Expression, Localization and Functional Role for Synaptotagmins in Pancreatic Acinar Cells

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Summary

Secretagogue–induced changes in intracellular Ca\(^{2+}\) play a pivotal role in secretion in pancreatic acini yet the molecules that respond to Ca\(^{2+}\) are uncertain. Zymogen granule (ZG) exocytosis is regulated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. In nerve and endocrine cells, Ca\(^{2+}\)-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 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 endo-lysosomal 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 Ca\(^{2+}\) 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 4 fold, demonstrating synaptotagmin is inserted into the apical membrane during ZG fusion. Collectively, these data indicate that acini express synaptotagmin 1 and support that it plays a functional role in secretion whereas synaptotagmin 3 has an alternative role in endo-lysosomal membrane trafficking.

Key words: pancreatic acinar cells, zymogen granules, synaptotagmin, secretion, exocytosis
INTRODUCTION

Pancreatic acinar cells are a prototypical model of exocrine secretory cells that undergo stimulated exocytosis of digestive enzymes which are necessary for the assimilation of the diet. Multiple intracellular signals including diacylglycerol, cAMP and inositol triphosphate (IP3)-mediated release of Ca\(^{2+}\) have been shown to stimulate acinar exocytosis. However, the pivotal role of Ca\(^{2+}\) in the overall secretory response is underscored by findings that double knockout of the type 2 and 3 IP3 receptors fully abolished secretagogue-induced changes in cellular Ca\(^{2+}\) and resulted in a complete lack of salivary and pancreatic acinar secretion (11). Although the critical importance of Ca\(^{2+}\) signaling in acinar exocytosis is well established, the exocytic regulatory proteins that respond to elevated Ca\(^{2+}\) in the cytoplasm are largely uncertain.

Synaptotagmins are a family of membrane proteins composed of 15 known isoforms distributed widely in neuronal and nonneuronal tissues (for comprehensive reviews see ref 4,39,45). Family members have the same basic structure consisting of a short N-terminal intravesicular domain followed by a transmembrane domain and a large C-terminal cytoplasmic segment which 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 Ca\(^{2+}\) binding sites that mediate Ca\(^{2+}\)-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 3 and C2B binds 2 Ca\(^{2+}\) ions. Although all isoforms have C2 domains, only 8 show Ca\(^{2+}\)-dependent phospholipid binding activity – 1, 2, 3, 5, 6, 7, 9 and 10. Additionally, the affinity for Ca\(^{2+}\) is in the micromolar range but differs between isoforms; for example, the ubiquitously expressed synaptotagmin 7 has a higher affinity for Ca\(^{2+}\) than the neuronal synaptotagmin 1. The absence
of Ca\textsuperscript{2+}-binding activity in other isoforms is due to one or more point mutations in the 5 aspartic
acid residues that coordinate Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+} concentrations and syntaxins at higher concentrations, whereas, synaptotagmins 3 and 7 bind both phospholipids and syntaxins at low micromolar Ca\textsuperscript{2+} concentrations.

Synaptotagmin 1 is the most studied and best characterized isoform and is thought to be the primary Ca\textsuperscript{2+} sensing protein for exocytosis of synaptic vesicles in neurons. The affinity of synaptotagmin 1 for Ca\textsuperscript{2+} becomes enhanced upon interaction of the C2 domains with acidic phospholipids, specifically phosphatidylserine (PS), which is important in Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+}-independent and Ca\textsuperscript{2+}-dependent interactions with SNARE proteins (31,32). In the absence of Ca\textsuperscript{2+}, 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 Ca\textsuperscript{2+}-regulated manner (31,32) as well as with heterotrimeric SNARE complexes containing VAMP 2 (4).

