Synaptotagmin III Isoform Is Compartmentalized in Pancreatic β-Cells and Has a Functional Role in Exocytosis

Hilary Brown, Björn Meister, Jude Deeney, Barbara E. Corkey, Shao-Nian Yang, Olof Larsson, C.J. Rhodes, Susumu Seino, Per-Olof Berggren, and Gabriel Fried

Synaptotagmin is involved in Ca^{2+}-regulated secretion and has been suggested to serve as a general Ca^{2+} sensor on the membrane of secretory vesicles in neuronal cells. Insulin exocytosis from the pancreatic β-cell is an example of a Ca^{2+}-dependent secretory process. Previous studies of pancreatic β-cells were unable to show presence of synaptotagmin I. We now present biochemical and immunohistochemical data showing that synaptotagmin III is present in pancreatic β-cells as well as in the insulin-secreting cell line HIT-T15 and in rat insulinoma. By subcellular fractionation, we found synaptotagmin III in high-density fractions together with insulin and secretogranin I, indicating colocalization of synaptotagmin III and insulin in secretory granules. We could also show that blockade of synaptotagmin III by a specific antibody inhibited Ca^{2+}-induced changes in β-cell membrane capacitance, suggesting that synaptotagmin III is part of the functional protein complex regulating β-cell exocytosis. The synaptotagmin III antibody did not affect the activity of the voltage-gated L-type Ca^{2+}-channel. These findings are compatible with the view that synaptotagmin III, because of its distinct localization in the pancreatic β-cell, functionally modulates insulin exocytosis. This indicates that synaptotagmin may have a general role in the regulation of exocytosis not only in neuronal cells but also in endocrine cells. *Diabetes* 49:383–391, 2000

Synaptotagmins are highly conserved, integral membrane proteins that are widely distributed in neuronal and non-neuronal tissues. To date, 11 mammalian isoforms of synaptotagmin have been described (1–10). These multidomain proteins possess an intravesicular amino terminus that can be glycosylated, a single transmembrane region containing multiple palmitoylated cysteines followed by a spacer region, a cytoplasmic region that mainly consists of two highly conserved C2 domains (C2A and C2B), and a conserved COOH-terminus (11–13). The C2 domains of synaptotagmin are homologous to those found in the Ca^{2+}-dependent form of protein kinase C and to other proteins that interact with phospholipids in a Ca^{2+}-dependent manner, such as rabphilin 3A and phospholipase A2 (12–19).

Recent work has shown that the synaptic vesicle protein synaptotagmin is involved in Ca^{2+}-regulated secretion (20–31). The proposed role for synaptotagmin in synaptic vesicle exocytosis is as a Ca^{2+} sensor on the vesicle membrane (19,21,26,28). Synaptotagmin is suggested to interact with plasma membrane proteins syntaxin (32) and SNAP-25 (33), as well as with other proteins, to form a "core complex" or prefusion particle (34–36). At cytoplasmic Ca^{2+} concentrations appropriate for exocytosis to occur, the Ca^{2+}-sensitive cytoplasmic domain on synaptotagmin may undergo a conformational change and thereby trigger fusion via an interaction with other proteins of the vesicle and plasma membrane (30). In support of this proposed role, mutational studies of the synaptotagmin I gene in various models have shown major disturbances in excitation-secretion coupling. In particular, the fast component of Ca^{2+}-induced exocytosis was virtually abolished in a synaptotagmin I knockout mouse, suggesting that Ca^{2+} regulation in the synaptotagmin I knockout mouse is lacking and that synaptotagmin I is essential for competent excitation-secretion coupling (26,37–40).

The pancreatic β-cell contains insulin secretory granules (41) from which insulin is released in response to stimulation by glucose and other secretagogues. The stimulus-secretion coupling in the β-cell involves a complex interaction between different signal transduction pathways (42–46), and it is well-accepted that Ca^{2+} influx has a fundamental role in this process (47–49).

In a previous study of the endocrine pancreas, we unexpectedly found that neither synaptotagmin I or II nor their mRNAs were present in pancreatic β-cells, even though other proteins known to interact with synaptotagmin were present (50). Both the fact that the synaptotagmin III isoform was cloned and the fact that the mRNA was shown to be expressed in the endocrine pancreas (4) point to the possibility of this isoform of synaptotagmin having a role in insulin exocytosis. Indeed, data have been published suggesting a functional role for synaptotagmin III in insulin release (51). We have

From the Departments of Molecular Medicine, The Rolf Luft Centre for Diabetes Research (H.B., J.D., S.-N.Y., O.L., P.-O.B.), Neuroscience (H.B., B.M.), Woman and Child Health (G.F.), and Physiology and Pharmacology (G.F.), Karolinska Institute, Stockholm, Sweden; the Diabetes and Metabolism Unit (J.D., B.E.C.), Evans Department of Medicine, Boston University School of Medicine, Boston, Massachusetts; the Departments of Internal Medicine and Pharmacology (C.J.R.), Center for Diabetes Research, University of Texas Southwestern Medical Center, Dallas, Texas; and the Department of Molecular Medicine (S.S.), Chiba University, Chiba, Japan.

Address correspondence and reprint requests to Gabriel Fried, Department of Physiology and Pharmacology, Karolinska Institute, S-171 77 Stockholm, Sweden. E-mail: gabriel.fried@fyfa.ki.se.

Received for publication 26 January 1999 and accepted in revised form 16 November 1999.

FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; LRSC, lissamine-rhodamine; MES, 2-[*N*-morpholino]ethanesulfonic acid; PBS, phosphate-buffered saline; synaptotagmin III-LI, synaptotagmin III–like immunoreactivity.

now investigated in more detail the cellular distribution and functional role of synaptotagmin III in rat pancreatic islets, rat insulinoma, and the insulin-secreting clonal cell line HIT T-15 (derived from hamster insulinoma) (52).

RESEARCH DESIGN AND METHODS

Immunofluorescence histochemistry and confocal microscopy. Male Sprague-Dawley rats (B & K Universal, Stockholm, Sweden; 150–200 g) were anesthetized with sodium pentobarbital (Mebumal; 40 mg/kg i.p.) and perfused via the ascending aorta with Ca^{2+}-free Tyrode's solution at 37°C followed by an ice-cold mixture of formalin-picric acid (4% paraformaldehyde and 0.4% picric acid in 0.16 mol/l phosphate buffer, pH 6.9). The pancreas was rapidly removed, immediately fixed by immersion in the same fixative for 90 min, and rinsed for at least 24 h in a 0.1 mol/l phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% bacitracin, and 0.01% sodium azide. Sections were cut at 10 µm in a cryostat and processed for indirect immunofluorescence. Briefly, sections were incubated in rabbit antiserum to synaptotagmin I/II, III, and IV (diluted 1:200, 1:200, and 1:100, respectively) for 18–22 h at 4°C. The sections were then washed in phosphate-buffered saline (PBS) for 30 min and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit (diluted 1:40; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies. After a further wash in PBS, the sections were mounted in 0.1% *p*-phenylenediamine dissolved in PBS and glycerol (1:3). Sections were double-labeled by combining rabbit antiserum to synaptotagmin I/II, III, and IV with guinea-pig antiserum to insulin (diluted 1:2,000; UCB-Bioproducts, Braine-l'Alleud, Belgium), mouse monoclonal antibodies to glucagon (diluted 1:1,000; Novo Nordisk, Bagsvaerd, Denmark), mouse monoclonal antibodies to somatostatin (diluted 1:200) (53), or guinea-pig antiserum to pancreatic polypeptide (diluted 1:24,000; Linco Research, St. Charles, MO). The combinations were visualized using a mixture of FITC-conjugated donkey anti-rabbit and lissamine-rhodamine (LRSC)-conjugated goat anti-guinea-pig (1:40) or goat anti-mouse (1:40; Jackson ImmunoResearch Laboratories) secondary antibodies. Controls were performed replacing the primary antibody with PBS. Sections were examined in a Bio-Rad MRC-600 laser scanning confocal imaging system equipped with a krypton/argon mixed gas laser and a Nikon Optiphot II microscope. The standard K1/K2 dual channel filter sets combined with an excitation filter (488 DF 10 for FITC-induced fluorescence and 568 DF 10 for LRSC-induced fluorescence) were used to examine the immunoreactivity. The images were produced using a Tektronix Phaser IIsd printer.

