane fractions and colocalized with an endolysosomal marker. A potential functional role for synaptotagmin 1 in secretion was indicated by results that introduction of synaptotagmin 1 C2AB domain into permeabilized acini inhibited Ca2+-dependent exocytosis by 35%. In contrast, constructs of synaptotagmin 3 had no effect. Confirmation of these findings was achieved by incubating intact acini with an antibody specific to the intraluminal domain of synaptotagmin 1, which is externalized following exocytosis. Externalized synaptotagmin 1 was detected exclusively along the apical membrane. Treatment with CCK-8 (100 pM, 5 min) enhanced immunoreactivity by fourfold, demonstrating that intraluminal domain that contains two C2 domains: C2A and C2B. These C2 domains are found in tandem and provide the only homology shared among family members. The C2 domains function as Ca2+-binding sites that mediate Ca2+-dependent interactions of synaptotagmin with other molecules including phospholipids and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (31, 32). The C2A domain binds three and C2B binds two Ca2+ ions. Although all isoforms have C2 domains, only eight show Ca2+-dependent phospholipid binding activity: 1, 2, 3, 5, 6, 7, 9, and 10. Additionally, the affinity for Ca2+ is in the micromolar range but differs between isoforms; for example, the ubiquitously expressed synaptotagmin 7 has a higher affinity for Ca2+ than the neuronal synaptotagmin 1. The absence of Ca2+-binding activity in other isoforms is due to one or more point mutations in the five aspartic acid residues that coordinate Ca2+-binding in each of the C2 domains. The binding affinity for phospholipids and the SNARE protein syntaxin also differs among family members. In vitro, synaptotagmins 1, 2, and 5 bind phospholipids at low micromolar Ca2+ concentrations and syntaxins at higher concentrations, whereas synaptotagmins 3 and 7 bind both phospholipids and syntaxins at low micromolar Ca2+ concentrations.

Synaptotagmin 1 is the most studied and best characterized isoform and is thought to be the primary Ca2+ sensing protein for exocytosis of synaptic vesicles in neurons. The affinity of synaptotagmin 1 for Ca2+ becomes enhanced upon interaction of the C2 domains with acidic phospholipids, specifically phosphatidylserine, which is important in Ca2+-triggered vesicle fusion (4, 31, 32, 39, 45). Synaptotagmin 1 is thought to regulate synaptic vesicle docking at the plasma membrane as well as fusion pore expansion dynamics (31, 32). In vitro, synaptotagmins have been shown to undergo Ca2+-independent and Ca2+-dependent interactions with SNARE proteins (31, 32). In the absence of Ca2+, synaptotagmin 1 was coisolated with SNARE complexes from brain. Synaptotagmin 1 was also shown to bind to t-SNARE proteins, syntaxin and SNAP 25, in a Ca2+-regulated manner (31, 32) as well as with heterotrimeric SNARE complexes containing vesicle-associated membrane protein (VAMP) 2 (4).

In addition to exocytic proteins, synaptotagmin 1 binds to the clathrin adaptor protein-2 (AP-2) with high affinity (29, 55,
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61), indicating a role in endocytosis as well (30, 38, 40). The C2B domain of synaptotagmin 1 binds to AP-2 (9) in a Ca^{2+}-independent manner (56). Synaptotagmins 1 through 7 interact with AP-2 with high affinity (29, 61) and binding of synaptotagmin and AP-2 is stimulated by peptides with a tyrosine-based endocytic sorting motif (22).

Although originally described as a neuronal specific isoform, Musch et al. (36) reported that synaptotagmin 1 is also present in intestinal epithelial cells. Synaptotagmin 1 was highly localized to the apical membrane of enterocytes in the small intestine and was likewise present in a colon carcinoma cell line (Caco-2BBe) (36, 37). Expression of synaptotagmin 1 was confirmed by RT-PCR, immunoblotting, small interfering RNA (siRNA) studies and by direct sequencing of the immunoprecipitated protein. Although a specific role for synaptotagmin 1 in exocytosis was not investigated, the protein was shown to regulate Ca^{2+}- and Ca^{2+}-induced endocytosis of the sodium/hydrogen exchanger 3 (NHE3) (36).

Other synaptotagmin isoforms have been implicated in regulated exocytosis in nonneuronal tissues including endocrine cells (14, 35), fibroblasts (33, 41), and sperm cells (34). Synaptotagmin 3 was localized to the synaptic plasma membrane in nerve cells, but not on synaptic vesicles (3), and is a high-affinity Ca^{2+} sensor in neurotransmitter release (47). However, in pancreatic beta cells, synaptotagmin 3 is found on the plasma membrane (20) as well as secretory granules (35) and plays a role in Ca^{2+}-induced insulin exocytosis (14). Additionally, synaptotagmin 3 is needed for the formation and delivery of internalized cargo from early endosomes to the perinuclear recycling compartment in mast cells (17). Like synaptotagmin 3, synaptotagmin 7 has also been found to have different subcellular localizations between cell types. Synaptotagmin 7 was shown on lysosomes in fibroblasts where it regulates Ca^{2+}-dependent lysosomal exocytosis to repair the plasma membrane (33). In neurons, synaptotagmin 7 was found at the plasma membrane where it regulates Ca^{2+}-dependent secretory vesicle exocytosis (46). Additionally, synaptotagmin 7 is known to have several splice variants (9, 45, 46), which may potentially account for these different subcellular localizations.

