Antibody inhibition of synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1 reduces rapid exocytosis in insulin-secreting cells

Jenny Vikman¹, Xiaosong Ma¹, Gregory H Hockerman², Patrik Rorsman^{1,3} and Lena Eliasson¹

¹Islet Cell Exocytosis, Department of Clinical Sciences in Malmö, Clinical Research Centre (CRC), Building 91, Floor 11, Lund University, Ing. 72 UMAS, SE-20502 Malmö, Sweden

²Department of Medical Chemistry and Molecular Pharmacology, Purdue University, Indiana, USA

³OCDEM, University of Oxford, Old Road Campus, Oxford OX3 7 LJ, United Kingdom

(Requests for offprints should be addressed to L. Eliasson; Email: Lena.Eliasson@med.lu.se)

Abstract

SNARE-proteins (soluble NSF-attachment protein receptor) are important for Ca^{2+} -dependent exocytosis. We have used capacitance measurements and confocal imaging to dissect the role of synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1 in rapid exocytosis in insulin-secreting pancreatic β -cells. Following immunoneutralization of syntaxin 1 and SNAP-25, exocytosis was strongly reduced and associated with a marked reduction in the size of the readily releasable pool (RRP) by 65% and 86% in the presence of the anti-SNAP-25 and anti-syntaxin 1 antibodies respectively. The size of the immediately releasable pool (IRP), a subset of RRP in close association with the voltage-dependent Ca^{2+} -channels, was reduced to an equal extent. The reduction in IRP correlated with slowed release kinetics and the time constant (τ) increased from a control value of 16 to 36 ms and 51 ms after inclusion of anti-SNAP-25 and anti-syntaxin 1 antibodies respectively in the pipette solution. We further show that SNAP-25 and syntaxin 1 aggregate in clusters along the plasma membrane. The size of these clusters was estimated to be ~ 300 nm and every β -cell contained ~ 400 SNAP-25/syntaxin 1 clusters. Whereas the inhibitory action of the anti-syntaxin 1 antibody on exocytosis could be attributed almost entirely to suppression of the voltage-dependent Ca^{2+} -current ($\sim 40\%$), the effect of the anti-SNAP-25 antibody was not mediated by decreased Ca^{2+} -entry and is more likely due to a direct interference with the exocytotic machinery. Our data are consistent with the concept that both syntaxin 1 and SNAP-25 are required for rapid exocytosis in β -cells.

Journal of Molecular Endocrinology (2006) **36**, 503–515

Introduction

Insulin is released from the pancreatic β -cell in response to elevated blood glucose by Ca^{2+} -dependent exocytosis (Lang 1999, Rorsman & Renström 2003). Exocytosis in the insulin-secreting pancreatic β -cell resembles that of neurons, and the SNARE (soluble NSF-attachment protein receptor)-proteins syntaxin 1 and synaptosomal protein of 25 kDa (SNAP-25) have been shown to be present in pancreatic islets (Jacobsson *et al.* 1994, Martin *et al.* 1995, Nagamatsu *et al.* 1999) and in insulin-secreting cell lines (Jacobsson *et al.* 1994, Martin *et al.* 1995, Oho *et al.* 1995, Wheeler *et al.* 1996). The requirement for syntaxin 1 in insulin secretion has previously been demonstrated by inclusion of antibodies against syntaxin 1A (Martin *et al.* 1995, Yang *et al.* 1999) or peptides against the H-3 region of syntaxin 1A (Martin *et al.* 1998), which reduce insulin secretion in permeabilized β -cells. In other studies, using insulin-secreting cell lines, overexpression of syntaxin 1

(Nagamatsu *et al.* 1999, Ohara-Imaizumi *et al.* 2001, Kang *et al.* 2002) or SNAP-25 (Ohara-Imaizumi *et al.* 2001) results in lower secretion and reduced Ca^{2+} -current amplitude (Ji *et al.* 2002, Kang *et al.* 2002).

A current unifying model of regulated exocytosis postulates that syntaxin 1 and SNAP-25 associate with the plasma membrane and that vesicle-associated membrane protein (VAMP)/synaptobrevin associate with the vesicular membrane. SNAP-25 is peripherally attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein, while VAMP and syntaxin 1 are inserted in the membrane by a carboxyterminal transmembrane domain. Aggregation of the SNARE-proteins give rise to a ternary complex that promotes fusion between vesicular and plasma membrane by pulling the membranes in close contact with each other (Lin & Scheller 2000).

The SNARE-complex is assembled during the priming process preparing granules for Ca^{2+} -dependent exocytosis (Xu *et al.* 1998, 1999).