Cell culture. Monolayers of the clonal pancreatic β-cell HIT-T15 were cultured in Nunc dishes, using RPMI 1640 medium supplemented with 10% fetal bovine serum, streptomycin (50 µg/ml), and penicillin (50 U/ml), and kept at 37°C in a humidified incubator with 95% air/5% CO_2 (52,54). Cells were used between passages 64 and 85.

Insulin release and radioimmunoassay. To verify that the HIT-T15 cells release insulin, secretion studies were performed. Briefly, HIT-T15 cells were cultured in 24-well plates, washed several times with a buffer containing 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.28 mmol/l $CaCl_2$, 25 mmol/l HEPES, and 0.1% bovine serum albumin and incubated for 1 h at 37°C. The buffer was replaced with the same buffer containing either 0 glucose, 10 mmol/l glucose, or 25 mmol/l KCl, incubated for a further 30 min, and then sampled for insulin release and assayed by radioimmunoassay (55).

Ca^{2+} current recordings. Whole-cell currents were recorded as previously described (56) in pancreatic β-cells from adult obese hyperglycemic mice (gene symbol *ob*/*ob*). Pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal programmable puller (DMZ Universal Puller, Zeitz-Instrumente, Augsburg, Germany). Typical electrode resistance was 3–5 MΩ. Electrodes were filled with a standard internal solution with or without anti-synaptotagmin III (1:100, 15 µg/ml). The standard internal solution contained the following (in mmol/l): 150.0 *N*-methyl-D-glucamine, 10.0 EGTA, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 5.0 HEPES, and 3.0 Mg-ATP (pH 7.2 adjusted with HCl). The cells were bathed in a solution containing the following (inmmol/l): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 10.0 $CaCl_2$, 5.0 HEPES, and 10.0 tetraethylammonium (pH 7.4 adjusted with NaOH). After obtaining a seal, the holding potential was set at –70 mV during the course of an experiment, and depolarizing voltage pulses (70 mV, 100 ms, 0.05 Hz) were applied. The resulting currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA.). All recordings were made at room temperature (~22°C). The amplitude of whole-cell Ca^{2+} currents was normalized to the capacitance of the cells. Acquisition and analysis of data were done using the software program pCLAMP (Axon Instruments).

Capacitance recordings. To record membrane capacitance, a 20-mV, 500-Hz sine wave was added to a holding potential of –70 mV using an EPC-9 patch clamp amplifier (HEKA Elektronik, Lamprecht/Pfalz, Germany). Changes in membrane capacitance were monitored using the locking extension of the Pulse software (HEKA) and were carried out at 32–34°C. The extracellular solution contained (in mmol/l): 138.0 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5.0 HEPES, pH-adjusted to 7.15 with KOH, resulting in a free Ca^{2+} concentration of ~500 nmol/l. The pipette solution contained the synaptotagmin III antibody at a 1:200 dilution (7.5 µg/ml) or a rabbit control IgG antibody (7.5 µg/ml).

Equilibrium sucrose density gradient of HIT-T15 cells. HIT-T15 cells were grown to 70% confluency in 80 cm^2 Nunc flasks, homogenized in the presence of protease inhibitors, and fractionated by equilibrium density gradient centrifugation, essentially as described (57). Briefly, the cells were detached from the flasks using a 2-min incubation in PBS with 10 mmol/l EDTA at 37°C, pelleted by centrifugation, and resuspended in homogenization buffer (4 mmol/l HEPES, pH 7.4, 1 mmol/l $MgCl_2$, 250 mmol/l sucrose, 0.005% DNAse, and the protease inhibitors: 4 µg/ml of pepstatin A, leupeptin, antipain and Aprotinin, 0.4 mmol/l phenylmethylsulfonyl fluoride, and 10 mmol/l benzamidine). The cells were homogenized using 40 strokes of a Teflon/glass homogenizer. A postnuclear supernatant (S1) was produced by centrifugation of the homogenate at 5,000*g* for 10 min at 4°C, using a TLA 100.2 Beckman rotor. The S1 was supplemented with EDTA to a final concentration of 1.5 mmol/l, loaded onto a linear sucrose density gradient (0.45–2.0 mol/l), and centrifuged at 4°C for 18 h at 100,000*g* in a SW51 Beckman rotor. Then seven fractions (700 µl) were collected. The molarity of each fraction was determined, and the fractions were analyzed by SDS-PAGE and Western blotting, as described below.

β-Cell granule preparation from rat insulinoma. Transplantable rat insulinoma was propagated in NEDH (New England Deaconess Hospital) rats, which were obtained from an in-house breeding colony at the Joslin Diabetes Center. The subcellular fractionation of the harvested insulinoma by differential and density gradient centrifugation was performed as described previously (41,58). Insulinoma tissue was homogenized at 4°C in 0.27 mol/l sucrose, 10 mmol/l HEPES, and 1 mmol/l EGTA, pH 6.5, using a Potter-Elvehjem homogenizer, 8–10 strokes. The homogenate was then centrifuged at 1,700*g* for 7 min at 4°C to remove cell debris and nuclei. The supernatant was applied to an iso-osmotic discontinuous density gradient composed of 19.2% (wt/vol) Nycodenz (Nyegaard Diagnostica, Oslo, Norway) mixed with 0.27 mol/l sucrose in the proportions 1:0, 1:1, and 1:3 (5 ml of each) and then centrifuged at 4°C in a Beckman SW 28 rotor at 100,000*g* for 60 min. Three fractions consisting of insulin secretory granules, smooth membrane, and cytosol were obtained. The cytosol fraction was further centrifuged at 150,000*g* and the supernatant collected. The band consisting of enriched β-cell granules was removed from the interface between the high- and medium-density solutions and then washed in the homogenization buffer. The granule fraction was mixed with eight volumes of 27% (vol/vol) Percoll (Pharmacia Biotech, Uppsala, Sweden) in 0.27 mol/l sucrose/10 mmol/l 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 6.5, and centrifuged at 35,000*g* at 4°C for 45 min using a Sorvall SS34 rotor. The purified β-cell granule fraction was recovered and washed 5–6 times in 0.25 mol/l sucrose/10 mmol/l MES, pH 6.5, to remove the gradient material. The fractions were analyzed by SDS-PAGE and Western blotting as described below.

SDS-PAGE and immunoblotting. Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Samples were denatured for 5 min at 100°C in SDS-PAGE sample buffer. Equal amounts of protein were loaded and subjected to analysis on a 7.5% polyacrylamide SDS-PAGE gel. The proteins were transferred to 0.2 µm nitrocellulose (Schleicher and Schuell, Dassel, Germany) in 20% methanol, 20 mmol/l Tris, 150 mmol/l glycine, and 0.05% SDS for 12–14 h at 350 mA (59). Nonspecific binding of the primary antibody was blocked by incubation in 5% dried milk in buffer A (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, containing 0.05% Tween-20). The blots were subsequently probed overnight at 4°C with rabbit anti-synaptotagmin I (diluted 1:2,000), purified rabbit anti-GST synaptotagmin III (diluted 1:1,000), rabbit anti-synaptotagmin IV (diluted 1:1,000), mouse monoclonal anti-secretogranin I (diluted 1:1,000), and rabbit anti-synaptophysin (diluted 1:2,000) in buffer A containing 5% dried milk. The blots were washed with buffer A, incubated for 1 h at room temperature with peroxidase-conjugated goat-anti-rabbit IgG (diluted 1:2,000; Organon Teknika, Durham, NC) in buffer A containing 5% dried milk, and finally washed with buffer A. Immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system and Hyperfilm (Amersham).