In addition to exocytic proteins, synaptotagmin 1 binds to the clathrin adaptor protein-2 (AP-2) with high affinity (29,55,61) indicating a role in endocytosis as well (30,38,40). The C2B domain of synaptotagmin 1 binds to AP-2 (9) in a Ca\textsuperscript{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. reported that synaptotagmin 1 is also present in intestinal epithelial cells (36). 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, 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 cAMP- and Ca\(^{2+}\)-induced endocytosis of the sodium hydrogen exchanger 3 (NHE3) (36).

Other synaptotagmin isoforms have been implicated in regulated exocytosis in non-neuronal 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\textsuperscript{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 located near the apical plasma membrane. Immunofluorescence showed partial colocalization of synaptotagmin 1 and vesicle associated membrane protein (VAMP) 2 on zymogen granules. In contrast, synaptotagmin 3 was found on acinar membrane and microsomal fractions but was absent on zymogen granules. 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\textsuperscript{2+}-stimulated secretion. Conversely, introduction of recombinant synaptotagmin 3 cytoplasmic domain in permeabilized cells had no effect on Ca\textsuperscript{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 (cat # 105 002) and monoclonal anti-VAMP 2 (cat # 104 211) were purchased from Synaptic Systems. Monoclonal anti-LAMP 1 (cat # VAM-EN001) was purchased from Assay Designs. An Alexa 488-conjugated anti-LAMP 1 (cat # 121608) was purchased from BioLegend. A polyclonal antibody to the N-terminal domain of synaptotagmin 1 (cat # 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, phenylmethanesulfonyl 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. Phadebas amylase assay kit, sucrose, MOPS, chloroform and isopropanol were purchased from Fisher Scientific. Trizol, Alexa Fluor 488- and 546-conjugated rabbit and mouse secondary antibodies, Alexa Fluor-647 conjugated Phalloidin, ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) and Image-iT FX Signal Enhancer were purchased from Invitrogen. Protein determination reagent was purchased from Bio-Rad. 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 O.C.T. 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).
Reverse Transcription Polymerase Chain Reaction (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 using a Polytron. Total RNA was isolated using chloroform, isopropanol and RNAeasy mini kit from QIAGEN. Acinar RNA was further purified using the Poly(A) Purist kit from Ambion. Primer pairs used were synaptotagmin 1 5’-CGGCAAACTGACTGTTGTCATTC-3’ (sense) and 5’-GCCCCAGTGCTGTTGAACCA-3’ (antisense), synaptotagmin 3 5’-CCCCCTGACCGTCCACTC-3’ (sense) and 5’-TCTCCACGCTCTCCGAG-3’ (antisense), synaptotagmin 6 5’-TCCCTACTATGTGGGCGC-3’ (sense) and 5’-GGGTTCCTCTTTGAGGAAGTT-3’ (antisense) and synaptotagmin 7 5’-CTTACGTCTCCTCC-3’ (sense) and 5’-GCATGGCTTCAGATCTCC-3’ (antisense). Reverse transcription (RT) of RNA was performed 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 sec, 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, 137 NaCl, 4.7 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, and adjusted to pH 7.48 and were maintained at 37°C for 30 min before performing assays.
**Immunofluorescence Microscopy.** After the indicated treatments, lobules or isolated acini were gently pelleted and fixed in 4% formaldehyde in 1× PBS for 2 h or 30 min, respectively, at room temperature. Lobules and acini were then dehydrated using a progressive sucrose gradient, resuspended in TissueTek O.C.T. compound, and then quick frozen in liquid nitrogen-cooled isopentane for cryosectioning. Percoll purified zymogen granules were harvested and fixed in 4% formaldehyde in 1× PBS for 1 h at room temperature, rinsed 2 times with 1× PBS, resuspended in TissueTek O.C.T. compound and quick frozen in liquid nitrogen-cooled isopentane for cryosectioning. 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 synaptotagmin3 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-synaptotagmin1 polyclonal antibody (1:20), anti-synaptotagmin3 polyclonal antibody (1:50), anti-VAMP 2 monoclonal antibody (1:20), or anti-LAMP1 monoclonal antibody (1:50). Alexa Fluor 647-conjugated phalloidin was added at 10 units/200 μl, in 1× PBS for 20 min at room temperature after secondary incubation and rinsing. Slides were mounted 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 TX-100 containing anti-synaptotagmin1 (C-terminus antibody) and placed on a wheel to be labeled.
in suspension at 4°C for 2 h. Acini were rinsed once with ice cold 1× PBS, fixed in 2% formaldehyde for 10 min at room temperature, rinsed 3 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. Brightfield images were captured 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 using Volocity software and were processed with Volocity, ImageJ, or Photoshop software for publication. Confocal images were captured using a Nikon A1R high-speed confocal microscope, a Plan Apo VC x 60 oil objective with a numerical aperture of 1.4, a side-mounted scanhead, argon gas, DPSS, and diode lasers, interface control unit, and workstation running NIS-Elements C imaging software. Confocal images were processed for publication using Photoshop software.

