SNAP-25 Is Expressed in Islets of Langerhans and Is Involved in Insulin Release

Karin Sadoul,* Jochen Lang,‡ Cesare Montecucco,§ Ulrich Weller,‖ Romano Regazzi,‡
Stefan Catsicas,¶ Claes B. Wollheim,‡ and Philippe A. Halban*

*Laboratoires de Recherche Louis Jeantet, Centre Médical Universitaire, 1211 Geneva 4, Switzerland; ‡Division de Biochimie Clinique, Département de Médecine, Centre Médical Universitaire, 1211 Geneva 4, Switzerland; §Centro Consiglio Nazionale delle Ricerche Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, 35121 Padova, Italy; ‖Institut für medizinische Mikrobiologie, Johannes Gutenberg Universität, 55101 Mainz, Germany; and ¶Glaxo Institute for Molecular Biology S.A., 1228 Plan-les-Ouates, Geneva, Switzerland

Abstract. SNAP-25 is known as a neuron specific molecule involved in the fusion of small synaptic vesicles with the presynaptic plasma membrane. By immunolocalization and Western blot analysis, it is now shown that SNAP-25 is also expressed in pancreatic endocrine cells. Botulinum neurotoxins (BoNT) A and E were used to study the role of SNAP-25 in insulin secretion. These neurotoxins inhibit transmitter release by cleaving SNAP-25 in neurons.

Cells from a pancreatic B cell line (HIT) and primary rat islet cells were permeabilized with streptolysin-O to allow toxin entry. SNAP-25 was cleaved by BoNT/A and BoNT/E, resulting in a molecular mass shift of ~1 and 3 kD, respectively. Cleavage was accompanied by an inhibition of Ca^{++}-stimulated insulin release in both cell types. In HIT cells, a concentration of 30–40 nM BoNT/E gave maximal inhibition of stimulated insulin secretion of ~60%, coinciding with essentially complete cleavage of SNAP-25. Half maximal effects in terms of cleavage and inhibition of insulin release were obtained at a concentration of 5–10 nM. The A type toxin showed maximal and half-maximal effects at concentrations of 4 and 2 nM, respectively. In conclusion, the results suggest a role for SNAP-25 in fusion of dense core secretory granules with the plasma membrane in an endocrine cell type—the pancreatic B cell.

I N pancreatic B cells, proinsulin is sorted in the *trans*-Golgi network for delivery to secretory granules where it is processed to insulin (23, 40). Insulin is packed and stored in large dense core granules (LDCG)[1] and is released when exocytosis is stimulated by secretagogues such as glucose (23). Current understanding of B cell stimulus-secretion coupling suggests that nutrient stimuli cause depolarization of the cell membrane, which leads to an influx of Ca^{++} triggering the fusion of granules with the plasma membrane (48). Although sensitivity to glucose is a peculiarity of the pancreatic B cell, the other steps leading from the *trans*-Golgi network to LDCGs and the fusion of granules with the plasma membrane are, most probably, common to all cells possessing the regulated secretory pathway.

The molecular mechanism for docking and fusion of LDCGs in endocrine cells has not so far been studied. Conversely, recent data from Söllner et al. have established a detailed model for docking and fusion of small synaptic vesicles (SSV) in neuronal cells (57, 58). Using cosedimentation and immunoprecipitation techniques they could identify a 20-S fusion complex. The complex is formed when a brain membrane detergent extract is mixed with NSF (*N*-ethyl-maleimid-sensitive factor) and SNAPs (soluble NSF attachment proteins) in the presence of non-hydrolyzable ATP. Besides NSF and SNAPs this complex contains VAMP/synaptobrevin, syntaxin, and SNAP-25 (the name is coincidental and stands in this instance for synaptosomal-associated protein of 25 kD). VAMP/synaptobrevin is an integral membrane protein of the synaptic vesicles (8, 64) and syntaxin is an integral membrane protein located on the plasma membrane (9). SNAP-25 lacks a transmembrane domain but is attached to the plasma membrane by palmitoylation of cysteine residues in the central domain of the molecule (26, 42). Additional proof for the involvement of VAMP/synaptobrevin, syntaxin, and SNAP-25 in the fusion process

1. *Abbreviations used in this paper:* BoNT, botulinum neurotoxin; LDCG, large dense core granule; LDCV, large dense core vesicle; NSF, N-ethyl-maleimid-sensitive factor; SLMV, synaptic-like microvesicle; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 kD; SSV, small synaptic vesicle.

came from studies using the clostridial neurotoxins, tetanus, and botulinum serotypes A-G (BoNT/A-BoNT/G) (35, 38). These potent neurotoxins are metallo endoproteases, which block transmitter release at the synapse by cleaving VAMP/synaptobrevin, syntaxin, or SNAP-25 (10, 13, 14, 52, 53, 55, 56).

So far it is not clear to what extent features of this model can be applied to regulated exocytosis of different kinds of vesicles, including LDCGs, or whether it is restricted to neurotransmitter release from SSVs in nerve terminals.

In the present study we show that SNAP-25, previously thought to be a neuron specific protein (42), is also expressed in pancreatic endocrine cells including insulin producing B cells. As in neurons SNAP-25 is found in the detergent soluble fraction of a crude preparation of total cellular membranes. In neurons and neurosecretory cells SNAP-25 is cleaved at a site 9 and 26 amino acid residues from the carboxy terminus by BoNT/A and BoNT/E, respectively (10, 55), which results in inhibition of transmitter release. We therefore used these toxins as tools to investigate the role of SNAP-25 in insulin secretion. BoNT/A and BoNT/E were found to hydrolyze SNAP-25 in a B cell line (HIT) and in primary islet cells accompanied by diminished Ca^{++}-induced insulin release.

Materials and Methods

Primary Cells

Primary pancreatic islet cells and pure islet B and non-B cells were obtained as described previously (50). In brief, pancreata from six rats were digested with collagenase in Ca^{++} containing Hanks buffer and islets of Langerhans were separated from exocrine tissue by discontinuous density gradient centrifugation (Histopaque 1077 from Sigma Chem. Co., St. Louis, MO). Islets were trypsinized to obtain a single cell suspension and cultured in RPMI, 10% FCS, 50 µg/ml gentamycin on glass coverslips coated with bovine corneal endothelial cell matrix (Eldan-Tech Ltd., Jerusalem, Israel) for immunofluorescence or in bovine corneal endothelial cell matrix coated 96-well plates (Eldan-Tech Ltd.) for toxin treatment.