Antibodies. Synaptotagmin III antibody was raised in a rabbit against rat synaptotagmin III (GST fusion protein). Briefly, the coding sequence of rat synaptotagmin III (nucleotide 94–1900) was fused in frame to the COOH terminus of glutathione S-transferase (GST) by ligating into the Sma site of pGEX4T-2 (Pharmacia Biotech). Synaptotagmin III fusion protein was expressed in JM109 cells and purified according to the manufacturer's instructions. Anti-synaptotagmin III antiserum was generated by immunizing a rabbit with purified recombinant GST-synaptotagmin III. The resulting antibody was purified by affinity chromatography through protein A-Sepharose CL-4B (Pharmacia Biotech). The antiserum for synaptotagmin IV was raised in a rabbit against the peptide of mouse synaptotagmin IV (SSQKKFGGDDKSEVKGKTAL) (amino acids 68–87) and was IgG purified. Rabbit polyclonal synaptotagmin I/II antibody was a kind gift from Drs. R.H. Scheller and A.J. Bean (60). Mouse monoclonal antibodies to secretogranin I

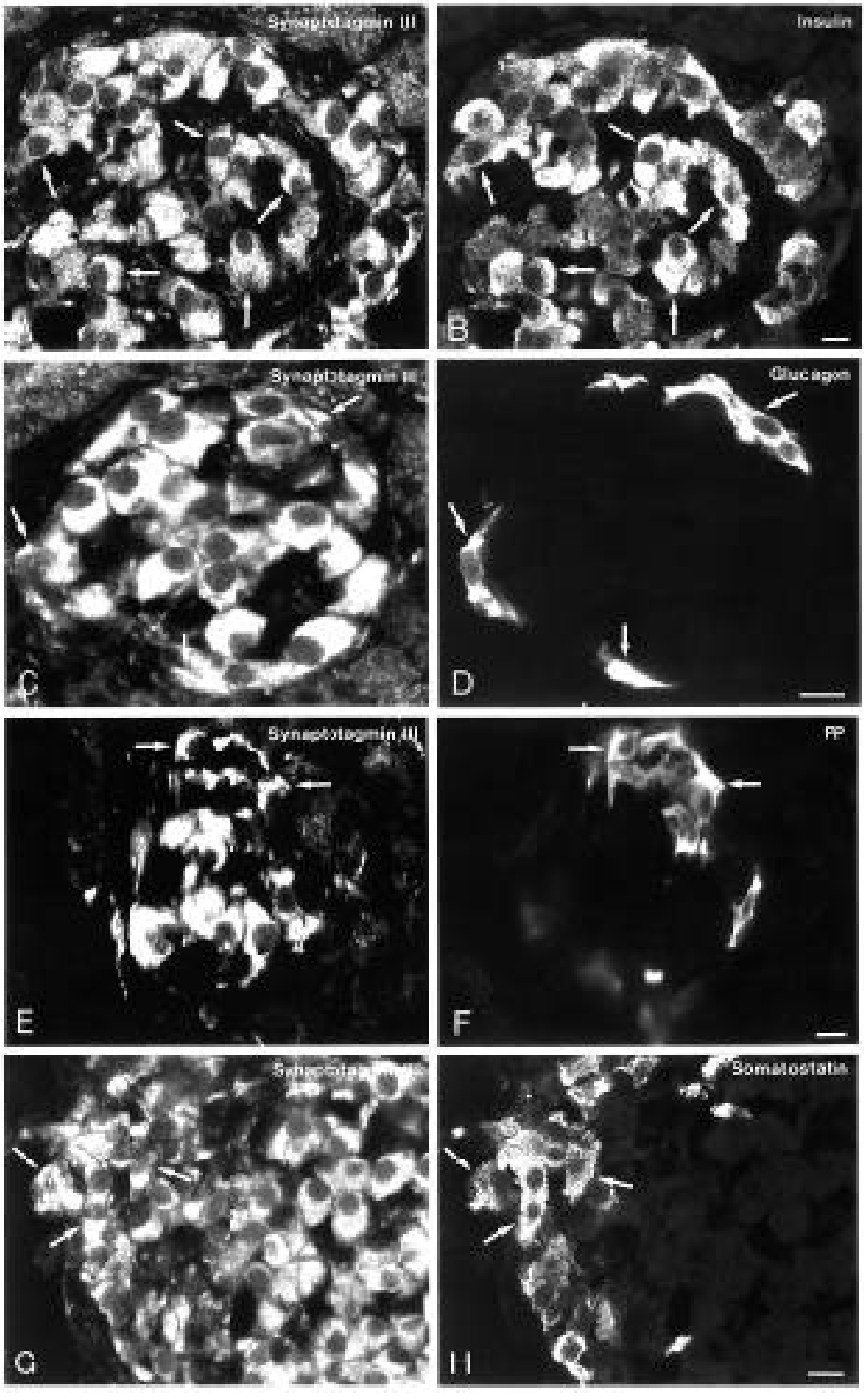

FIG. 1. Immunofluorescence images of sections of rat pancreatic islets obtained with confocal laser microscopy after direct double-labeling combining antiserum to synaptotagmin III (*A*, *C*, *E*, and *G*) with antiserum to insulin (*B*), glucagon (*D*), pancreatic polypeptide (PP) (*F*), or somatostatin (*H*). Strong synaptotagmin III-LI is present in all cells of the rat endocrine pancreas. Note the granular appearance of synaptotagmin III-LI. Synaptotagmin III-LI is colocalized with insulin, glucagon, PP, and somatostatin (see arrows). Bar = 10 µm.

were a kind gift from Dr. P. Rosa, Milan, and Dr. W.B. Huttner, Heidelberg. Rabbit polyclonal antiserum to synaptophysin was a kind gift from Dr. R. Jahn, Yale University. Antibodies were used at dilutions from 1:200 to 1:1,000 and, when reused, were stored in 5% milk containing 0.1% sodium azide.

RESULTS

Immunohistochemistry of rat pancreatic islets. Incubation with antiserum to synaptotagmin III showed intense synaptotagmin III-like immunoreactivity (LI) in all cells of the islets of Langerhans (Fig. 1*A*, *C*, *E*, and *G*). There was only weak synaptotagmin III-LI in nerve terminals present in the islets. Confocal microscopy further revealed that synaptotagmin III-LI was granular in appearance within the cytoplasm of all endocrine cells (Fig. 1*A*, *C*, *E*, and *G*). Omission of primary antiserum did not show any fluorescence signal in the rat pancreas (data not shown). Moreover, this antibody did not cross-react with rat synaptotagmin I and II (51). Direct double-labeling demonstrated granular synaptotagmin III-LI in islet cells containing insulin (compare Fig. 1*A* with Fig. 1*B*), glucagon (compare Fig. 1*C* with Fig. 1*D*), pancreatic polypeptide (compare Fig. 1*E* with Fig. 1*F*), and somatostatin (compare Fig. 1*G* with Fig. 1*H*). Incubation with antiserum raised to synaptotagmin IV gave intense synaptotagmin IV-LI in some of the peripherally located endocrine cells of the islet of Langerhans (Fig. 2*A*, *C*, *E*, and *G*). Confocal microscopy further revealed that synaptotagmin IV had a granular appearance within the cytoplasm of these cells (Fig. 2*A*, *C*, *E*, and *G*). Unlike synaptotagmin III, nerve fibers or terminals showed no synaptotagmin IV-LI. Omission of primary antiserum gave no immunofluorescence in the rat pancreas (data not shown). Direct double-labeling demonstrated that there was virtually no synaptotagmin IV-LI in islet cells containing insulin (compare Fig. 2*A* with Fig. 2*B*) or pancreatic polypeptide (compare Fig. 2*C* with Fig. 2*D*). However, intense synaptotagmin IV-LI was evident in all somatostatin-containing cells (compare Fig. 2*E* with Fig. 2*F*), and a slightly less intense signal was seen in occasional glucagon-containing cells (compare Fig. 2*G* with Fig. 2*H*) . In agreement with our previous data (50), incubation with rabbit polyclonal antiserum to synaptotagmin I showed an intense synaptotagmin I-LI in some of the peripherally located endocrine cells of the islets of Langerhans and an intense signal in many nerve fibers and terminals present within the islets as well as surrounding blood vessels. Confocal microscopy revealed a granular appearance in the endocrine cells, and direct double-labeling showed that synaptotagmin I-LI was present only in the somatostatin-containing cells (data not shown).