Quantification of Immunofluorescence Images. Ten, confocal, z-series images collected using identical parameters with threshold set at 10x background from three separate tissue preparations were analyzed 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 PMSF, 1.28 benzamadine 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. Zymogen granules
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 phenylmethylsulfonyl 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 cytosolic fractions.

**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 pM PFO. PFO is a
cholesterol-dependent cytolysin 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 using
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 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 (GST) Fusion Proteins. GST 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 reverse transcription-PCR (RT-PCR) indicated that the neuronal specific isoform, synaptotagmin 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 Zymogen Granules 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 zymogen granule membranes (ZGM). Further analysis following fractionation of the pancreas detected the 72 kDa band in the post nuclear supernatant (PNS), zymogen granules (ZGs), ZGM, NaCO₃-washed ZGM (WZGM), microsomal (Mic) and NaCO₃-washed microsomal (WMic) fractions (Fig 2B). An extremely faint band was also sometimes detected in ZG content (ZGC) and cytosol (Cyto) 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 it is an integral membrane protein. Based on 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 approximately 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 so 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 sub-apical
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). Based on our
(8,58) and others (2,13) 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 3Aa-d). As previously shown, VAMP 2 was extensively
localized within and above the actin web along the plasma membrane (Fig 3Af,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 3Ae,e’). Some minor diffuse synaptotagmin 1 staining was also detected in
basal cytoplasmic regions 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 3Ag,g’) as well as areas of clear overlap of
immunoreactivity within the actin web (Fig 3Ah,h’). The extent of colocalization between
fluorophores is most clearly displayed by examining sequential images of 3-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 antiserum was demonstrated by preabsorbing 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 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 (54) inhibitory construct of synaptotagmin 1 containing the C2A and C2B Ca$^{2+}$-binding domains but lacking the N terminal transmembrane anchoring sequence of synaptotagmin 1 (synaptotagmin C2AB) into PFO-permeabilized acinar cells and its effects on Ca$^{2+}$-stimulated amylase release evaluated. Preliminary experiments measuring the Ca$^{2+}$-sensitivity of amylase secretion from PFO permeabilized cells indicated maximum secretion was achieved at 3 μM free Ca$^{2+}$. Thus, permeabilized acini were preincubated with increasing concentrations of C2AB for 15 min and then stimulated with 3μM free Ca$^{2+}$ for 30 min. The C2AB construct inhibited Ca$^{2+}$-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 Ca$^{2+}$ was 25.5 ± 3.7% versus 35.0 ± 0.4% seen at 3 μM Ca$^{2+}$ (mean ± S.D., 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 Ca$^{2+}$) significantly inhibited (data not shown). This narrow concentration-response to the synaptotagmin protein is similar to our previous studies in permeabilized acini using soluble SNARE proteins (59), the SNARE clamping protein complexin 2 (8), CRHSP-28 (50) or cysteine string protein (58) to modulate secretion. A C2AB mutant construct replacing 4 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 μg/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 Ca²⁺-stimulated secretion at concentrations as high as 400 µg/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 N-terminal intraluminal domain of synaptotagmin 1 which 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 ± 84 cubic micrometers) versus 2 min CCK-8 (100 pM) stimulation (4147 ± 538 cubic micrometers), revealed a 4 fold 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, Levius et al. isolated and sequenced synaptotagmin 1 from rat parotid acini (28). Likewise, synaptotagmin 1 was demonstrated in mouse and rat parotid, submandibular and pancreatic acinar cells by immunoblotting (7,26,28). Furthermore, Zhao et al. reported synaptotagmin 1 protein levels in pancreatic acinar cells are reduced whereas synaptotagmin 3 protein levels are increased in SERCA2+/− mice which have decreased levels of cytosolic Ca^{2+} (62). 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-reacted with the SLPs because NaCO_3-washing of ZGMs significantly enhanced immunoreactivity. Unlike traditional synaptotagmins, SLPs are peripheral membrane proteins which are
presumably removed by NaCO₃-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 synaptotagmin 1 is externalized during acinar stimulation and thereby recognized by an N-terminal specific antibody to synaptotagmin 1 in intact acini clearly supports the specific expression and localization of synaptotagmin 1 in acinar cells.