Exocytosis of insulin-containing large dense core vesicles (LDCVs) is preceded by several priming events, which render the vesicles ready for fusion with the plasma membrane (Rorsman *et al.* 2000, Barg *et al.* 2002). The group of primed LDCVs is referred to as the readily releasable pool (RRP). A subset of RRP vesicles appear tightly associated with the L-type voltage-dependent Ca^{2+} -channels and their fusion with the plasma membrane gives rise to a very fast component of exocytosis (immediately releasable pool, IRP; Barg *et al.* 2001, 2002). It has been hypothesized (Wiser *et al.* 1999) that IRP vesicles are tethered to the L-type Ca^{2+} -channels via a multiprotein complex named excitosome, comprising the SNARE-proteins as well as other exocytotic proteins including synaptotagmin.

Although extensive studies demonstrating the importance of SNAP-25 and syntaxin 1 for insulin secretion have been performed previously, none of them has addressed the temporal aspects and the role for the transition of the LDCVs in β -cell between different functional granule pools. Here we have performed high-resolution capacitance measurements on single primary β -cells following intracellular application of antibodies against SNAP-25 and syntaxin 1. This approach has enabled us to establish the role(s) of these SNARE-proteins in Ca^{2+} -dependent rapid exocytosis in the pancreatic β -cells. We report that both SNAP-25 and syntaxin 1 are required for rapid exocytosis. In addition, syntaxin 1 is required for the refilling of IRP/RRP by granule mobilization from the reserve pool and Ca^{2+} -channel function.

Materials and methods

Cells and cell culture

Pancreatic β -cells were isolated from NMRI mice or Sprague–Dawley rats (Bomholtgaard, Ry, Denmark) according to procedures described previously (Barg *et al.* 2001) and approved by the Lund, Malmö ethics committee. The mouse or rat islet cell suspensions were plated on plastic Petri dishes or (for immunocytochemistry) glass coverslips and maintained in tissue culture for 1–2 days prior to experiments. The tissue culture medium used for culturing primary β -cells consisted of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin. Rat insulinoma cells (INS-1; Asfari *et al.* 1992) were cultured with RPMI 1640 media containing 10% FCS (w/v), 50 mM 2-mercaptoethanol, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Immunocytochemistry

After culture (15–24 h), the cells were fixed for 5 min in 3% paraformaldehyde in K-PIPES (Sigma, Stockholm,

Sweden) followed by 10 min in 3% paraformaldehyde supplemented with 100 mM NaB_4O_7 . Finally, they were permeabilized using 0.1% Triton X-100 for 30 min. Non-specific binding was suppressed by incubating the cells for 15 min with 5% normal donkey serum. The cells were subsequently incubated for 2 h in the presence of primary antibodies in different combinations as described in the text. The following antibodies were used: mouse monoclonal anti-SNAP-25 (dilution 1:100, clone 71.1; Synaptic Systems, Göttingen, Germany), rabbit polyclonal anti-SNAP-25 (dilution 1:200; StressGen, Biotechnologies Corp., Victoria, Canada), rabbit anti-syntaxin 1 (dilution 1:100; StressGen Biotechnologies Corp.) and guinea-pig polyclonal anti-insulin (B 65–1, dilution 1:1000; Euro-diagnostica, Malmö, Sweden). The anti-insulin was used to ascertain the identification of the pancreatic β -cells. Cy3- and Cy5-conjugated donkey anti-mouse, anti-rat and anti-rabbit (Jackson Immuno, Westgrove, PA, USA) were used to label the detected sites. In one set of experiments (Fig. 6D), INS-1 cells were transiently transfected with pEGFP-N1/ α 1c-DHPi (Liu *et al.* 2003) using Effectene Transfection Regent (QIAGEN, Germany) before fixation and incubation with the monoclonal SNAP-25 antibody as above. Excitation of EGFP, Cy-3 and Cy-5 was performed using the 488 nm, 543 nm and 633 nm lines respectively of a Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany). Emitted light passed through a BP505–530 (EGFP), a BP565–615 filter (Cy-3) or an LP650 filter (Cy-5) and was visualized using a $63\times/1.3\text{NA}$ oil objective. The samples were scanned sequentially with the appropriate settings for each label to minimize crosstalk. No visual detection could be observed when immunolabelling was performed with the secondary antibodies (Cy-3 and Cy-5) in the absence of primary antibodies and unspecific binding of the secondary antibodies can thus be excluded. The Zeiss LSM Image examiner (version 2.80.1123) was used to evaluate overlap of double labelling and to perform line scans.