Immunohistochemistry of HIT-T15 cells. HIT-T15 cells typically cluster together in islet-like aggregates. Moderate synaptotagmin I-LI was detected in only a few cells within the aggregate, and these cells coincided with the cells that showed insulin-LI (data not shown). Synaptotagmin III-LI exhibited a distinctly granular appearance and was present in virtually all cells within an aggregate, whereas intense insulin-LI was detected only in a few cells. Weak synaptotagmin IV-LI was present in most cells of the aggregate, and again only a few cells showed insulin-LI (data not shown).

Synaptotagmin isoforms in subcellular fractions of insulin-secreting HIT-T15 cells. Before fractionation, intact HIT-T15 cells were shown to functionally release insulin in response to 10 mmol/l glucose and 25 mmol/l KCl. Basal insulin secretion was 30 μU/ml medium. Secretion was increased by 30% in the presence of 10 mmol/l glucose and by 50% in the presence of 25 mmol/l KCl ($n = 3$, $P < 0.01$). All three isoforms of synaptotagmin were present in the subcellular fractions derived from HIT-T15 cells, but with different distribution patterns. Synaptotagmin I/II was present mainly in lower-density fractions, with a maximum in fraction 3 (0.89 mol/l sucrose). Synaptophysin, a marker for small synaptic-like vesicles, was shown to have a similar distribution to synaptotagmin I/II, peaking in fraction 3 (Fig. 3). Insulin storage granules were assumed to be present in high-density fractions 5–7, with a maximum in fraction 5–7 (1.31–1.7 mol/l sucrose) based on the distribution of secretogranin I and insulin (Fig. 3). Synaptotagmin III was present at 63 kDa in high-density fractions 4–7 with a distribution similar to that of secretogranin I and insulin (Fig. 3). Also, in 50-μg homogenates of the insulin-secreting cell lines RINm5F and INS-1, as well as in rat brain and mouse brain, positive bands at 63 kDa were detected with synaptotagmin III antiserum (data not shown). Weak immunostaining for synaptotagmin IV was detected in both low-density (fractions 2–3) and high-density (fractions 5–6) fractions (Fig. 3).

Synaptotagmin isoforms in insulinoma **β**-cell granule preparation. Synaptotagmin III was shown to be highly enriched in the granule fraction of the preparation at 63 kDa, and a weak band was also detected in the mixed membrane fraction (Fig. 4). Synaptotagmin IV antiserum gave a band at 66 kDa in all fractions of this preparation (Fig. 4). In addition, the granule fraction showed a band at 46 kDa. Synaptotagmin I was not detected in any of these fractions.

Effect of anti-synaptotagmin III on cell membrane capacitance and L-type Ca^{2+}-channel activity. Cell capacitance was recorded as a measure of insulin exocytosis at the single-cell level. In the presence of anti-synaptotagmin III, the increase in cell capacitance was significantly less than that evoked in control cells in the presence of rabbit IgG (Fig. 5). There was no effect of anti-synaptotagmin III on whole-cell Ca^{2+} currents (Fig. 6).

DISCUSSION

Synaptotagmin III-LI was present in pancreatic β-cells; in insulin-secreting cell lines such as HIT-T15 cells, RINm5F cells, and INS-1 cells; and in a transplantable insulinoma. This finding is in agreement with data showing expression of synaptotagmin III mRNA in HIT-T15, RINm5F, and MIN6 cells (4). No synaptotagmin I/II was detected in the β-cells of the rat pancreatic islet. This agrees with previous results demonstrating no mRNA for synaptotagmin A and B (I and II) in either mouse β-cells or the clonal insulin-secreting RINm5F cells (50) and argues against a function for synaptotagmin I in insulin release from normal β-cells. However, synaptotagmin I was present in peripherally located cells of the islet that also stain for somatostatin (δ-cells) (50). Furthermore, synaptotagmin IV was present in all δ-cells, as well as in some glucagon-containing α-cells, but was not detected in β-cells. Therefore, it may be suggested that neither synaptotagmin I/II nor synaptotagmin IV appear to be involved in the regulation of insulin release in the normal islet but may be involved in regulated release from δ- and/or α-cells. Previous in situ hybridization experiments in rat brain and PC12 cells report that synaptotagmin IV strongly overlaps with synaptotagmin I, and the immunohistochem-