In the present study, we determined the synaptotagmin isoforms present in acinar cells and began to elucidate their role in Ca^{2+}-dependent exocytosis. Here we identify the once thought neuronal specific synaptotagmin 1 along with synaptotagmins 3, 6, and 7 in pancreatic acini. Results indicate that synaptotagmin 1 is found on a subpopulation of zymogen granules (ZGs) located near the apical plasma membrane. Immunofluorescence showed partial colocalization of synaptotagmin 1 and VAMP-2 on ZGs. In contrast, synaptotagmin 3 was found on acinar membrane and microsomal fractions but was absent on ZGs. Synaptotagmin 3 localized to perinuclear and apical regions in acini and particularly localized with a lysosomal membrane protein, LAMP-1. Use of recombinant synaptotagmin 1 C2AB domain in permeabilized cells significantly inhibited Ca^{2+}-stimulated secretion. Conversely, introduction of recombinant synaptotagmin 3 cytoplasmic domain in permeabilized cells had no effect on Ca^{2+}-stimulated secretion. Collectively these data provide molecular evidence of synaptotagmin 1 function outside of the nervous system.

Materials and Methods

Antibodies. Polyclonal anti-synaptotagmin 1 (catalog no. 105 002) and monoclonal anti-VAMP-2 (catalog no. 104 211) were purchased from Synaptic Systems. Monoclonal anti-LAMP-1 (catalog no. VAM-EN001) was purchased from Assay Designs. An Alexa 488-conjugated anti-LAMP-1 (catalog no. S2177) was purchased from Sigma. A polyclonal antibody to the cytosolic C2AB domain of recombinant synaptotagmin 1 was a generous gift from T. Martin at the University of Wisconsin. A polyclonal antibody for synaptotagmin 3 was a generous gift from M. Takahashi from Kitasato University in Japan.

Other reagents. Soybean trypsin inhibitor, benzamidine, phenylmethylsulfonfyl fluoride, goat serum, cold-water fish gelatin, and Triton X-100 were purchased from Sigma-Aldrich, essential amino acid solution from Gibco, and a protease inhibitor cocktail containing AEBSF, aprotinin, EDTA, leupeptin, and E64 from Calbiochem. Peroxidase-conjugated donkey anti-rabbit IgG, Percoll, and glutathione Sepharose high-performance beads were from GE Healthcare. Easy-A Hi-Fi PCR cloning enzyme was purchased from Stratagene. TissueTek optimal cutting temperature (OCT) compound was purchased from Sakura Finetek. The perfringolysin O (PFO) bacterial expression plasmid was a kind gift from A. Johnson and A. P. Heuck at the University of Texas and University of Massachusetts-Amherst, respectively (23, 24).

RT-PCR. Total RNA was isolated from brain and pancreas and poly(A)-RNA was purified from pancreatic acinar cells. Tissues were homogenized in Trizol by use of a Polytron. Total RNA was isolated by using chloroform, isopropanol, and an RNAeasy mini kit from Qiagen. Acinar RNA was further purified by using the Poly(A) Purist kit from Ambion. Primer pairs used were synaptotagmin 1 5'-CCCCTGAAAACCTGACGCCGCTGTTTCACC-3' (sense) and 5'-CCCTGAGCCCTCGGAG-3' (antisense), synaptotagmin 6 5'-TCCCTAATGTGATGCGGGCCG-3' (sense) and 5'-GGGCTCTCCTCTTGAGAGTT-3' (antisense) and synaptotagmin 7 5'-CTTATGAAGCCTACATGAGCC-3' (sense) and 5'-GAACCTCGTCATGACCACTTC-3' (antisense). Reverse transcription (RT) of RNA was performed by using the RETROscript kit from Ambion. Polymerase chain reaction (PCR) was performed on a MJ Mini Personal Thermo Cycler (Bio-Rad) with an initial denaturation of 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 55.4°C for 1 min, 72°C for 1-min amplification, and a final extension of 72°C for 5 min.

Isolation of pancreatic acini. The University of Wisconsin Committee on Use and Care of Animals approved all studies involving animals. Pancreatic acinar cells were isolated from adult male Sprague-Dawley rats by collagenase digestion as described previously (50). Acini were suspended in HEPES buffer consisting of (in mM) 10 HEPES, 1.6 KCl, 0.56 MgCl2, 1.28 CaCl2, 0.6 Na2HPO4, 5.5 D-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 BSA, gassed with 100% O2, adjusted to pH 7.48, and maintained at 37°C for 30 min before performance of assays.