Immunoblot analysis

Subcellular fractionation was performed using INS-1 cells as previously described (Dotta *et al.* 1998). Approximately 30–50 million cells were washed 3 times with ice-cold homogenization medium (HM; 250 mM sucrose, 5 mM HEPES, 0.5 mM EGTA and 0.2 mM PEFA Block (Sigma) adjusted to pH 7.4 with KOH) and then detached from the tissue culture flask by a plastic policeman into 3–5 ml HM. The cells were homogenized under nitrogen pressure (350 psi) in a cell disruption bomb (Parr Instruments Company, Moline, IL, USA) for 15 min at 4 °C. After centrifugation at 700 *g* for 15 min at 4 °C, the resulting post-nuclear supernatant was diluted to 5 ml with HM and mixed with Percoll

and sucrose to give a final concentration of 15% v/v Percoll and 250 mM sucrose. A self-generating gradient was formed by spinning the sample at 48 000 *g* for 25 min at 4 °C in a fixed angle rotor (Beckman, TI 70-1, 22 800 r.p.m.). Two opaque bands were visible at the top and bottom corresponding to the plasma membrane and secretory granules respectively. These bands were collected manually with a pipette. Each fraction was washed twice with 4 volumes HM and re-centrifuged at 150 000 *g* at 4 °C for 30 min. A cytosolic fraction was achieved by centrifuging the post-nuclear supernatant at 150 000 *g* at 4 °C for 60 min and then collecting the supernatant. The fractions were sonicated and stored at -20 °C pending full analysis.

For western blot analysis, the plasma membrane, cytosolic and granular fractions as well as the homogenate (40 µg/lane) were loaded on 7.5% or 12.5% SDS-PAGE gels, separated by electrophoresis and blotted onto nitrocellulose membranes (Biorad, Sweden). Blots were incubated overnight at 4 °C with mouse monoclonal anti-SNAP-25 (1:5000, clone 71-1) or rabbit anti-syntaxin 1 (1:1000, Alomone, Jerusalem, Israel) and treated with horseradish peroxidase-conjugated secondary antibodies (1:50 000, Pierce, Rockford, IL, USA) for 60 min. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection.

The plasma membrane fraction was identified by the presence of Na⁺/K⁺-ATPase using western blot analysis (mouse monoclonal anti-Na⁺/K⁺-ATPase α-1, 1:2000, Upstate Biotechnology, Lake Placid, NY, USA) and the granular fraction was confirmed by measuring the insulin content using a rat insulin ELISA (Mercordia, Uppsala, Sweden). Insulin concentration was more than eightfold higher in the granular fraction compared with the plasma membrane fraction.

Electrophysiology

Whole-cell currents were recorded using an EPC-9 patch-clamp amplifier and the software Pulse (Heka Elektronik, Lamprecht/Pfalz, Germany; ver 8.31). Exocytosis was detected as changes in cell capacitance using the software-based lock-in application (which adds a sine wave with a frequency of 500–1000 Hz to the holding potential) of the amplifier. Exocytosis was elicited by trains of ten 500 ms voltage-clamp depolarizations from -70 to 0 mV applied at 1 Hz or by single depolarizations of variable pulse duration (5–450 ms). Patch electrodes were pulled from borosilicate glass capillaries, coated with Sylgard (Dow Corning, Midland, MI, USA) and fire-polished. The pipette resistance was 3–6 MΩ when the pipettes were filled with the intracellular solutions specified below. Experiments were conducted using the standard whole-cell configuration, which allowed intracellular application of the antibodies. To allow complete influx of the antibodies, the first

depolarization was applied ≥2 min after establishment of the whole-cell configuration (Barg *et al.* 1999).

The standard extracellular solution consisted of (in mM) 118 NaCl, 20 tetraethyl-ammonium chloride (TEA-Cl; to block voltage-gated K⁺-currents), 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 5 glucose and 5 Hepes (pH 7.4 using NaOH). The pipette solution contained (in mM) 125 Cs-Glut, 10 NaCl, 10 CsCl, 1 MgCl₂, 0.05 EGTA, 3 Mg-ATP, 10 Hepes (pH 7.15 using CsOH), and 0.1 cAMP. Where indicated, the pipette solutions were supplemented with either a rabbit polyclonal anti-syntaxin 1 (diluted 1:100; StressGen, Biotechnologies Corp.), a rabbit anti-SNAP-25 (diluted 1:200; StressGen, Biotechnologies Corp.) or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In one series of experiments the intracellular solution was supplemented with Fab-fragments of the rabbit polyclonal antibody against syntaxin 1. Fab-fragments were generated by papain digestion using ImmunoPure Fab Preparation kit (Pierce). Before inclusion in the pipette solution, the Fab-fragments were concentrated in Micron 30 concentrators (Millipore, Billerica, MA, USA).