For Western blot analysis islet B cells were sorted from non-B cells by size and FAD auto fluorescence using a fluorescence-activated cell sorter (50). This method has been shown to yield one population consisting of 95% B cells and a second with 93% non-B cells as shown by classical double immunofluorescence techniques (50). Isolated cells were lysed immediately in SDS-sample buffer for gel electrophoresis. To obtain exocrine cells the pellet obtained after Histopaque density gradient centrifugation, containing exocrine acini, was washed twice with PBS and trypsinized to obtain a single cell suspension. Exocrine cells were counted and lysed immediately in SDS-sample buffer for gel electrophoresis.

Cell Lines

AtT-20 (pituitary corticotroph), FAO (hepatoma), and COS (kidney) cells were grown in DMEM, 10% FCS. RIN cells (46) were cultured in RPMI 1640, 10% FCS, 2.05 mM glutamine; HIT cells (23, 42) in RPMI 1640, 10% FCS, 2.05 mM glutamine, 32.5 µM glutathione, 0.1 µM seleneous acid and INS-1 cells (4) in RPMI 1640, 10% FCS, 10 mM Hepes, 2.05 mM glutamine, 1 mM Na-pyruvate, 0.05 mM β-mercaptoethanol.

Fractionation of Cell Lysates

HIT cells were resuspended in PBS, 0.01% EDTA, and counted. Cells were lysed on ice in hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA 1 mM PMSF, pH 7.4) using a dounce homogenizer. After low speed centrifugation (2,500 rpm, 10 min, 4°C, Heraeus Biofuge) to eliminate nuclei, the postnuclear supernatant was centrifuged at 30,000 g, 30 min, 4°C to obtain a crude membrane pellet and cytosol. The membrane pellet was washed once in high salt buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 M NaCl, pH 7.4) before solubilization with a detergent containing buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 150

mM NaCl, 1% Triton X-100, pH 7.4). Solubilized membranes were centrifuged at 100,000 g, 1 h, 4°C to separate Triton soluble membrane components from insoluble material. The remaining pellet was resuspended directly in SDS-sample buffer for gel electrophoreses. All volumes were kept constant starting with 10^6 cells/100 µl hypotonic buffer.

Immunofluorescence

Cryosections of adult rat pancreas were fixed for 10 min at room temperature in PBS, 4% paraformaldehyde blocked for 15 min with Dulbecco's phosphate buffered saline (DPBS), 5% BSA and incubated for 1 h in DPBS, 0.1% BSA, 0.3% Triton X-100, containing a polyclonal antibody (diluted 1:200) against a synthetic peptide of the 12 carboxy terminal amino acid residues of SNAP-25 prepared as described (42). Sections were washed and incubated with an FITC-labeled secondary antibody (dilution of 1:80, Sigma catalogue number F-0382) for 1 h. Finally sections were mounted with mounting medium (Vectashield, Vector Laboratories Inc., Burlingame, CA) and viewed using an Axiophot fluorescence microscope. Control sections were incubated in parallel, omitting the first antibody.

Primary islet cells obtained as described above were cultured for 5 d before immunofluorescence staining. Cells were fixed for 10 min at room temperature in PBS, 4% paraformaldehyde, permeabilized in acetone for 30 s at room temperature, blocked for 15 min in DPBS, 5% BSA before incubation with a monoclonal antibody against SNAP-25 (diluted 1:1,000, Sternberger Monoclonals Incorporated, Baltimore, MD) for 1 h in DPBS, 1% BSA. Cells were washed twice in DPBS, incubated with an FITC-labeled secondary antibody (1:1,000, Antibodies Inc. catalogue number 41-150), and then visualized with an Axiophot fluorescence microscope. Control cells were treated identically without addition of the first antibody.

Streptolysin-O Permeabilization and BoNT Treatment

HIT cells were grown in 96-well plates (1.5 × 10^5 cells/200 µl/well) for 3 or 4 d before permeabilization. Permeabilization was performed as described previously (22). In brief, cells were washed once with 100 µl Krebs buffer without Ca^{++}, and then with 100 µl glutamate buffer (140 mM K⁺-glutamate, 5 mM NaCl, 7 mM $MgSO_4$, 20 mM Hepes, 0.4 mM EGTA, pH 7) at 37°C. Cells were then permeabilized with 50 µl streptolysin-O (activated with a final concentration of 5 mM DTT for 5 min a 37°C) in glutamate buffer (1.5 U/ml) for 7 min at 37°C, followed by an incubation period of 10 min at 37°C with 50 µl glutamate buffer, low Ca^{++} (glutamate buffer containing 2.5 mM Na_2ATP, 0.5 mM $CaCl_2$, 10.2 mM EGTA, resulting in a final concentration of 0.1 µM free Ca^{++}). For measuring insulin secretion, cells were incubated in 50 µl glutamate buffer containing 2.5 mM Na_2ATP, 10.2 mM EGTA and various concentrations of Ca^{++} (0.5 mM $CaCl_2$ = 0.1 µM free Ca^{++}, 7 mM $CaCl_2$ = 1 µM free Ca^{++}, 9.8 mM $CaCl_2$ = 10 µM free Ca^{++}, 10 mM $CaCl_2$ = 40 µM free Ca^{++}) for 7 min at 37°C. The concentration of free Ca^{++} has been estimated using a Ca^{++} concentration curve established with a Ca^{++} electrode and the same buffer composition as in our assay system (47). Incubation medium was taken for radioimmuno assay to measure insulin concentrations (25) (human insulin standard and anti-insulin serum from Novo-Nordisk, Bagsvaerd, Denmark) and cells were lysed in SDS sample buffer for gel electrophoresis.