Immunofluorescence microscopy. After the indicated treatments, lobules or isolated acini were gently pelleted and fixed in 4% paraformaldehyde in 1x PBS for 2 h or 30 min, respectively, at room temperature. Lobules and acini were then dehydrated by using a progressive sucrose gradient, resuspended in TissueTek OCT compound, and then quick frozen in liquid nitrogen-cooled isopentane for

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Immunofluorescence microscopy was conducted on 9-μm-thick cryostat sections as detailed previously (49, 51) with the exception of the utilization of Image-iT FX Signal Enhancer according to manufacturer’s instructions followed by combining the blocking and addition of primary antibody into one step with a 2-h simultaneous incubation. Antigen-competition studies were conducted by preincubating synaptotagmin 3 antibody with a 10-fold molar excess of peptide antigen for 2 h at 4°C, prior to a 2 h incubation with the tissue. The buffer used for blocking and incubation steps contained 1× PBS, 3% bovine serum albumin, 2% goat serum, 0.7% cold-water fish skin gelatin, and 0.2% Triton X-100. Sections were incubated with anti-synaptotagmin 1 polyclonal antibody (1:20), anti-synaptotagmin 3 polyclonal antibody (1:50), anti-VAMP-2 monoclonal antibody (1:20), or anti-LAMP-1 monoclonal antibody (1:50). Alexa Fluor 647-conjugated phallolidin was added at 10 units/200 μl, in 1× PBS for 20 min at room temperature after secondary incubation and rinsing. Slides were mounted by using Prolong Antifade with DAPI to label nuclei. For external cell surface labeling of acinar cell whole mounts isolated acini were treated as indicated and immediately immersed in an ice bath at 4°C. Acini were allowed to settle and transferred to ice-cold blocking buffer devoid of Triton X-100 containing anti-synaptotagmin 1 (COOH-terminus antibody) and placed on a wheel to be labeled in suspension at 4°C for 2 h. Acini were rinsed with ice-cold 1× PBS, fixed in 2% formaldehyde for 10 min at room temperature, rinsed three times with 1× PBS, and labeled with secondary antibody for 1 h at room temperature. Rinsed acini were resuspended in 1× PBS and layered onto a coverslip for confocal evaluation.

For immunofluorescence measurements utilizing multiple fluorophores, excitation was captured at the appropriate wavelength to ensure no overlapping excitation between channels. Bright-field images were captured by using a Nikon Eclipse TE2000 microscope, a PlanApo ×100 oil objective with a numerical aperture of 1.4, and a Hamamatsu Orca camera. Images were deconvolved by use of Volocity software and were processed for publication with Volocity, ImageJ, or Photoshop software. Confocal images were captured with a Nikon A1R high-speed confocal microscope, a Plan Apo VC ×60 oil objective with a numerical aperture of 1.4, a side-mounted scanhead, argon gas, diode-pumped solid-state laser and diode lasers, interface control unit, and workstation running NIS-Elements C imaging software. Confocal images were processed for publication with Photoshop software.

Quantification of immunofluorescence images. Ten confocal z-series images collected by using identical parameters with threshold set at 10× background from three separate tissue preparations were analyzed by using volume measurement on NIS-Elements C imaging software, which enables the estimate of the volume of threshold objects by offering a mean intensity of the selected channel throughout the Z-stack.

Tissue fractionation. Acini were suspended in a lysis buffer containing (in mM) 50 Tris (pH 7.4), 5 EDTA, 10 tetrasodium pyrophosphate, 1 PMSP, 1.28 benzamidine, and protease inhibitor cocktail and sonicated. Soluble and membrane fractions were separated by centrifugation at 100,000 g for 30 min at 4°C. The pellet was resuspended in the same buffer containing 0.2% Triton X-100 and sonicated. Membrane fractions were washed in a 0.1 M sodium carbonate (pH 11.0) and placed on ice for 30 min. Sodium carbonate-soluble and -insoluble proteins were separated by centrifugation at 100,000 g for 1 h at 4°C. The pellet was resuspended in the same buffer containing 0.2% Triton X-100 and sonicated. ZGs were purified by Percoll density centrifugation as previously described (59). Briefly, rat pancreases were minced in 5 vol of a homogenization buffer containing (in mM) 10 MOPS (pH 6.8), 250 sucrose, 0.1 MgCl₂, 0.1 PMSP, and 1 benzamidine and homogenized. 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 at 4°C to separate microsomal and cytotoxic fractions.

Acinar cell permeabilization. Acini were suspended in a permeabilization buffer containing (in mM) 20 PIPES (pH 6.6), 139 K⁺-glutamate, 1.78 MgCl₂, 2 Mg-ATP, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 35 pM PFO. PFO is a cholesterol-dependent cytolsin that assembles to create large (25 nm) aqueous pores in cell membranes (23, 24). PFO was allowed to bind to intact cells on ice for 15 min, and excess unbound PFO was removed by washing at 4°C in the same buffer without PFO. Acini were aliquoted into prechilled microcentrifuge tubes containing the indicated amounts of recombinant proteins. The cell suspension was then diluted with an equal volume of the same buffer. Cell suspensions were immersed in a 37°C water bath and incubated with gentle mixing for 15 min. To the cell suspension, the indicated amounts of recombinant protein and the same buffer containing enough CaCl₂ to create the desired final concentration of free Ca²⁺ were added. The quantity of CaCl₂ added to the buffer was calculated on the basis of dissociation constants by use of WEBMAXCLITE v1.15 software. Cell suspensions were immersed in a 37°C water bath and incubated with gentle mixing for 30 min. Cells were then cooled in an ice bath for 3 min and centrifuged at 12,000 g for 1 min. Amylase activity in the medium was determined by using a Phadebas assay kit. Data were calculated as the percent of total cellular amylase present in an equal amount of cells measured at the start of the experiment.