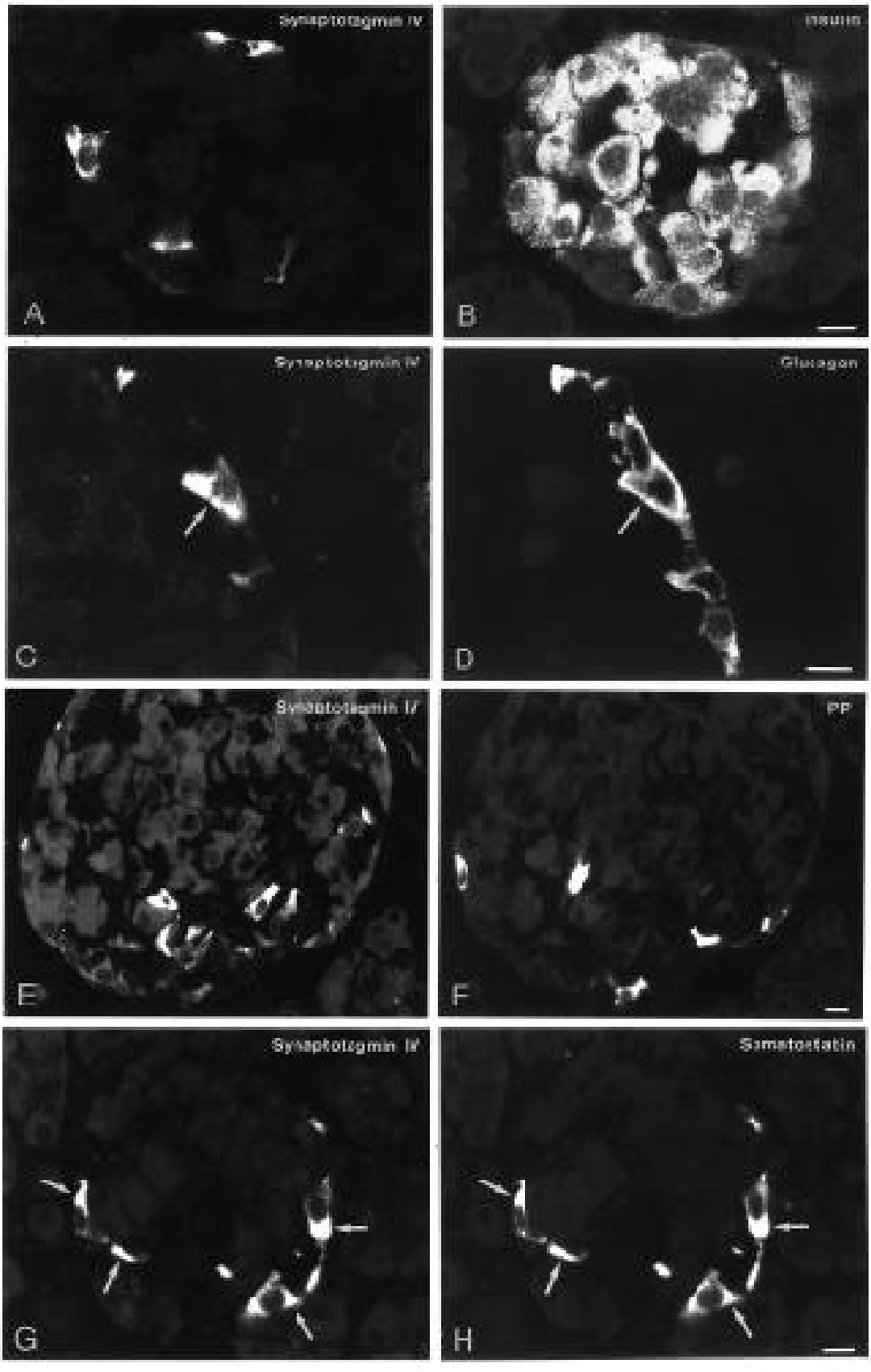

FIG. 2. Immunofluorescence images of sections of rat pancreatic islets obtained with confocal laser microscopy after direct double-labeling combining antiserum to synaptotagmin IV (*A*, *C*, *E*, and *G*) with antibodies to insulin (*B*), glucagon (*D*), pancreatic polypeptide (PP) (*F*), or somatostatin (*H*). Synaptotagmin IV-LI is present mainly in peripheral cells and absent in central cells. There is no presence of synaptotagmin IV-LI in insulin or PP cells. Note the colocalization of synaptotagmin IV-LI in single glucagon cells and in all somatostatin-containing cells (see arrows). Bar = 10 µm.

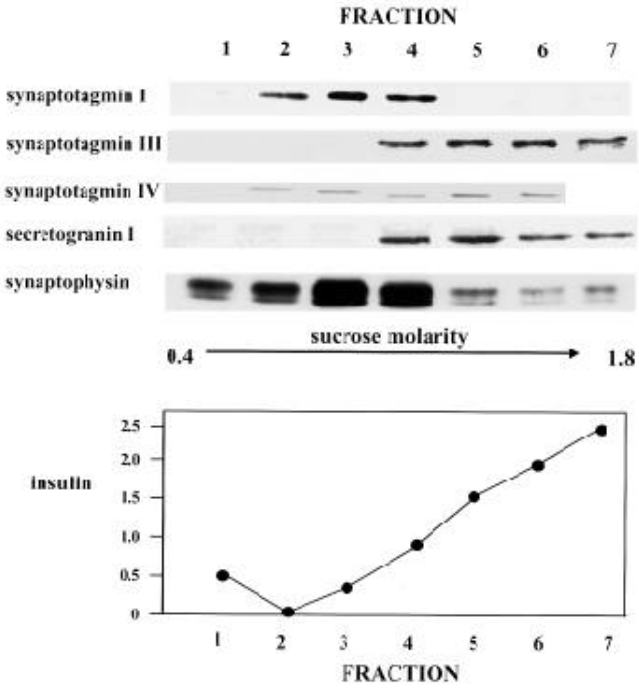

FIG. 3. Subcellular distribution of synaptotagmin I, III, and IV, synaptophysin (p38), secretogranin I, and insulin in HIT-T15 cells. A postnuclear supernatant (25 µg) was loaded onto a linear sucrose density gradient (0.45–2.0 mol/l) and centrifuged at 110,000*g*. Seven fractions were collected. The fractions correspond to the following sucrose molarity: 1 (0.42 mol/l), 2 (0.65 mol/l), 3 (0.89 mol/l), 4 (1.08 mol/l), 5 (1.31 mol/l), 6 (1.54 mol/l), 7 (1.75 mol/l). Twenty-five micrograms of protein from each fraction was analyzed by SDS-PAGE/Western blotting, and the immunoreactive bands were visualized with ECL. One representative experiment is shown (n = 5).

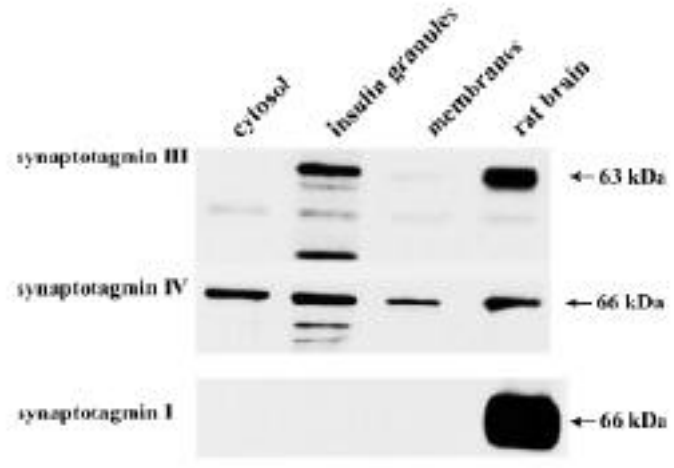

FIG. 4. Subcellular distribution of synaptotagmin I, III, and IV in fractions isolated from rat insulinoma. A postnuclear supernatant was loaded onto a discontinuous Nycodenz/sucrose density gradient and centrifuged at 100,000*g*. Three fractions were collected, and after purification (see RESEARCH DESIGN AND METHODS), 10 µg of each fraction was analyzed by SDS-PAGE/Western blotting. Immunoreactive bands were visualized with ECL. One representative experiment is shown (n = 5).

istry results in the δ-cells agree with this observation (61). However, we did not detect synaptotagmin IV in nerve fibers and terminals even though synaptotagmin I was present there. Furthermore, we were not able to detect any synaptotagmin I/II in any of the α-cell populations. Pancreatic polypeptide–containing cells, like β-cells, showed neither synaptotagmin I nor synaptotagmin IV. In HIT-T15 cells, the immunohistochemical staining for synaptotagmin IV gave a weak signal in all cells.

The subcellular fractionation experiments of HIT-T15 cells showed that synaptotagmin III was present with maxima in high-density fractions 5, 6, and 7, which also contained the endocrine granule marker secretogranin I (62,63) and insulin, suggesting that synaptotagmin III colocalizes with insulin. Likewise, results obtained from the fractionation of the insulinoma showed that synaptotagmin III was highly enriched in the β-granule fraction. These data are in agreement with results from MIN-6 cells (51) and suggest that synaptotagmin III is colocalized with insulin-secreting granules. These data, together with those of Mizuta et al. (51), indicate that synaptotagmin III may be the isoform that participates in the regulation of insulin release. Immunoblotting of the HIT-T15 cells showed weak bands at the predicted molecular weight of 46 kDa. In the insulinoma fractions, there was a band in the granule fraction at 46 kDa and another strong band at 66 kDa in all fractions, including the cytosol. Bands of the same molecular weight were also detected in rat and mouse brain homogenates.