BoNT treatment of primary cells was performed identically except that the washing step with glutamate buffer was omitted and a 10 × less concentrated streptolysin-O solution was used. The radioimmuno assay for insulin from primary cells was performed using a rat insulin standard (Novo-Nordisk).

BoNTs were obtained as described (54) and activated with a final concentration of 10 mM DTT at 37°C for 30 min before use. Toxins were included during the permeabilization and preincubation periods.

Western Blot Analysis

SDS gel electrophoresis was performed under reducing conditions using 10% polyacrylamide gels according to Laemmli et al. (27). Loading quantities were as mentioned in figure legends or one fifth of a well from 96-well plates for toxin-treated cells. Transfer of proteins to nitrocellulose was performed as described (63). The nitrocellulose membranes were then blocked for 1 h at room temperature in TBS, 5% dry milk (BioRad Labs., Hercules, CA) before incubation with the first antibody in TBS, 5% dry milk, 0.1% Tween-20 overnight at 4°C. The polyclonal and monoclonal antibody against SNAP-25 as well as the polyclonal antibody against actin (generous gift from Dr. Gabbiani, Geneva, Switzerland) and the monoclonal antibody against the heat shock protein HSP 70 (StressGen, Victoria, B.C., Canada, catalogue number SPA-820) were diluted 1:1,000. The primary antibody was

then visualized using a horseradish peroxidase–coupled secondary antibody combined with the ECL detection procedure from Amersham International (Amersham, Bucks, UK) or with an alkaline phosphatase–coupled secondary antibody and a color substrate (66 μl nitro-blue tetrazolium, 50 mg/ml 70% dimethylformamide and 33 μl 5-bromo-4-chloro-3-indolylphosphate, 50 mg/ml dimethylformamide in 10 ml 10 mM Tris, 100 mM NaCl, 5 mM MgCl$_2$, pH 9.5).
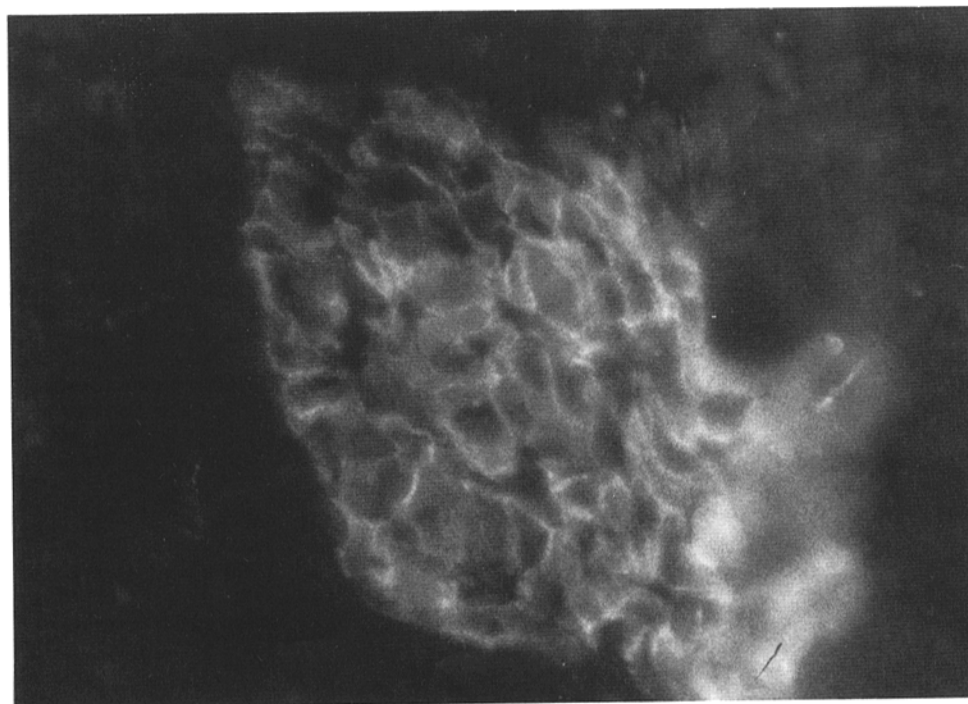
Results

Expression of SNAP-25 in the Islet of Langerhans

SNAP-25 expression was studied by immunofluorescence on cryosections of adult rat pancreas. Fig. 1 shows the typical
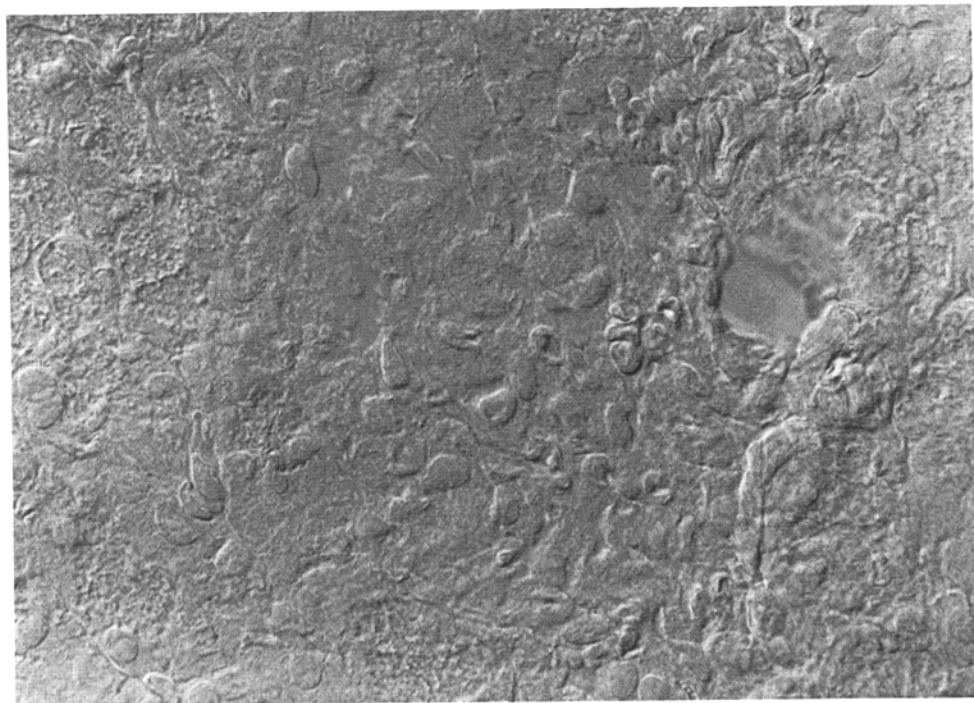
Figure 1. Cryosection of adult rat pancreas stained with a polyclonal antibody against SNAP-25. Nomarski (a) and fluorescence image (b). Note the staining of the islet of Langerhans and the absence of fluorescence in the surrounding exocrine tissue. Bar, 10 μm.