Glutathione S-transferase fusion proteins. Glutathione S-transferase fusion proteins of synaptotagmin 1 and 3 were purified by glutathione affinity chromatography and released from the beads by thrombin cleavage as previously described (18).

RESULTS

Synaptotagmins 1, 3, 6, and 7 are present in pancreatic acinar cells. Synaptotagmins-1, 2, 3, 5, 6, 7, 9, and 10 are known to bind to Ca²⁺ at physiological levels (29); thus we investigated which isoforms are expressed in pancreatic acinar cells. Initial studies using RT-PCR indicated that the neuronal specific isoform, synaptotagamin 1, was present at very high levels in brain but surprisingly also detected in whole pancreas and isolated pancreatic acinar cells (Fig. 1). Additionally, synaptotagmins 3 and 6 were also present in brain, pancreas, and isolated acinar cells. Synaptotagmin 7 was present in...
pancreas and isolated acinar cells but absent in brain. Synaptotagmins 2, 4, and 5 were found in brain but absent in isolated acinar cells (data not shown). We were unsuccessful at detecting synaptotagmins 8 and 9 in control samples and did not analyze synaptotagmin 10.

Synaptotagmin 1 localizes to ZGs and the apical membrane of acini. Immunoblotting with an antibody raised against the C2AB domain of synaptotagmin 1 revealed multiple bands in a brain lysate likely reflecting the homology of C2 domains between synaptotagmin isoforms (Fig. 2A). However, this antiserum detected a single 72-kDa band in an acinar membrane (MEM) fraction and on ZG membranes (ZGM). Further analysis following fractionation of the pancreas detected the 72-kDa band in the postnuclear supernatant (PNS), ZGs, ZGM, NaCO3-washed ZGM (WZGM), microsomal, and NaCO3-washed microsomal fractions (Fig. 2B). An extremely faint band was also sometimes detected in ZG content (ZGC) and cytosol fractions. We attributed this faint band to some slight contamination from membranes because NaCO3 (pH 11) washing of microsomes or ZGM significantly enriched the 72-kDa band, indicating that it is an integral membrane protein. On the basis of the size of the 72-kDa band as well as RT-PCR results, we utilized a synaptotagmin 1-specific antibody raised against amino acids 120–131 of the protein. As anticipated, strong immunoreactivity was detected in brain at ~72 kDa. Similar to results with the C2AB antibody, analysis of acinar membrane fractions revealed a faint band at 72 kDa (data not shown). Likewise, the 72-kDa immunoreactivity was also in ZGs, was significantly enriched in ZGM fractions, and further enriched when membranes were washed in NaCO3 (pH 11) (WZGM) (Fig. 2C). No immunoreactivity was present in ZGC.

Immunofluorescence localization of synaptotagmin 1 in paraformaldehyde-fixed cryostat sections of isolated pancreatic acini showed distinct staining at the very apical and subapical regions of the acini but was not apparent deeper within the cytoplasm (Fig. 2D). Confirming the presence of synaptotagmin 1 on ZGs, immunofluorescence microscopy on paraformaldehyde-fixed cryostat sections of Percoll-purified ZGs revealed immunoreactivity on the granules (Fig. 2D). Identical results were obtained with the anti-C2AB antibody (data not shown). On the basis of our (8, 58) and others (2, 3) previous findings that VAMP-2 localizes to the more apical regions of cytoplasm, we triple-labeled VAMP-2 and synaptotagmin 1 together with actin filaments in cryostat sections of isolated acini (Fig. 3A, a–d). As previously shown, VAMP-2 was extensively localized within and above the actin web along the plasma membrane (Fig. 3A, f and f'). Similarly, synaptotagmin 1 was also apically localized, with strong immunoreactivity present along the apical membrane and a more sparse punctuate localization below the actin filaments within the apical cytoplasm (Fig. 3A, e and e'). Some minor diffuse synaptotagmin 1 staining was also detected in basal cytoplasmic regions.
Fig. 3. Synaptotagmin 1 colocalizes with vesicle-associated membrane protein (VAMP) 2. Rat pancreatic acini (A) and Percoll-purified ZGs (C) were fixed in 4% paraformaldehyde and bright-field immunofluorescence microscopy was conducted on 9-μm-thick sections of tissue. Synaptotagmin 1-isoform specific (1:100) and VAMP-2-specific (1:50) immunoreactivity were detected by using Alexa Fluor 488-conjugated anti-rabbit IgG (1:250) and Alexa Fluor 546-conjugated anti-mouse IgG, respectively. Actin filaments (Actin) were identified with Alexa Fluor 647-conjugated phalloidin and label the apical-most aspects of the acini. A and B: pseudo-colors were applied postcollection as indicated. White boxes in (A, e–h) correspond to the subsequent magnifications in (A, e–h’). B: sequential reconstructed 3-dimensional images rotated along the z-plane of the magnifications in (A, e–h’). Arrows indicate 3D direction: green, x; red, y; blue, z. C: arrows and arrowheads denote areas of colocalization and areas lacking colocalization, respectively. Scale bars, 7 μM. Images are a single representative experiment performed on 3 separate tissue preparations. Nuclei are labeled in blue with DAPI and mark the basolateral region of the acini. Images in Ad and Cl show corresponding differential interference contrast images.
of the acini, especially in areas where actin filaments were present. An incomplete but significant overlap between VAMP-2 and synaptotagmin 1 was detected at distinct areas along the apical plasma membrane (Fig. 3A, g and g') as well as areas of clear overlap of immunoreactivity within the actin web (Fig. 3A, h and h').