Synaptotagmins are considered to be integral membrane proteins, and the significance of the presence of synaptotagmin IV immunoreactivity in the cytosol is difficult to explain. It could be related to the fact that the C2 domains of synaptotagmin IV differ significantly from the other isoforms, even though there is a region that satisfies the criteria for a transmembrane region (64). In addition, there is great divergence in the spacer region of synaptotagmin IV when compared with any of the other synaptotagmins. Synaptotagmin I was present in the subcellular fractions of HIT-T15 cells, with maxima in low-density fractions that also contained the vesicle membrane protein synaptophysin. Previous subcellular fractionation experiments using pancreatic islets have suggested the presence of small, γ-aminobutyric acid–containing vesicles (57,65). Synaptophysin is a marker for small vesicles (66), and the distribution pattern of synaptotagmin I in the HIT-T15 cells could be compatible with a possible role for this isoform in regulating release from small synaptic-like vesicles in these cells. Interestingly, the immunohistochemistry of the HIT-T15 cells showed that synaptotagmin I/II-LI was present, but in relatively few cells that also exhibited strong insulin-LI. Because the fractionation clearly showed that synaptotagmin I and synaptotagmin III are present in different compartments, it seems conceivable that in these cells, synaptotagmin I and III have specialized functions. Synaptotagmin I was not identified, however, in the fractions of the transplanted insulinoma. It is evident that in the β-cell of the rat islet, neither synaptotagmin I nor synaptotagmin IV are highly expressed. It could therefore be implied that HIT-T15 cells and the transplanted insulinoma, although a suitable model for studies of many aspects of β-cell physiology, do not perfectly mimic primary β-cells. Indeed, insulin-secreting cell lines can consist of several cell populations that can secrete insulin, glucagon, and somatostatin (67).

Ca^{2+}-dependent neurotransmitter release has been suggested to consist of at least two components: a major fast component insensitive to Sr^{2+} and one slow component potentiated by Sr^{2+} (68). Based on the different Ca^{2+} and Sr^{2+}

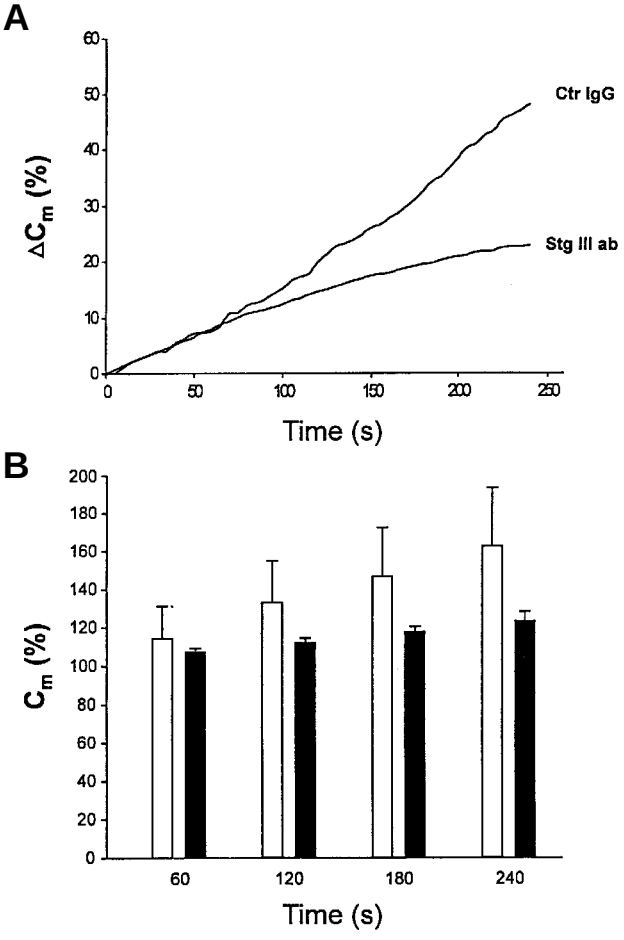

FIG. 5. Anti-synaptotagmin III antibody inhibits Ca^{2+}-induced changes in cell membrane capacitance. *A*: Example traces demonstrating changes in cell capacitance (ΔC_m) monitored for 4 min from single pancreatic β-cells following inclusion of an antibody against synaptotagmin III and a rabbit IgG control antibody in the pipette. *B*: Compiled data on ΔC_m at 1, 2, 3, and 4 min in the presence of anti-synaptotagmin III (■) or rabbit IgG control (□) antibody. Linear regression analysis on each recording from the two sets of experiments resulted in a *P* value <0.05 (Student's *t* test for unpaired data) between synaptotagmin III (0.091 ± 0.016) and control IgG (0.261 ± 0.073) antibody-treated cells (n = 9).

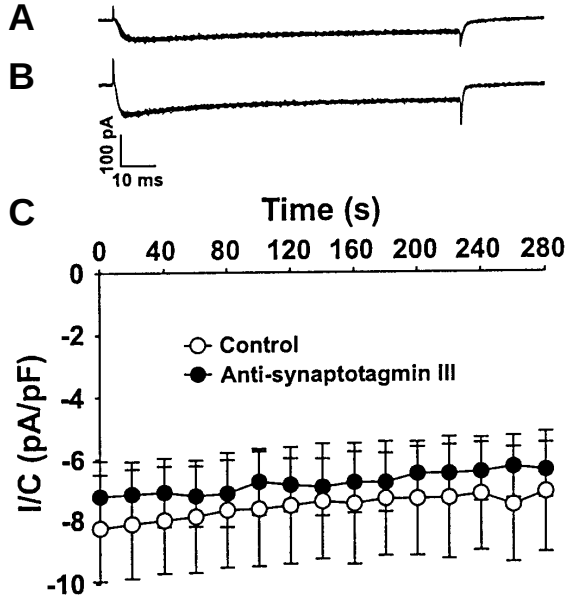

FIG. 6. Effect of anti-synaptotagmin III antibody on L-type Ca^{2+} currents in mouse pancreatic β-cells. Whole-cell patch-clamp recordings with 15 superimposed L-type Ca^{2+} current traces, evoked by depolarizing voltage steps from –70 mV to 0 mV (100 ms, 0.05 Hz), showed no significant difference in currents between cells filled with the standard internal solution (*A*) and those filled with anti-synaptotagmin III antibody (1:100) (*B*). *C*: Compiled data on changes in current amplitude show that L-type Ca^{2+} currents in cells intracellularly perfused with anti-synaptotagmin III antibody (n = 7) are not significantly different in amplitude from those in control cells (n = 6). The data are presented as means ± SE. Statistical differences were evaluated by one-way analysis of variance followed by a least significant difference test.

requirements for phospholipid binding, it has been suggested that different synaptotagmin isoforms localized on the same vesicle could perform distinct functions, with synaptotagmin I being a Ca^{2+}-sensor for fast release and synaptotagmin III a sensor for slow release (7). Since there is evidence for graded Ca^{2+} requirements for release of, for example, large and small vesicles in neurons (69–72), this hypothesis may generally be valid for cells with different types of vesicles. This would imply that different synaptotagmin isoforms may differentially regulate release from several classes of vesicles. The present results indeed demonstrate that synaptotagmin isoforms are distinctly compartmentalized in pancreatic endocrine cells, implying that there may be functionally separate roles for these isoforms. With regard to the β-cell, the ionic requirements and temporal characteristics for insulin release have been fairly well established (73–75). Characteristics of release for both insulin from β-cells (76) and catecholamines from adrenal chromaffin cells (77,78) suggest that the major component of release from large endocrine vesicles is slow. The requirements of synaptotagmin for Ca^{2+} and Sr^{2+} in phospholipid binding would therefore be compatible with a role for synaptotagmin III in regulating release of insulin from insulin storage granules; indeed, Sr^{2+}-stimulated insulin release has been described previously (79). Additional evidence that lends support for a role of synaptotagmin III in insulin release is the observation that the various synaptotagmin isoforms have different Ca^{2+} concentration dependencies for the interaction with syntaxin at the plasma membrane. Isoforms I and II require >200 µmol/l Ca^{2+} to interact with syntaxin, whereas synaptotagmin III requires <10 µmol/l (7). Since it has been suggested that Ca^{2+}-dependent secretion of insulin initially requires a localized concentration of <10 µmol/l (76), this again is compatible with a possible role for synaptotagmin III in insulin release. The presence of synaptotagmin III in pancreatic islet cells and in insulin-secreting HIT-T15 cells therefore strongly suggests that synaptotagmin III may be a Ca^{2+} sensor for slow insulin release in β-cells. Indeed, such a role for synaptotagmin III is further supported by experiments showing blockade of insulin release by anti-synaptotagmin III antibody in permeabilized insulin-secreting MIN6 cells (51). When performing more detailed studies of the exocytosis machinery at the single-cell level at an intracellular Ca^{2+} concentration of ~500 nmol/l, we could in the present study show that insulin release was blocked by anti-synaptotagmin III antibody. From the Ca^{2+} current recordings, it is suggested that this effect could not be explained by interference of the antibody