The current study, demonstrates synaptotagmins 1, 3, 6 and 7 at the mRNA level in pancreatic acini, and expression of synaptotagmins 1 and 3 proteins were 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 to control fusion pore opening during exocytosis (43).

The current 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 Grimberg et al. (17) showing a perinuclear staining of synaptotagmin 3. Moreover, the colocalization of LAMP 1 and synaptotagmin 3 is consistent with an endo-lysosomal 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 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 acini (12). The delay in secretory inhibition seen with C2AB (Fig 6C) may reflect that the protein was unable to interact with SNAREs and/or phospholipids in the docked state and poised to undergo exocytosis. Additionally, the secretory assays are conducted by shifting the cells from an ice-bath to 37°C in order to initiate secretion. This significant period of warming likely delays the kinetics of protein interactions.

The inhibitory response achieved with the RK/KK mutant, although diminished in comparison to the wild-type protein, was unexpected as the full-length RK/KK mutant of synaptotagmin 1 failed to rescue Ca²⁺-triggered exocytosis in PC12 cells devoid of synaptotagmin 1 and 9 (32). However, *in vitro* studies utilizing the RK/KK cytoplasmic mutant demonstrated no effects on phosphatidyl serine or PtdIns(4,5)P₂ binding but did show reduced
Ca\(^{2+}\)-sensitivity for t-SNARE binding and Ca\(^{2+}\)-dependent liposome fusion (32). Thus, it is conceivable that the inhibitory effects of the RK/KK mutant were mediated by Ca\(^{2+}\)-regulated PtdIns(4,5)P\(_2\) binding.

The lack of effect of the synaptotagmin 3 cytoplasmic domain on acinar secretion is consistent with its subcellular localization in acini. However, these results are also unanticipated as the single C2A domain of synaptotagmin 3 inhibits exocytosis in permeabilized PC12 cells and this activity is strongly correlated with its Ca\(^{2+}\)-dependent PtdIns(4,5)P\(_2\) 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 current 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 N-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 due to 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 min, which is sustained in the presence of secretagogue (21). This pattern of exocytosis was first described using sequential secretory measures in isolated cells (44) and later quantified at the cellular level by FM143 labeling, membrane capacitance measures and DIC 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 Ca$^{2+}$-evoked transmitter release by controlling SNARE function (4,31,32,39,45). Upon Ca$^{2+}$ binding to the C2 domains, synaptotagmin penetrates the plasma membrane containing anionic phospholipids such as phosphotidylserine (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 pre-fusion 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 current 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 approximately 50 fold larger diameter of ZGs.