The extent of colocalization between fluorophores is most clearly displayed by examining sequential images of three-dimensional reconstructed z-series rotated in space along the z-plane (Fig. 3B). Immunofluorescence of synaptotagmin 1 and VAMP-2 in Percoll-purified ZGs confirmed their colocalization on ZGs; however, some independent staining of each molecule was clearly evident (Fig. 3C).

Synaptotagmin 3 localizes to acinar membrane and microsomal fractions. Consistent with its characterization in beta cells (14, 35), immunoblotting with a synaptotagmin 3 specific antibody raised against the cytoplasmic domain of the protein revealed a 63-kDa band in brain lysates, which is in agreement with the predicted molecular mass of synaptotagmin 3 (Fig. 4A). Immunoreactivity was also detected in acinar membrane fractions. Following tissue fractionation, the 63-kDa band was present in microsomal fractions devoid of ZGs and was enhanced following NaCO₃ (pH 11) washing. Conversely no immunoreactivity was seen in PNS, ZG, ZGM, ZGC, or cytosolic fractions. Immunofluorescence localization of synaptotagmin 3 in cryostat sections of isolated acini revealed staining in a punctuate pattern extending throughout the apical cytoplasm (Fig. 4B). Areas of immunoreactivity were also noted along the cytoplasmic side of the actin web with little or no localization evident on the apical plasma membrane (Fig. 4C). Specificity of the synaptotagmin 3 antisera was demonstrated by preabsorption with synaptotagmin 3 antigen prior to immunofluorescence (Fig. 4D). Consistent with previous reports that synaptotagmin 3 localized on endosomes in RBL mast cells (17), significant colocalization with lysosomal-associated membrane protein 1 (LAMP-1) was detected in acinar cells (Fig. 5). This pattern of localization suggests that synaptotagmin 3 is not likely to be directly involved in acinar ZG exocytosis.

The C2AB domain of synaptotagmin 1 inhibits Ca²⁺-stimulated exocytosis. To begin to identify a functional role for synaptotagmin in Ca²⁺-stimulated digestive enzyme secretion, we introduced a previously characterized (34) inhibitory construct of synaptotagmin 1 containing the C2A and C2B Ca²⁺-binding domains but lacking the NH²-terminal transmembrane anchoring sequence of synaptotagmin 1 (synaptotagmin C2AB) into PFO-permeabilized acinar cells and evaluated its effects on Ca²⁺-stimulated amylase release. Preliminary experiments measuring the Ca²⁺-sensitivity of amylase secretion from PFO permeabilized cells indicated that maximum secretion was achieved at 3 μM free Ca²⁺. Thus permeabilized acini...
were preincubated with increasing concentrations of C2AB for 15 min and then stimulated with 3 μM free Ca2⁺ for 30 min. The C2AB construct inhibited Ca2⁺-stimulated secretion in a concentration-dependent manner with a significant 22% inhibition achieved at 300 μg/ml and a maximal inhibition of 35% achieved at 400 μg/ml (Fig. 6). The maximal C2AB-mediated secretory inhibition measured at 1 μM Ca2⁺ was 25.5 ± 3.7 vs. 35.0 ± 0.4% seen at 3 μM Ca2⁺ (means ± SD, n = 2 independent experiments performed in triplicate) (data not shown). Concentrations of C2AB as high as 500 μg/ml did not further inhibit secretion nor was basal secretion (≤10 nM free Ca2⁺) significantly inhibited (data not shown). This narrow concentration response to the synaptotagmin protein is similar to our previous studies in permeabilized acini using soluble

Fig. 5. Synaptotagmin 3 colocalizes with LAMP-1 in perinuclear and apical regions of acini. Bright-field immunofluorescence microscopy was conducted on 9-μm-thick sections from 4% paraformaldehyde-fixed acini. Synaptotagmin 3 (1:100) and LAMP-1 (1:100) immunoreactivity were detected by use of Alexa Fluor 488-conjugated anti-rabbit IgG (1:250) and Alexa Fluor 546-conjugated anti-mouse IgG (1:250), respectively. Arrows and arrowheads denote areas of overlap and lack of overlap, respectively. Scale bars, 7 μM. Images are a single representative experiment performed on 3 separate tissue preparations. Nuclei are labeled in blue with DAPI, Far right image shows corresponding DIC.