staining obtained using a polyclonal antibody against a carboxy-terminal peptide of SNAP-25. SNAP-25 is expressed in the islet of Langerhans but not in the surrounding exocrine tissue. As expected, a cross section of a nerve in the same tissue slice was also SNAP-25 positive (not shown).

Immunofluorescence of SNAP-25 in Primary Islet Cells in Culture

Indirect immunofluorescence studies were performed using 5-d-old monolayer cultures of primary islet cells from adult rat pancreas. As shown in Fig. 2, the staining pattern of a monoclonal antibody against SNAP-25 (as well as the polyclonal antibody, not shown) on permeabilized primary islet cells in culture also suggests a cell membrane–associated localization of SNAP-25. An enrichment of SNAP-25 in an intracellular, possibly Golgi, region was often visible as observed previously for PC 12 cells (51).

Western Blot Analysis of SNAP-25 in Cells of Different Origin and in Different Cellular Fractions

Western blots were performed using the polyclonal antibody against SNAP-25 to examine the presence of SNAP-25 in endocrine and non-endocrine cells. SNAP-25 was strongly expressed in primary islet B and non-B cells (Fig. 3 a, upper panel, lanes 3 and 2 respectively), which together build up the endocrine pancreas (39). SNAP-25 was also expressed in different pancreatic B cell lines, RIN, (Fig. 3 a, upper panel, lane 4) INS-1 (Fig. 3 a, upper panel, lanes 5–7), HIT (Figs. 3 b and 4), in a pancreatic non-B cell line (α-TC, glucagon-producing cells [not shown]) and in a pituitary corticotroph cell line (AtT-20, Fig. 3 a, upper panel, lane 1). The apparent molecular mass of 25 kD corresponds exactly to that of SNAP-25 from brain extract (not shown). By contrast SNAP-25 could not be detected in primary pancreatic exocrine cells, in a liver cell line (FAO), or COS cells (kidney) (Fig. 3 a, upper panel, lanes 8, 9, 10, respectively). Fig. 3 a, lower panel, shows the corresponding actin staining used to estimate protein quantity, rather than cell number, loaded in each lane. These results show that SNAP-25 expression is not confined to the nervous system but is also found in the pancreas where it is restricted to endocrine cells.

Analyses of whole cell extracts cannot distinguish between soluble and membrane-bound SNAP-25. Subcellular fractionation was thus used to this end. The results show that SNAP-25 in HIT cells has the biochemical properties of a molecule tightly associated with cellular membranes, as has been observed for SNAP-25 in neurons (42). In contrast to actin which under our assay conditions is found exclusively in the cytosol, and the heat shock protein HSP 70 which is found mainly in the cytosol and to a lesser extent in the nuclear fraction, SNAP-25 can only be detected in the detergent soluble fraction of a crude preparation of total cellular membranes and not in the nuclear fraction, the cytosol, the high salt membrane wash, or the Triton insoluble membrane fraction (Fig. 3 b).

BoNT/E Cleaves SNAP-25 and Inhibits Insulin Release from HIT Cells

To investigate whether SNAP-25 is involved in exocytosis of insulin containing LDCGs we used HIT cells, a pancreatic

B cell line. HIT cells can be permeabilized with streptolysin-O to allow exchange of macromolecules (3, 29). Permeabilization is necessary because BoNTs cannot enter primary B cells or B cell lines as, in contrast to neurons, these cells lack the receptor for the toxins (12, 34). Permeabilized HIT cells can still be stimulated to secrete insulin by raising the Ca^{++} concentration (22, 29).

Treatment of permeabilized HIT cells with BoNT/E results in cleavage of SNAP-25, visible as a decrease in apparent molecular mass of ∼3 kD, shown in Fig. 4 a, right panel. The effect on insulin secretion is shown in Fig. 4 a, left panel. At a concentration of 30–40 nM the maximal inhibitory effect of ∼60% is reached, which coincides with nearly complete cleavage of SNAP-25 (Fig. 4 a). A concentration of 5–10 nM BoNT/E gives half maximal effects for both cleavage of SNAP-25 as well as inhibition of insulin release. SNAP-25 is not hydrolyzed when cells are only permeabilized (Fig. 4 a, right panel) or treated with toxin which has been heat inactivated before use (not shown). The heat-inactivated toxin also does not change the Ca^{++}-response (not shown).

The effect of 40 nM BoNT/E on insulin secretion at various Ca^{++} concentrations is illustrated in Fig. 5. Whereas insulin release under low Ca^{++} conditions is not significantly altered by the toxin, the inhibitory effect on Ca^{++}-evoked release at an intermediate Ca^{++} concentration is almost complete. At high Ca^{++} concentrations inhibition was around 75% (this series of experiments has been performed with another batch of toxin as compared to Fig. 4 a, where maximal inhibition did not exceed 60%).

Effect of BoNT/A on SNAP-25 and Insulin Release in HIT Cells

Treatment of permeabilized HIT cells with BoNT/A resulted in a decrease in the apparent molecular mass of SNAP-25 of ∼1 kD (Fig. 4 b, right panel). Maximal cleavage of SNAP-25 is obtained at a concentration of 4 nM BoNT/A, which is accompanied by a significant decrease in Ca^{++}-stimulated insulin release (Fig. 4 b, left panel). Half maximal concentration for cleavage of SNAP-25 is ∼2 nM (Fig. 4 b, right panel). As shown in Fig. 4 b, the heat-inactivated toxin does not cleave SNAP-25 nor does it influence insulin secretion. Once maximal cleavage of SNAP-25 is obtained, inhibition of Ca^{++}-evoked release could not be augmented any further by raising the toxin concentration up to 64 nM (not shown).