with the Ca^{2+}-channel protein. Although this may indicate that synaptotagmin III does not interact with the voltage-gated L-type Ca^{2+} channel in the β-cell, we cannot exclude that the antibody interacts with an epitope on synaptotagmin III that is not involved in possible interaction with the Ca^{2+}-channel protein complex.

Synaptotagmin III may thus serve as a Ca^{2+} sensor in regulated release of insulin from the pancreatic β-cell. Future studies should address the presence of other isoforms of synaptotagmin in these cells. In addition, electron microscopy and functional studies should be able to identify their subcellular localization and precise role in the regulation of insulin granule docking and fusion.

ACKNOWLEDGMENTS

This study was supported by the Swedish Medical Research Council (14X-7164, 03X-09890, 03X-09890, 03XS-12708, 19X-00034), U.S. Public Health Service Grant DK-35914, the Swedish Diabetes Association, Novo Nordisk Foundation, the Nordic Insulin Foundation, Juvenile Diabetes Foundation, Berth von Kantzows Foundation, and Karolinska Institutets Fonder.

We thank Dr. R. Jahn and Dr. R. Scheller for their kind gifts of antisera.

REFERENCES

1. Matthew WD, Tsavaler L, Reichardt LF: Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J Cell Biol* 91:257–269, 1981
2. Geppert M, Archer BT, Südhof TC: Synaptotagmin II: a novel differentially distributed form of synaptotagmin. *J Biol Chem* 266:13548–13552, 1991
3. Hilbush BS, Morgan JI: A third synaptotagmin gene, Syt3, in the mouse. *Proc Natl Acad Sci U S A* 91:8195–8199, 1994
4. Mizuta M, Inagaki N, Nemoto Y, Matsukura S, Takahashi M, Seino S: Synaptotagmin III is a novel isoform of rat synaptotagmin expressed in endocrine and neuronal cells. *J Biol Chem* 269:11675–11678, 1994
5. Ullrich B, Li C, Zhang JZ, McMahon H, Anderson RG, Geppert M, Südhof TC: Functional properties of multiple synaptotagmins in brain. *Neuron* 13:1281–1291, 1994
6. Hudson AW, Birnbaum MJ: Identification of a nonneuronal isoform of synaptotagmin. *Proc Natl Acad Sci U S A* 92:5895–5899, 1995
7. Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Südhof TC: Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* 375:594–599, 1995
8. Marquéze B, Boudier JA, Mizuta M, Inagaki N, Seino S, Seagar M: Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. *J Neurosci* 15:4906–4917, 1995
9. Ullrich B, Südhof TC: Differential distributions of novel synaptotagmins: comparison to synapsins. *Neuropharmacology* 34:1371–1377, 1995
10. von Poser C, Ichtchenko K, Shao X, Rizo J, Südhof TC: The evolutionary pressure to inactivate: a subclass of synaptotagmins with an amino acid substitution that abolishes Ca2+ binding. *J Biol Chem* 272:14314–14319, 1997
11. Perin MS, Brose N, Jahn R, Südhof TC: Domain structure of synaptotagmin (p65) [Erratum published in *J Biol Chem* 266:10018, 1991]. *J Biol Chem* 266:623–629, 1991
12. Perin MS: The COOH terminus of synaptotagmin mediates interaction with the neurexins. *J Biol Chem* 269:8576–8581, 1994
13. Sutton RB, Davletov BA, Berghuis AM, Südhof TC, Sprang SR: Structure of the first C2 domain of synaptotagmin I: a novel Ca2+/phospholipid-binding fold. *Cell* 80:929–938, 1995
14. Davletov BA, Südhof TC: A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. *J Biol Chem* 268:26386–26390, 1993
15. Chapman ER, Jahn R: Calcium-dependent interaction of the cytoplasmic region of synaptotagmin with membranes: autonomous function of a single C2-homologous domain. *J Biol Chem* 269:5735–5741, 1994
16. Brose N, Hofmann K, Hata Y, Südhof TC: Mammalian homologues of Caenorhabditis elegans unc-13 gene define novel family of C2-domain proteins. *J Biol Chem* 270:25273–25280, 1995
17. Miyazaki M, Kaibuchi K, Shirataki H, Kohno H, Ueyama T, Nishikawa J, Takai Y: Rabphilin-3A binds to a M(r) 115,000 polypeptide in a phosphatidylserine- and Ca(2+)-dependent manner. *Brain Res Mol Brain Res* 28:29–36, 1995
18. Shao X, Davletov BA, Sutton RB, Südhof TC, Rizo J: Bipartite Ca2+-binding motif in C2 domains of synaptotagmin and protein kinase C. *Science* 273:248–251, 1996
19. Südhof TC, Rizo J: Synaptotagmins: C2-domain proteins that regulate membrane traffic. *Neuron* 17:379–388, 1996
20. Südhof TC, Jahn R: Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* 6:665–677, 1991
21. Brose N, Petrenko AG, Südhof TC, Jahn R: Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science* 256:1021–1025, 1992
22. Jahn R, Südhof TC: Synaptic vesicle traffic: rush hour in the nerve terminal. *J Neurochem* 61:12–21, 1993
23. Elferink LA, Peterson MR, Scheller RH: A role for synaptotagmin (p65) in regulated exocytosis. *Cell* 72:153–159, 1993
24. O'Connor V, Augustine GJ, Betz H: Synaptic vesicle exocytosis: molecules and models. *Cell* 76:785–787, 1994
25. Bennett MK, Scheller RH: Molecular correlates of synaptic vesicle docking and fusion. *Curr Opin Neurobiol* 4:324–329, 1994
26. Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC: Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. *Cell* 79:717–727, 1994
27. Littleton JT, Bellen HJ: Synaptotagmin controls and modulates synaptic-vesicle fusion in a Ca(2+)-dependent manner. *Trends Neurosci* 18:177–183, 1995
28. Kelly RB: Neural transmission: synaptotagmin is just a calcium sensor. *Curr Biol* 5:257–259, 1995
29. Fried G: Synaptic vesicles and release of transmitters: new insights at the molecular level. *Acta Physiol Scand* 154:1–15, 1995
30. Südhof TC: The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* 375:645–653, 1995
31. Bauerfeind R, Jelinek R, Huttner WB: Synaptotagmin I- and II-deficient PC12 cells exhibit calcium-independent, depolarization-induced neurotransmitter release from synaptic-like microvesicles. *FEBS Lett* 364:328–334, 1995
32. Bennett MK, Garcia-Arraras JE, Elferink LA, Peterson K, Fleming AM, Hazuka CD, Scheller RH: The syntaxin family of vesicular transport receptors. *Cell* 74:863–873, 1993
33. Hess DT, Slater TM, Wilson MC, Skene JH: The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. *J Neurosci* 12:4634–4641, 1992
34. Rothman JE, Orci L: Molecular dissection of the secretory pathway. *Nature* 355:409–415, 1992
35. Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE: A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75:409–418, 1993
36. Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE: SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362:318–324, 1993
37. Nonet ML, Grundahl K, Meyer BJ, Rand JB: Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin. *Cell* 73:1291–1305, 1993
38. Broadie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL: Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proc Natl Acad Sci U S A* 91:10727–10731, 1994
39. DiAntonio A, Schwarz TL: The effect on synaptic physiology of synaptotagmin mutations in Drosophila. *Neuron* 12:909–920, 1994
40. Littleton JT, Stern M, Perin M, Bellen HJ: Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. *Proc Natl Acad Sci U S A* 91:10888–10892, 1994
41. Hutton JC: The insulin secretory granule. *Diabetologia* 32:271–281, 1989
42. Efendic S, Kindmark H, Berggren PO: Mechanisms involved in the regulation of the insulin secretory process. *J Intern Med* 735:9–22, 1991
43. Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic beta-cell signal transduction. *Ann Rev Biochem* 64:689–719, 1995
44. Brun T, Roche E, Assimacopoulos-Jeannet F, Corkey BE, Kim KH, Prentki M: Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic beta-cell nutrient signaling. *Diabetes* 45:190–198, 1996
45. Prentki M: New insights into pancreatic beta-cell metabolic signaling in insulin secretion. *Eur J Endocrinol* 134:272–286, 1996
46. Asanuma N, Aizawa T, Sato Y, Schermerhorn T, Komatsu M, Sharp GW, Hashizume K: Two signaling pathways, from the upper glycolytic flux and from the mitochondria, converge to potentiate insulin release. *Endocrinology* 138:751–755, 1997
47. Berggren PO, Larsson O: Ca2+ and pancreatic B-cell function. *Biochem Soc Transact* 22:12–18, 1994