Fig. 6. Synaptotagmin 1 but not synaptotagmin 3 C2AB domain modulates Ca2⁺-stimulated amylase release. A: isolated acini were permeabilized with perfringolysin O (PFO) and preincubated with the indicated concentrations of recombinant synaptotagmin 1 C2AB for 15 min. Amylase secretion was measured after an additional 30 min under basal (≤10 nM free Ca2⁺) or Ca2⁺-stimulated (3 μM) conditions. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. Basal secretion was not altered by maximal concentrations of synaptotagmin 1 C2AB protein (data not shown). Data are means ± SE of a single representative experiment performed in triplicate. B: means ± SE of 3 independent experiments each performed in triplicate expressed as a % maximal secretion. The RK/KK mutant (R199A/K200A/K297A/K301A) was added at 400 μg/ml. Statistical significance (* P <0.05) was determined by a paired t-test. C: isolated acini were permeabilized with PFO and preincubated with 400 μg/ml recombinant synaptotagmin 1 for 15 min. Amylase secretion was measured after an additional 30 min under Ca2⁺-stimulated (≤10 nM free Ca2⁺) or Ca2⁺-stimulated (3 μM) conditions. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. Basal secretion was not altered by maximal concentrations of synaptotagmin 3 protein. Data are means ± SE of 3 independent experiments performed in triplicate. Note that no concentrations were statistically significant from control (Con), which was determined by a paired t-test.
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SNARE proteins (59), the SNARE clamping protein complex 2 (8), CRHSP-28 (50), or cysteine string protein (58) to modulate secretion. A C2AB mutant construct replacing four basic amino acids with alanine at positions R199A, K200A within the C2A domain and K297A, K301A within the C2B domain (RK/KK), known to have reduced sensitivity of Ca\(^{2+}\)-dependent SNARE binding but retain normal phospholipid binding, was also utilized (32). Preincubation of cells with the RK/KK mutant (400 \(\mu\)g/ml) also inhibited Ca\(^{2+}\)-stimulated secretion by 25%, which was significantly less than that achieved with wild-type C2AB protein (Fig. 6B). Analysis of the time course of C2AB-mediated inhibition of secretion revealed only 6% inhibition at 10 min followed by 46 and 34% inhibition at 15 and 30 min, respectively (Fig. 6C). Consistent with a previous study demonstrating the ability of the C2AB construct to inhibit exocytosis in PC12 cells (54), these findings suggest that preincubation of acini with the C2AB construct arrests the SNARE complex prior to exocytosis. In contrast to the synaptotagmin 1 C2AB and in accordance with its absence within the acinar secretory pathway, the cytoplasmic domain of synaptotagmin 3 had no significant effects on Ca\(^{2+}\)-stimulated secretion at concentrations as high as 400 \(\mu\)g/ml (Fig. 6D). Basal secretion was not altered by synaptotagmin 3 (data not shown).

**Synaptotagmin 1 enters the plasma membrane during exocytosis.** The effects of the C2AB construct to inhibit secretion together with the apical localization of synaptotagmin 1 supported a role for the protein in secretion. Thus, the dynamics of synaptotagmin 1 trafficking to the apical membrane was analyzed by incubating intact acini with an antibody specific to the NH\(_2\)-terminal intraluminal domain of synaptotagmin 1 that is externalized following exocytosis (29, 55). Consistent with the immunofluorescence localization of synaptotagmin 1 in acinar cryostat sections (Figs. 2 and 3), surface labeling of intact acini showed immunoreactivity exclusively along the apical membrane (Fig. 7). Moreover, this immunoreactivity was markedly enhanced upon acinar stimulation by CCK-8. Quantification of basal immunoreactivity (997 \(\pm\) 84 \(\mu\)m\(^3\)) vs. 2 min CCK-8 (100 pM) stimulation (4,147 \(\pm\) 538 \(\mu\)m\(^3\)) revealed a fourfold increase at the apical membrane indicating that synaptotagmin 1 enters the plasma membrane during secretion.