Although hydrolysis of SNAP-25 by BoNT/A is almost complete and very reproducible at all concentrations above 4 nM, the effect on insulin secretion was highly variable and always less efficient than the E type toxin (we therefore did not study the effect of BoNT/A at intermediate Ca^{++} concentrations). Inhibition values varied from almost no inhibition to a maximum of 45% between different experiments (not shown). Basal release however was never influenced by the toxin (not shown).

Effect of BoNT/A and BoNT/E in Primary Islet Cells

To verify that the effects of the toxins, observed in the pancreatic B cell line HIT, are not due to the transformed phenotype of these cells, toxin treatment was performed on streptolysin-O permeabilized primary rat islet cells. As in HIT cells cleavage of SNAP-25 was observed after BoNT/E
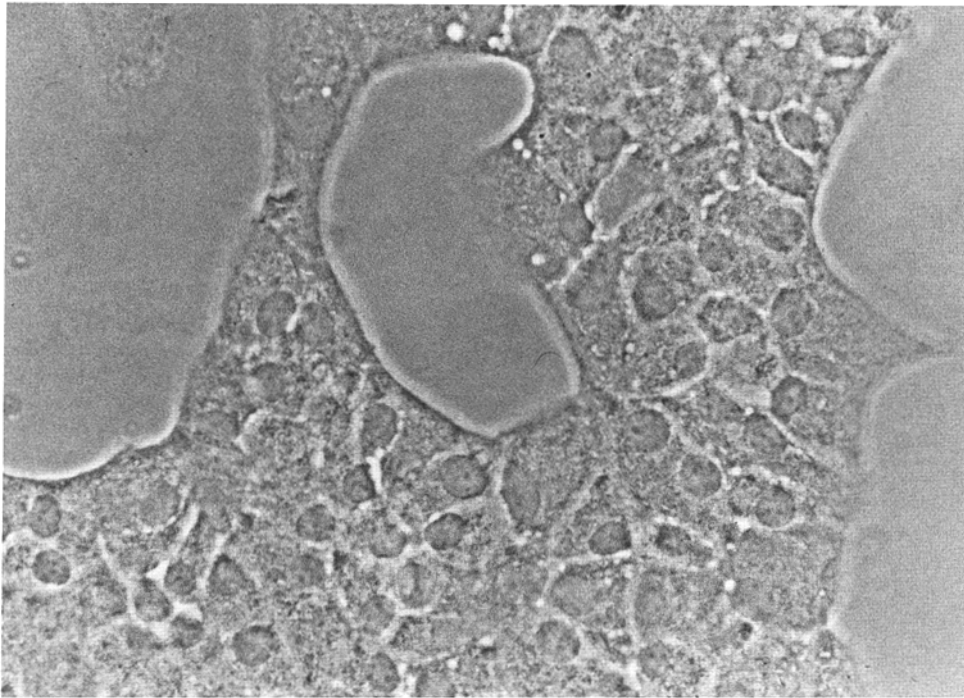
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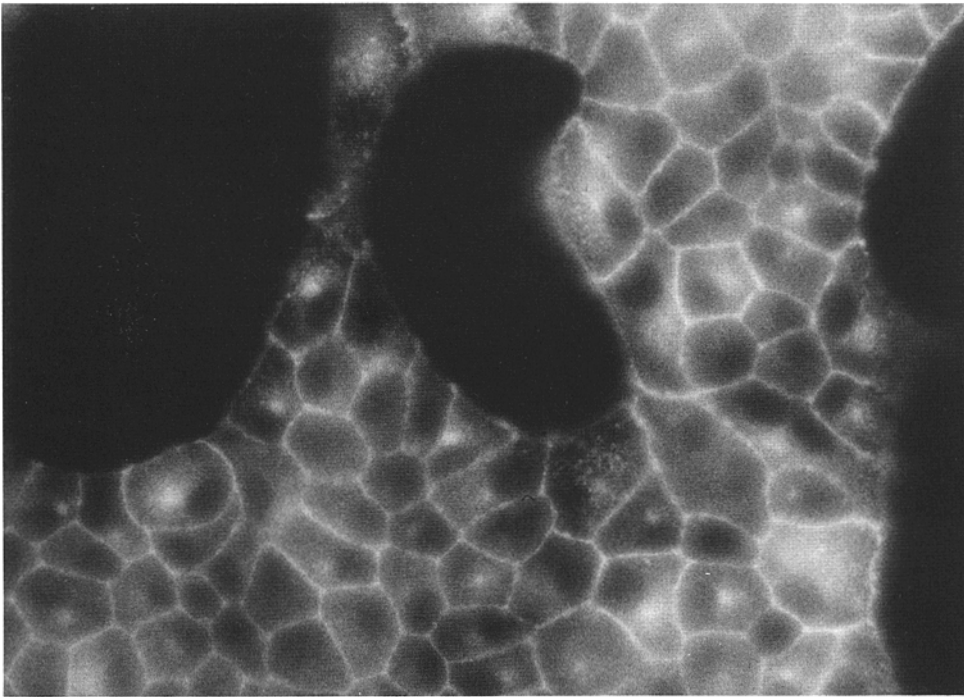
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Figure 2. Immunofluorescence staining of primary islet cells in culture using the monoclonal antibody against SNAP-25. Phase contrast (*a*) and fluorescence image (*b*). Bar, 10 μm.

and BoNT/A treatment, resulting in a mobility shift of 3 and 1 kD, respectively (not shown). Ca^{++}-induced insulin release was inhibited by 60 and 50%, respectively (Fig. 6).