Interestingly, and similar to our results, Chang et al. recently demonstrated 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 (36,37). We also found 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 current 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 if synaptotagmin 1 binds to AP-2
thus having a possible function in endocytosis in pancreatic acini.
Acknowledgements

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associated membrane protein 2- and 8-specific populations of zymogen granules with distinct


FIGURE LEGENDS

Figure 1. Synaptotagmins -1, -3, -6 and -7 are expressed in acinar cells. Synaptotagmin isoforms expression was analyzed by RT-PCR conducted on total RNA from whole pancreas (P) and brain (B) and poly(A)-RNA from isolated acini (A). Note that the lower panel shows a longer exposure demonstrating the synaptotagmin 1 band in isolated acini.

Figure 2. Synaptotagmin 1 localizes to the apical plasma membrane of acini and in purified zymogen granules. A, acinar membrane fractions (MEM) (150 µg) were prepared from isolated acini, and zymogen granule membranes (ZGM) (100 µg) were prepared by Percoll density centrifugation. These fractions together with a whole brain lysate (brain) (20 µg) were analyzed by immunoblotting with anti-synaptotagmin C2AB antibody (1:1000). B, immunoblotting with an anti-C2AB antibody (1:1000) of pancreatic tissue fractions: post nuclear supernatant (PNS), zymogen granules (ZG), zymogen granule membranes (ZGM), NaCO₃-washed zymogen granule membranes (WZGM), zymogen granule content (ZGC), cytosol (Cyto), microsomal fractions (MIC) and NaCO₃-washed microsomes. All lanes received 100 µg protein except brain and cytosol which received 50 µg. C, immunoblotting with an anti isoforms specific antibody raised against aa 120-131 of rat synaptotagmin 1 (1:1000) analyzing ZG (100 µg), ZGC (30 µg), ZGM (30 µg), WZGM (30 µg) and a whole brain lysate (brain) (10 µg). Not in panels A – C, a lighter exposure of brain is shown due to strong immunoreactivity. Data are a single representative experiment performed three times on separate tissue preparations yielding identical results. D,
brightfield immunofluorescence microscopy was conducted on 9 µM thick sections of paraformaldehyde fixed isolated pancreatic acini (a and b) and Percoll purified zymogen granules (c and d). Synaptotagmin 1 specific-antibody (1:100) was detected using Alexa Fluor 488-conjugated anti-rabbit IgG (1:250). Note the strong accumulation of synaptotagmin 1 along the apical aspect of the acini. Scale bars, 7 µM. Images are a single representative experiment performed on 3 separate tissue preparations. Nuclei are labeled in blue with DAPI. Images b and d show corresponding differential interference contrast images.

Figure 3. Synaptotagmin 1 colocalizes with VAMP 2. Rat pancreatic acini (A) and Percoll-purified zymogen granules (C) were fixed in 4% paraformaldehyde and brightfield 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 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 & B) Psuedo colors were applied post collection as indicated. White boxes in (Ae-h) correspond to the subsequent magnifications in (Ae’-h’). Panel B shows sequential reconstructed 3-dimensional images rotated along the z plane of the magnifications in (Ae’-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.
Figure 4. Synaptotagmin 3 is found on acinar membrane and microsomal fractions. A, acinar and pancreatic tissue fractions were analyzed by immunoblotting with anti-synaptotagmin 3 antibody (1:1000). A whole brain lysate (50 µg), acinar membrane fractions (Mem) prepared from isolated acini (75 µg) and the pancreatic tissue fractions: post nuclear supernatant (PNS), cytosol (Cyto), microsomal fraction (MIC), NaCO₃-washed microsomal fraction (WMIC) all at 75 µg/lane, zymogen granule (ZG), zymogen granule membrane (ZGM), NaCO₃-washed ZGM (WZGM) and zymogen granule content (ZGC) all at 50 µg/lane. Note that synaptotagmin 3 was present in the microsomal and acinar membrane fractions but absent from all ZG fractions and cytosol. Data are a single representative experiment performed three times on separate tissue preparations yielding identical results. B, rat pancreatic lobules were fixed in 4% paraformaldehyde. Brightfield immunofluorescence microscopy was conducted on 9 µM thick sections. Synaptotagmin 3 (1:100) immunoreactivity was detected using Alexa Fluor 488-conjugated anti-rabbit IgG (1:250). Actin filaments (Actin) were identified with Alexa Fluor 647-conjugated phalloidin and label the apical most aspects of the acini. Note the very punctuate staining in perinuclear regions of the acini. C, (a) identifies a magnification of the white box in (B) and b – e show reconstructed 3-dimensional images of the magnifications in (a). Arrows indicate 3D direction; green – x, red – y, blue – z. D, the specificity of the synaptotagmin 3 antiserum was demonstrated by preabsorbing with excess (5 µg/ml) synaptotagmin 3 antigen prior to immunofluorescence. Note, the absence of all synaptotagmin 3 immunoreactivity after preabsorbing with antigen. 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. Psuedo-red color was applied to the actin post collection.
Figure 5. Synaptotagmin 3 colocalizes with LAMP1 in perinuclear and apical regions of acini. Brightfield immunofluorescence microscopy was conducted on 9 µM thick sections from 4% paraformaldehyde fixed acini. Synaptotagmin 3 (1:100) and LAMP1 (1:100) immunoreactivity were detected using 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. Image to the far right of the panel show corresponding differential interference contrast.