**DISCUSSION**

Previous studies have provided evidence to support synaptotagmin isoforms in various exocrine tissues. Synaptotagmins 6 and 8 were found by RT-PCR and the products analyzed by Southern blotting in whole pancreas (29). Subsequently, synaptotagmins 1 and 3 were demonstrated in isolated pancreatic acinar cells by RT-PCR (62). In mouse parotid acinar cells, mRNAs to synaptotagmins-1, 2, 3, 4, 6, and 7 were shown (26). Likewise, synaptotagmins-1, 3, 4, 7, and 11 mRNAs were shown in rat parotid acini (25, 26, 28). At the protein level, Leviaus et al. (28) isolated and sequenced synaptotagmin 1 from rat parotid acini. Likewise, synaptotagmin 1 was demonstrated in mouse and rat parotid, submandibular, and pancreatic acinar cells by immunoblotting (7, 26, 28). Furthermore, Zhao et al. (62) reported synaptotagmin 1 protein levels in pancreatic acinar cells are reduced whereas synaptotagmin 3 protein levels are increased in SERCA\(^{2-/-}\) mice that have decreased levels of cytosolic Ca\(^{2+}\). Finally, an unidentified synaptotagmin isoform was also shown by immunoblotting in tick salivary glands (27). Thus, although several studies support synaptotagmin isoforms including synaptotagmin 1 are expressed in exocrine cells, little is understood of their subcellular localization or functional roles within these tissues.

In contrast to RT-PCR and immunoblotting, proteomic analysis of ZGs and ZGMs has not demonstrated any synaptotagmin family members; however, evidence for synaptotagmin-like proteins (SLPs) 1 and 4 was demonstrated on ZGMs (5, 42). The lack of detection of synaptotagmins may not be unexpected given the relatively low levels of synaptotagmin 1 detected by immunoblotting. It is unlikely that the antibody to the C2AB domain cross-reacts with the SLPs because NaCO\(_3\)-washing of ZGMs significantly enhanced immunoreactivity. Unlike traditional synaptotagmins, SLPs are peripheral membrane proteins that are presumably removed by NaCO\(_3\) washing. Additionally, the antigen for the synaptotagmin 1-specific antibody used for immunoblotting and immunofluorescence shows no sequence homology with the SLPs. Finally, our demonstration that the intraluminal domain of

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**References:**

synaptotagmin 1 is externalized during acinar stimulation and thereby recognized by an NH2-terminal specific antibody to synaptotagmin 1 in intact acini clearly supports the specific expression and localization of synaptotagmin 1 in acinar cells.

The present study demonstrates synaptotagmins 1, 3, 6, and 7 at the mRNA level in pancreatic acini. Expression of synaptotagmins 1 and 3 proteins was confirmed by immunoblotting. Using various antisera, we were unable to detect synaptotagmins 6 and 7 in acini. Relatively little is known about the function of synaptotagmin 6. It was shown to be expressed in sperm cells and to regulate acrosomal exocytosis (34). Multiple studies in various cell types have shown synaptotagmin 7 regulates Ca2⁺-dependent exocytosis (33, 41, 46); however, its subcellular localization (plasma membrane, lysosome or large dense core vesicles) (14, 33, 46, 57) and the number of alternative splice variants present in cells are controversial (10, 45, 46). Synaptotagmin 7 was reported to be one of the Ca2⁺ sensors for insulin exocytosis in β-cells (14), glucagon exocytosis in α-cells (19), and lysosomal exocytosis in fibroblasts (33, 41). In chromaffin cells, synaptotagmin 7 was also demonstrated control fusion pore opening during exocytosis (43).

The present findings show that synaptotagmin 3 is present in acinar microsomal fractions devoid of ZGs and partially colocalizes with LAMP-1, indicating an association with endosomes and/or lysosomes. In RBL mast cells, synaptotagmin 3 immunoreactivity copurified with early endosomal markers including annexin II, early endosomal antigen 1 (EEA1) and syntaxin 7 (17). It was postulated that synaptotagmin 3 is involved in a perinuclear endocytic recycling compartment involved in secretory granule maturation. Our results are similar to those of Grimberg et al. (17) showing a perinuclear staining of synaptotagmin 3. Moreover, the colocalization of LAMP-1 and synaptotagmin 3 is consistent with an endolysosomal localization of LAMP-1, which is known to traffic from the trans-Golgi network to an early endosomal compartment prior to delivery to lysosomes (16). Thus, as has been suggested in RBL cells, which also have large secretory granules, it is possible that synaptotagmin 3 has a role in the maturation of immature secretory granules and the trafficking of proteins through an early endosomal compartment (17).

Previous studies have shown that the cytoplasmic domain of synaptotagmin 1 acts as a clamp to inhibit neuronal exocytosis by disrupting the ability of endogenous synaptotagmin to bind t-SNAREs and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] (1, 54). Similar to the inhibitory effects of C2AB in PC12 cells (54), this construct significantly inhibited Ca2⁺-dependent secretion by 35% when introduced into permeabilized acini. Interestingly, this level of inhibition is consistent with the inhibitory effects of cleaving VAMP-2 with tetanus toxin prior to stimulating secretion in permeabilized PC12 cells and this activity is strongly correlated with its Ca2⁺-dependent PtdIns(4,5)P2 and SNARE binding activity (54). In contrast, the C2A domain of synaptotagmin 1 failed to inhibit exocytosis in PC12 cells but rather required the C2B domain for inhibition, establishing that the inhibitory activity of different synaptotagmin C2 domains cannot be predicted by their position in the molecule. Although the complete C2AB domain of synaptotagmin 3 was not tested in this earlier study, the present results may indicate that the tandem orientation of the C2 domains in a given isoform may also convey the inhibitory effects on exocytosis.