Discussion

Pancreatic endocrine cells have several features in common with neuronal cells (18), including sensitivity to nerve growth factor (45) and expression of neuronal proteins such as tyrosine hydroxylase (62), glutamic acid decarboxylase (6), and synaptophysin (37, 65). The current scientifc opinion about the origin of pancreatic endocrine cells, however, favors an endodermal lineage rather than development from the neuroectoderm (28). Several types of secretory vesicles,
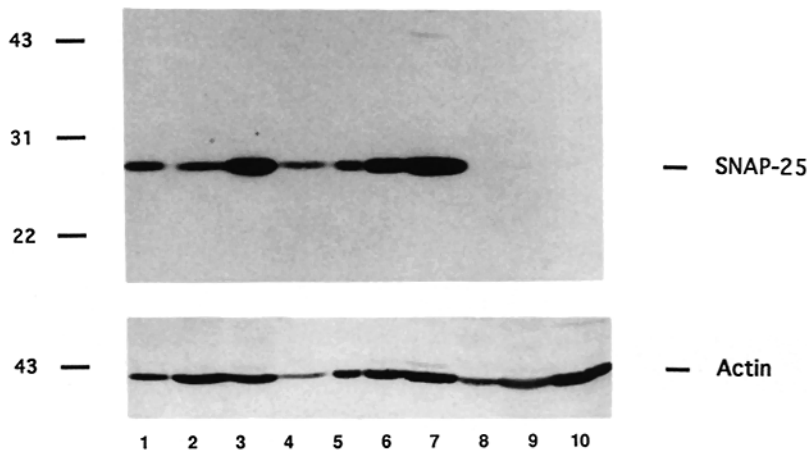
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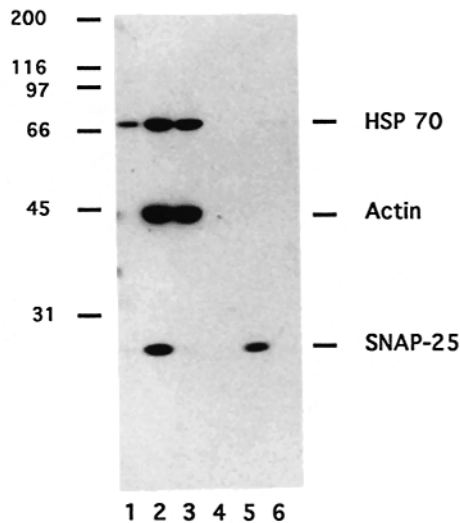
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Figure 3. Detection of SNAP-25 in different cells and different cellular fractions. (*a*) Western blot of lysates of different primary cells or cell lines, incubated with the polyclonal antibody against SNAP-25 (*upper panel*) or a polyclonal antibody against actin (*lower panel*). (Lane *1*) 1.5 × 10⁵ AtT-20 cells; (lane *2*) 4 × 10⁵ primary islet non–B cells; (lane *3*) 4 × 10⁵ primary islet B cells; (lane *4*) 1.5 × 10⁵ RIN cells; (lanes *5–7*) 1.5, 4, and 10 × 10⁵ INS-1 cells, respectively; (lane *8*) 4 × 10⁵ exocrine cells; (lane *9*) 4 × 10⁵ FAO cells; (lane *10*) 4 × 10⁵ COS cells. The primary islet cells were first sorted by autofluorescence-activated flow cytometry to obtain populations of B and non–B cells. (*b*) Western blot of different cellular fractions from HIT cells (10 μl/lane, corresponding to fractions obtained from 10⁵ cells) incubated with the monoclonal antibody against SNAP-25, a polyclonal antibody against actin and a monoclonal antibody against the heat shock protein HSP 70. (Lane *1*) Nuclear fraction; (lane *2*) postnuclear supernatant; (lane *3*) cytosol; (lane *4*) high salt membrane wash; (lane *5*) Triton X-100 soluble membrane fraction; (lane *6*) Triton X-100 insoluble membrane fraction.

different in terms of biogenesis, membrane composition, and secretory pathway, have been described both in neurons and endocrine cells. In addition to SSVs, used to store classical neurotransmitters including γ-amino butyric acid, glycine and acetylcholine, neurons, and neurosecretory cells (pheochromocytoma, adrenal chromaffin cells) also contain large dense core vesicles (LDCV) to store peptide neurotransmitters such as vasopressin and substance P (18). Insulin-containing LDCGs of pancreatic B cells resemble LDCVs of neurons. B cells contain also a second population of vesicles which resemble SSVs. These synaptic-like microvesicles (SLMV) contain γ-amino butyric acid (49). The membranes of LDCVs and SSVs differ in their protein composition (36). In addition, the molecular process of exocytosis of LDCVs and SSVs can be distinguished in frog motor neurons using α-latrotoxin. This toxin leads to a massive exocytosis of SSVs whereas the number of LDCVs in the same nerve end-

ing is not reduced (30). Similarities and differences of neurons vs endocrine cells and their different types of secretory vesicles raise the question whether regulated secretion in the two cellular systems follows the same molecular principles. We thus wanted to address the question whether the mechanism for insulin secretion from LDCGs resembles transmitter secretion from SSVs.