48. Larsson O, Kindmark H, Brändström R, Fredholm B, Berggren PO: Oscillations in K-ATP channel activity promote oscillations in cytoplasmic free Ca2+ concentration in the pancreatic beta cell. *Proc Natl Acad Sci U S A* 93:5161–5165, 1996
49. Nilsson T, Schultz V, Berggren PO, Corkey BE, Tornheim I: Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca2+ in glucose-stimulated pancreatic beta-cells. *Biochem J* 31:91–94, 1996
50. Jacobsson G, Bean AJ, Scheller RH, Juntti-Berggren L, Deeney JT, Berggren PO, Meister B: Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *Proc Natl Acad Sci U S A* 91:12487–12491, 1994
51. Mizuta M, Kurose T, Mike T, Shoji-Kasai Y, Takahashi M, Seino S, Matsukura S: Localization and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic β-cells. *Diabetes* 46:2002–2006, 1997
52. Santerre RF, Cook RA, Crisel RM, Sharp JD, Schmidt RJ, Williams DC, Wilson CP: Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. *Proc Natl Acad Sci U S A* 78:4339–4343, 1981
53. Buchan AMJ, Sikora LKJ, Levy JG, McIntosh CHS, Dyck I, Brown JC: An immunocytochemical investigation with monoclonal antibodies to somatostatin. *Histochemistry* 83:175–180, 1985
54. Hill RS, Boyd AE: Perifusion of a clonal cell line of Simian virus 40-transformed beta-cells: insulin secretory dynamics in response to glucose, 3-isobutyl-1-methylxanthine, and potassium. *Diabetes* 34:115–120, 1985
55. Grill V, Pigon J, Hartling SG, Binder C, Efendic S: Effects of dexamethasone on glucose-induced insulin and proinsulin release in low and high responders. *Metabolism* 39:251–258, 1990
56. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85–100, 1981
57. Reetz A, Solimena M, Matteoli M, Folli F, Takei K, De Camilli P: GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J* 10:1275–1284, 1991
58. Rhodes CJ, Brennan SO, Hutton JC: Proalbumin to albumin conversion by a proinsulin processing endopeptidase of insulin secretory granules. *J Biol Chem* 254:14240–14245, 1989
59. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350–4354, 1979
60. Wendland B, Miller KG, Schilling J, Scheller RH: Differential expression of the p65 gene family. *Neuron* 9:993–1007, 1991
61. Vician L, Lim IK, Ferguson G, Tocco G, Baudry M, Herschman HR: Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. *Proc Natl Acad Sci U S A* 92:2164–2168, 1995
62. Helle KB: Chromogranins: universal proteins in secretory organelles from Paramecium to man. *Neurochem Int* 17:165–175, 1990
63. Winkler H, Fischer-Colbrie R: The chromogranins A and B: the first 25 years and future perspectives. *Neuroscience* 49:497–528, 1992
64. Eisenberg D, Schwartz E, Komaromy M, Wall R: Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol* 179:125–142, 1984
65. Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P: A gamma-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic beta cells. *Proc Natl Acad Sci U S A* 90:5317–5321, 1993
66. Jahn R, Schiebler W, Ouimet CC, Greengard P: A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc Natl Acad Sci U S A* 82:4137–4141, 1985
67. Poitout V, Olson LK, Robertson RP: Insulin-secreting cell lines: classification, characteristics and potential applications. *Diabetes* 22:7–14, 1996
68. Goda Y, Stevens CF: Two components of transmitter release at a central synapse. *Proc Natl Acad Sci U S A* 91:12942–12946, 1994
69. Lundberg JM, Rudehill A, Sollevi A, Fried G, Wallin G: Frequency- and reserpine-dependent chemical coding of sympathetic transmission: differential release of noradrenaline and neuropeptide Y from pig spleen. *Neurosci Lett* 63:96–100, 1986
70. Iverfeldt K, Serfozo P, Diaz Arnesto LL, Bartfai T: Differential release of coexisting transmitters: frequency dependence of the efflux of substance P, thyrotropin releasing hormone and 3H-serotonin from tissue slices of rat ventral spinal cord. *Acta Physiol Scand* 137:63–71, 1989
71. Verhage M, McMahon HT, Ghijsen WE, Boomsma F, Scholten G, Wiegant VM, Nicholls DG: Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* 6:517–524, 1991
72. Franck J, Brodin E, Fried G: Differential release of endogenous 5-hydroxytryptamine, substance P and neurokinin A from ventral spinal cord in response to electrical stimulation. *J Neurochem* 61:704–711, 1993
73. Augustine GJ, Neher E: Calcium requirements for secretion in bovine chromaffin cells. *J Physiol (Lond)* 450:247–271, 1992
74. Theler JM, Mollard P, Guérinau N, Vacher P, Pralong WF, Schlegel W, Wollheim CB: Video imaging of cytosolic Ca2+ in pancreatic beta-cells stimulated by glucose, carbachol, and ATP. *J Biol Chem* 267:18110–18117, 1992
75. Ämmälä C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM, Rorsman P: Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *J Physiol (Lond)* 472:665–688, 1993
76. Bokvist K, Eliasson L, Ämmälä C, Renström E, Rorsman P: Co-localization of L-type Ca2+ channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic B-cells. *EMBO J* 14:50–57, 1995
77. Heinemann C, Chow RH, Neher E, Zucker RS: Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca2+. *Biophys J* 67:2546–2557, 1994
78. Neher E, von Ruden L: Depression and augmentation of quantal release in adrenal chromaffin cell. *Adv Second Messenger Phosphoprotein Res* 29:353–362, 1994
79. Henquin JC: Specificity of divalent cation requirement for insulin release: effects of strontium. *Pflügers Arch* 383:123–129, 1980