Because C2AB domains show considerable homology within synaptotagmin isoforms, we cannot conclusively rule out that synaptotagmin 1 C2AB was inhibiting the function of other synaptotagmins in acini. Taken together with the known function of synaptotagmin 1 in other secretory cell types, its subcellular localization in the secretory pathway of acini, and the rapid externalization of the NH2-terminal intraluminal domain at the apical membrane upon CCK-8 stimulation, these data clearly support a functional role for this molecule in the early stages of ZG exocytosis. Clearly, additional studies selectively ablating synaptotagmin expression in acini will be necessary to unambiguously identify its precise role in the acinar secretory pathway.

Our previous results showed acini express at least 2 populations of ZGs based on their expression of VAMP-2 or 8 (59). Synaptotagmin 1 significantly colocalized with VAMP-2; however, we were unable to colocalize VAMP-8 and synaptotagmin 1 because of cross-reactivity of the secondary antibodies against polyclonal antiserum. The VAMP-2-containing granules accumulate in the most apical aspects of the cytoplasm immediately below the plasma membrane (2, 8, 13, 59), whereas VAMP-8-positive granules are dispersed deeper within the apical cytoplasm (8, 59). Acinar secretion proceeds as an initial peak phase within the first 2 min of stimulation and then declines to a plateau phase over several minutes, which is sustained in the presence of secretagogue (21). This pattern of exocytosis was first described by using sequential secretory measures in isolated cells (44) and later quantified at the cellular level by FM143 labeling, membrane capacitance measures, and differential interference contrast microscopy (6). Using dextran labeling of sites of exocytosis, we previously found evidence to support that VAMP-2-positive ZGs mediate the initial secretory response (59), which is consistent with localization of VAMP-2 at the most apical aspects of acini.

In neurons, synaptic vesicle exocytosis is mediated by the vesicle SNAREs VAMP-2 and the target SNAREs syntaxin 1 and SNAP 25. Synaptotagmin 1 is crucial for rapid Ca2⁺-evoked transmitter release by controlling SNARE function (4,
Upon Ca\(^{2+}\) binding to the C2 domains, synaptotagmin penetrates the plasma membrane containing anionic phospholipids such as phosphatidylserine (31, 32). The C2 domains also mediate Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent interactions with syntaxin 1 and SNAP 25. Evidence suggests that Ca\(^{2+}\)-dependent membrane binding by synaptotagmin is itself insufficient to trigger fusion but that it also requires Ca\(^{2+}\)-dependent SNARE binding by synaptotagmin. An additional regulatory protein, complexin 1, also coordinates synaptotagmin function. Complexin 1 was originally thought to clamp SNARE complexes at a prefusion step, thereby inhibiting exocytosis (15, 60). Upon elevation of intracellular Ca\(^{2+}\), complexin 1 was proposed to be displaced from the SNARE complex by synaptotagmin 1, in turn allowing the final stages of neurotransmitter release to commence (15). More recent studies have shown that, in addition to this clamping activity, complexin 1 also directly facilitates the final stages of exocytosis (48, 52). We recently reported that acinar cells express complexin 2 and further found that introduction of recombinant complexin 2 into permeabilized acini inhibited Ca\(^{2+}\)-dependent exocytosis (8). Moreover, we showed that complexin 2 interacts with VAMP-2-positive granules. Taken together with the present results, it is conceivable that VAMP-2 ZGs together with synaptotagmin 1 and complexin 2 function in the early peak phase of the secretory response analogous to their concerted role in synaptic vesicle exocytosis. Clearly, however, the kinetics of ZG exocytosis are much slower than synaptic vesicles likely owing to differences in SNARE isoforms, Ca\(^{2+}\) release kinetics, and the ~50-fold larger diameter of ZGs.

Interestingly, and similar to our results, Chang and colleagues (36, 37) recently demonstrated that synaptotagmin 1 is present in intestinal epithelial cells by RT-PCR, immunoblotting and direct sequencing, and further showed synaptotagmin 1 localizes to the apical membrane by immunofluorescence. We also found that synaptotagmin 1 localized to the apical aspects of acini but surprisingly showed incomplete overlap with VAMP-2 on the plasma membrane. In contrast, synaptotagmin 1 and VAMP-2 showed considerable overlap in isolated ZGs. This incomplete overlap on the plasma membrane may indicate that synaptotagmin 1 is retained separate from VAMP-2 on the apical membrane following exocytosis. Synaptotagmin 1 was previously shown to bind to the clathrin adaptor AP-2, thus indicating a role in endocytosis (29).

In summary, the present study establishes the expression and subcellular localization of the previously described “neuronal specific” synaptotagmin 1 in acinar cells of the pancreas. Use of a synaptotagmin 1 truncation construct strongly suggests a functional role for the molecule in acinar secretion. In light of recent evidence implicating synaptotagmin 1 in enterocyte apical membrane endocytosis, it will be of major interest to determine whether synaptotagmin 1 binds to AP-2, thus having a possible function in endocytosis in pancreatic acini.

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