In the present study SNAP-25, known as a neuron specific protein (15, 21, 42, 43) involved in axonal elongation (41) and fusion of synaptc vesicles with the presynaptic membrane (13, 58), has been shown to be expressed in pancreatic endocrine cells. As in neurons (42) SNAP-25 is found in the detergent soluble fraction of a crude preparation of total cellular membranes from the insulinoma cell line HIT. SNAP-25 was however not detectable in cell lines such as FAO (hepatoma) or COS (kidney), which secrete proteins only by the constitutive pathway. Strikingly, SNAP-25 is also absent from
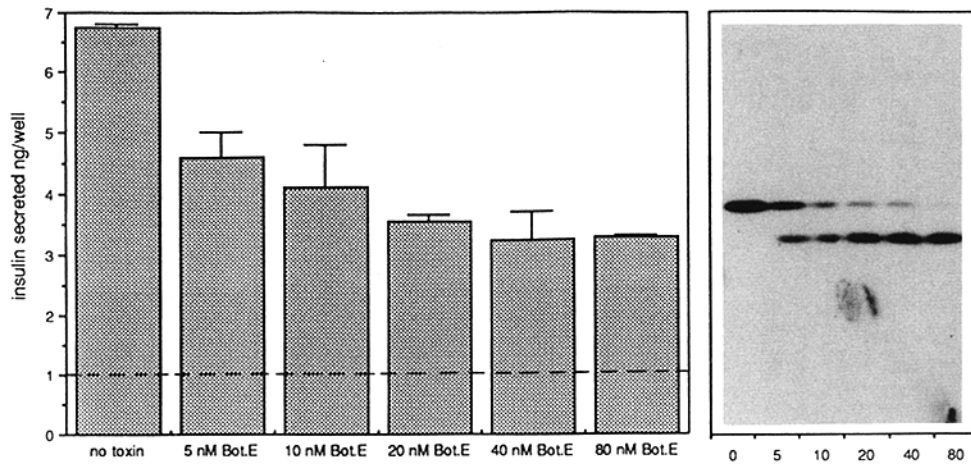
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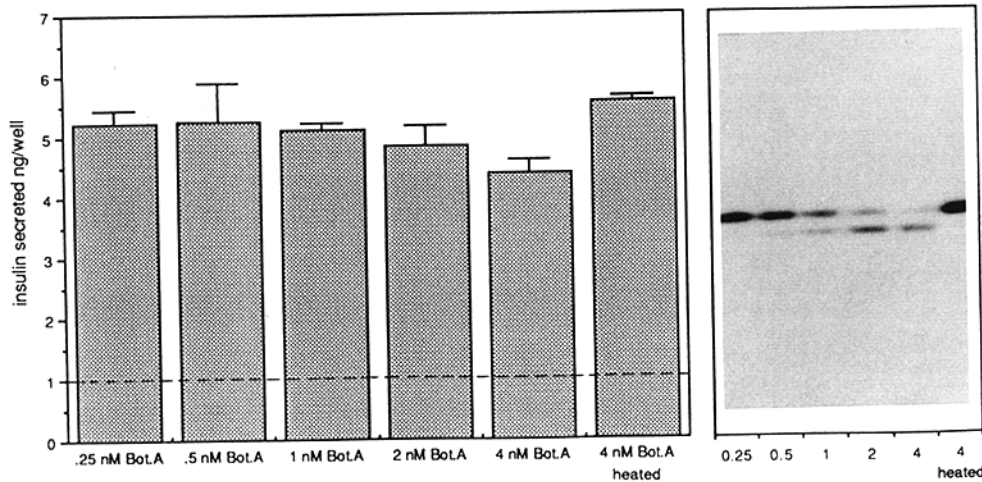
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Figure 4. Dose response curve for treatment of streptolysin-O permeabilized HIT cells with BoNT/E (*a*) or BoNT/A (*b*). Left panels show the effect of toxin treatment on Ca^{++}-induced insulin release (10 μM free Ca^{++}) (mean $\pm$ SD, $n = 3$). Dashed line indicates insulin release under low Ca^{++} condition (0.1 μM free Ca^{++}), which was not altered by the toxin. Right panels show corresponding Western blots of cell lysates using the monoclonal antibody against SNAP-25 to evaluate effect of toxin treatment on SNAP-25.
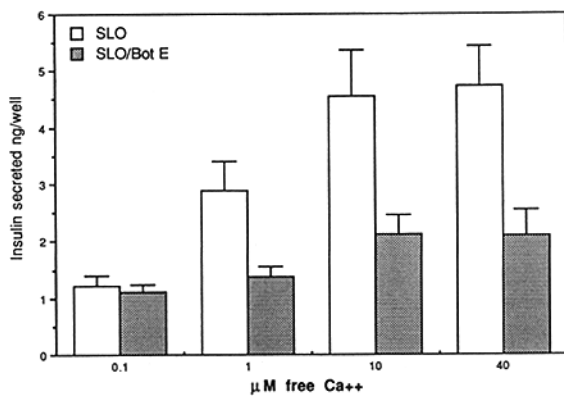

Figure 5. Effect of 40 nM BoNT/E on insulin secretion from permeabilized HIT cells at various free Ca^{++} concentrations (estimated using a Ca^{++} concentration curve measured with a Ca^{++} electrode). Mean $\pm$ SD; $n = 6$ from two independent experiments.
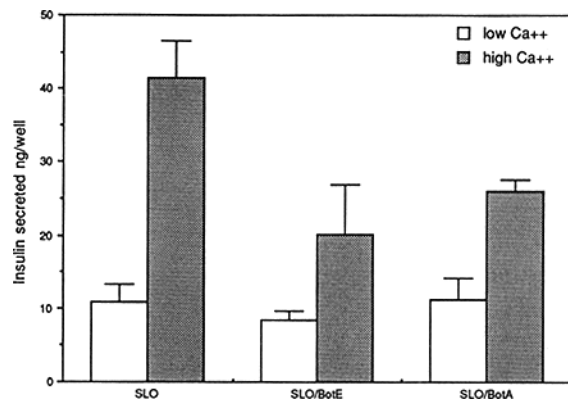

Figure 6. Effect of 40 nM BoNT/A and BoNT/E on insulin secretion from streptolysin-O permeabilized primary rat islet cells under low (0.1 μM free Ca^{++}) and high (10 μM free Ca^{++}) Ca^{++} conditions. Mean $\pm$ SD, $n = 3$.

pancreatic exocrine cells which surround the islets of Langerhans and possess a regulated secretory pathway for secretion from zymogen-containing granules (1).

By analogy to neurons, SNAP-25 in pancreatic B cells could be involved in secretion of γ-amino butyric acid from SLMVs rather than in exocytosis of insulin-containing LDCGs. The observation that release of vasopressin, a peptide neurotransmitter, stored in LDCVs, is inhibited by BoNT/A in isolated neurosecretory nerve terminals, makes an involvement of SNAP-25 in exocytosis of LDCGs, however, likely (17). We therefore used the neurotoxins BoNT/A and BoNT/E as tools to evaluate the role of SNAP-25 in secretion of insulin from LDCGs. Using permeabilized HIT cells and primary rat islet cells we have shown that, as in neuronal cells, SNAP-25 is hydrolyzed by these two toxins. The molecular weight shift and the fact that the polyclonal antibody directed against the 12 carboxy-terminal amino acids of SNAP-25 does not recognize the cleaved products (not shown) suggests that cleavage occurred at the expected sites, 9 and 26 residues from the carboxy terminus, respectively (10, 55).