Figure 6. Synaptotagmin 1 but not synaptotagmin 3 C2AB domain modulates Ca\textsuperscript{2+}-stimulated amylase release. A, isolated acini were permeabilized with 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 (\textlesssim 10 nM free Ca\textsuperscript{2+}) or Ca\textsuperscript{2+}-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 the mean ± S.E. from a single representative experiment performed in triplicate. Data in (B) show the mean ± S.E. 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 using 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 5, 10, 15 and 30 min under Ca\textsuperscript{2+}-stimulated (3 µM) conditions. Data are the mean ± S.D. of 2 independent experiments each performed in triplicate expressed as
a % of total cellular amylase. **D**, PFO permeabilized acini were preincubated with the indicated concentrations of recombinant synaptotagmin 3 for 15 min. Amylase secretion was measured after an additional 30 min under basal (≤10 nM free Ca\(^{2+}\)) or Ca\(^{2+}\)-stimulated (3µM) 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 the mean ± S.E. of 3 independent experiments performed in triplicate. Note that no concentrations were statistical significance from control (Con) which was determined using a paired *t* test.

**Figure 7. Synaptotagmin 1 positive granules are recruited to the apical plasma membrane of acini upon stimulation with CCK-8.** **A**, isolated acini were treated as control or with 100 pM CCK-8 for 5 min at 37 °C and then incubated for 2 hr at 4°C with anti-synaptotagmin 1 (1:20) to label externalized antigen. Cells were then fixed in 2% formaldehyde and immunoreactivity was detected postfixation, by use of Alexa Fluor 488-conjugated anti-rabbit IgG (1:250). Nuclei were labeled with DAPI. Each image is a reconstructed z-series obtained by confocal microscopy. Bars, 7 µM. All images are representative of multiple determinations performed on at least 3 separate tissue preparations. **B**, quantitative analysis measuring the volume of synaptotagmin 1 immunoreactivity in acini acquired from multiple reconstructed z-series images of single acini from 3 separate tissue preparations. Data are mean and SE (n = 10 for each experimental condition).
Figure 6

A

Amylase Secretion (% of Total)

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C2AB (µg/ml)

B

Amylase Secretion (% of Maximal)

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C

Amylase Secretion (% of Total)

Con C2AB

Time (min)

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D

Amylase Secretion (% of Total)

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Synaptotagmin 3 (µg/ml)
Figure 7

A

Con

CCK-8

Synaptotagmin

DIC

Overlay

B

\( \mu M \) Pixels above Threshold x 100

Con

CCK-8

p < 0.001