Cleavage of SNAP-25 by BoNT/A and BoNT/E resulted in inhibition of Ca^{++}-induced insulin release in HIT cells as well as in primary islet cells. The inhibition of BoNT/A varied from experiment to experiment and was generally less pronounced than that of BoNT/E. Insulin release under low Ca^{++} conditions was not affected by either toxin.

Neither toxin treatment resulted in complete inhibition of Ca^{++}-induced insulin secretion, although both toxins were able to hydrolyze SNAP-25 almost quantitatively. Note, however, that for both toxins a faint band of uncleaved SNAP-25 was always visible even when high toxin concentrations were used. It is not clear whether this is due to incomplete permeabilization, too short a treatment with toxin, or whether there is a small pool of SNAP-25 protected against cleavage as proposed by Niemann et al. (38). In fact a recent study by Hayashi et al. (24) has shown that BoNT/A and E have little effect on SNAP-25 when SNAP-25 is associated with VAMP and syntaxin in a complex assembled in vitro using the recombinant proteins. Thus similar complexes possibly present in the cell might protect small quantities of SNAP-25 from being cleaved by the toxins. The fact that almost complete cleavage of SNAP-25 results in only partial inhibition of insulin release is intriguing but has some precedents. It has been shown for instance that tetanus toxin, which can hydrolyze cellubrevin, inhibits only 35% of exocytosis of transferrin receptor containing vesicles, although cellubrevin is completely digested under the assay conditions (19). More important for our observations, however, is that several studies show only a partial inhibitory effect of BoNT/A on neurotransmitter release (45–60%), especially when permeabilized cells are used (2, 17, 31, 59, 60). BoNT/E has been used in only a few assay systems where it is usually more effective than the A type toxin (7, 11). As the proteolytic target of BoNT/A and BoNT/E was not known at the time of these previous studies, this is the first comparative study on inhibition of secretion with respect to cleavage of SNAP-25.

The only partial inhibition of insulin release caused by BoNT/A and BoNT/E could have several explanations. It is possible that even a very small number of uncleaved SNAP-25 molecules is sufficient to allow a substantial amount of fusion of insulin-containing granules to occur. It is also possible that another functionally equivalent molecule, not attacked by the toxin, can still function to give rise to part of the Ca^{++} response. Alternatively, BoNT/A and BoNT/E may attack additional proteins which are not completely cleaved under these conditions but which are implicated in insulin secretion. Recently it has been shown that BoNT/E hydrolyses denatured actin in vitro at several sites all characterized by Arg or Lys before the cleavage point (16). SNAP-25 is also cleaved after an arginine residue, between Arg180 and Ile181, but as there are other Arg-Ile peptide bonds in SNAP-25 which are not hydrolyzed by BoNT/E, the tertiary structure of the substrate must be essential for the action of the toxin (56). In any event, incubation of the Western blot with a polyclonal antibody against actin did not show any actin degradation products (not shown). There is another report suggesting that BoNT/A might cleave additional proteins besides SNAP-25. Steinhardt et al. showed that resealing of fibroblast plasma membranes by a vesicular mechanism is inhibited by BoNT/A (61). As fibroblasts do not express SNAP-25, inhibition could be due to cleavage of an isoform of SNAP-25 so far not detected with the available antibodies, or to cleavage of an unrelated protein.

An additional explanation for the partial inhibitory effect of the BoNTs could be that a number of insulin-containing granules are already docked at the plasma membrane, or primed to fuse, and therefore do not require SNAP-25 for subsequent fusion. The fact that basal insulin release is not influenced by the toxin supports this view, as such release might be due to occasional fusion of already docked granules. SNAP-25 would therefore be responsible for the first docking step of the insulin-containing granule to the plasma membrane (57). This would be in agreement with the model proposed by Söllner et al., which suggests that the first step in exocytosis is the docking of the secretory vesicle by binding of the integral vesicle protein synaptobrevin to its receptors, syntaxin and SNAP-25, in the plasma membrane.

The inhibitory effect of BoNT/E is somewhat more pronounced at intermediate Ca^{++} concentrations. In neurons it has been shown that a rise in intracellular Ca^{++}, induced by amino pyridines or Ca^{++}-ionophores, can counteract the poisoning effect of BoNT/A and E but not of BoNT/B, D, and F (5, 20, 32, 33). It may be that only the "calcium-SNAP-25" conformation is active in the secretion process and that the percent of molecules able to reach this conformation is lower when 9 amino acids at the carboxy terminus are missing (cleavage by BoNT/A) and yet lower when 26 amino acids are missing (cleavage by BoNT/E). High Ca^{++} concentrations used as stimulus for insulin secretion could push the equilibrium to the calcium conformation and thus shadow the toxin effect. This could also explain why treatment with BoNT/A in such a sensitive assay system was highly variable in terms of inhibition of insulin release and generally less effective than BoNT/E treatment. Recent in vitro studies on the binding properties of recombinant SNAP-25, VAMP, and syntaxin also support the notion that treatment with BoNT/E and more obviously with BoNT/A may result in only partial inhibition of exocytosis. It has been shown that SNAP-25, VAMP, and syntaxin assemble into a high affinity complex (44) resistant to SDS (24). SNAP-25 cleaved by BoNT/A or E can still associate with VAMP and syntaxin to form a high affinity complex. SDS resistance of this complex, how-

ever, is strongly diminished or completely abolished when BoNT/A or E treated SNAP-25 is used, respectively (24). Thus SNAP-25 lacking the last carboxy terminal amino acids might still be able to fulfill most of its role in the docking and/or fusion process although not as efficiently as intact SNAP-25. This might lead to the observation of a partial inhibition of exocytosis after BoNT/A and E treatment.

In conclusion our results demonstrate that SNAP-25 is expressed in pancreatic endocrine cells. Cleavage of SNAP-25 by BoNT/A and BoNT/E results in partial inhibition of Ca^{++}-dependent insulin release which suggests a role for SNAP-25 in exocytosis of endocrine LDCGs.

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