Data analysis

The density (*D*) of clusters was determined from the immunostainings using the formula (equation 1) (Williams 1977):

$$D = \frac{N}{O \cdot t} \quad (\text{Eq. 1}),$$

where *N* is the number of clusters in the focal plane, *O* is the profile perimeter and *t* the thickness of the optical slice. For our microscope, *t* was estimated, as suggested by Wilson (1995), to be ≈0.6 µm.

An upper limit of the real size of the clusters of SNAP-25 and syntaxin 1 was estimated using beads (Molecular Probes, Leiden, the Netherlands) with a diameter of 170 nm and in conjunction with the point-spread function (PSF) estimated as described elsewhere (Lang *et al.* 2001). PSF was calculated using the formula (equation 2):

$$PSF = [(d_{\text{measured}})^2 - (d_{\text{real}})^2]^{1/2} \quad (\text{Eq. 2}),$$

where *d*_{measured} and *d*_{real} represent the measured and actual size of the beads respectively. The 170 nm beads had a measured size of 293 nm and PSF was accordingly estimated to be 238 ± 5 nm. The real size of the clusters (*D*_{real}) was estimated by subtracting PSF from the measured size of the clusters (*D*_{measured}) (equation 3)

$$D_{\text{real}} = [(D_{\text{measured}})^2 - (PSF)^2]^{1/2} \quad (\text{Eq. 3}).$$

The activation of the Ca²⁺-current is described by equation 4

$$I = G * \frac{(V - V_r)}{1 + e^{-\left[\frac{(V - V_h)}{k}\right]}} \quad (\text{Eq. 4}),$$

where I is the peak current, G is the whole-cell conductance, V is the voltage of the depolarizing pulse, V_r is the reversal potential, V_h is the membrane potential at which activation is half-maximal and k is the slope coefficient.

Experimental data in Fig. 5 were approximated using a kinetic model, which considers the exchange of granules out from the immediately releasable pool (P) into a fused state, as has been described elsewhere (Eliasson *et al.* 2003). Briefly, the pool dynamics can be described by the differential equation (equation 5)

$$\frac{\delta P}{\delta t} = -\alpha_0(1 - e^{-\left[\frac{t}{\tau}\right]})^3 P \quad (\text{Eq. 5}),$$

Solutions to equation 5 are approximated to experimental data to derive the initial values of P corresponding to the maximal size of IRP, and the values of the rate constants τ and τ_0 , where the latter is derived from α_0 ($\tau_0 = 1/\alpha_0$), the decay rate of P (see Barg *et al.* 2001 for derivation).

Depolarization-evoked exocytotic responses are expressed as the net change in whole-cell capacitance (ΔC_m). Data are presented as mean values $\pm$ S.E.M. of the indicated number of experiments (n). Statistical significances were assessed using Student's t -test or ANOVA.

Results

Presence and distribution of SNAP-25 and syntaxin 1 in insulin-secreting cells

Using confocal immunofluorescence microscopy we demonstrate that SNAP-25 and syntaxin 1 are present in primary β -cells (Fig. 1A,B) and thus confirm observations made in other laboratories (Jacobsson *et al.* 1994, Martin *et al.* 1995, Sadoul *et al.* 1995, Wheeler *et al.* 1996, Gonelle-Gispert *et al.* 1999). The subcellular distribution of SNAP-25 and syntaxin 1 in insulin-secreting cells was investigated by immunoblotting using fractionated INS-1 cells. SNAP-25 immunoreactivity was mainly detected in the plasma membrane fraction (P), and to a lesser extent also in the granular fraction (G; Fig. 1C). Similar results were obtained when the fractions were immunoblotted against syntaxin 1; a 35 kDa-band was observed in both the P and G fractions, with the strongest band detected in the P fraction. The finding that Na^+/K^+ -ATPase immunoreactivity is confined to the plasma membrane fraction, with no detectable activity in the granular fraction, argues that the presence of SNAP-25 and syntaxin 1 in the granular fraction cannot be explained as contamination of this fraction with plasma membrane. Thus, it appears that insulin-containing secretory granules, by

analogy to what has previously also been detected in granules in nerve terminals (Walch-Solimena *et al.* 1995) and in chromaffin granules (Tagaya *et al.* 1995, 1996, Haynes *et al.* 1999), contain both syntaxin 1 and SNAP-25. This conclusion is also supported by earlier reports from insulin secreting cell lines suggesting the presence of SNAP-25 not only in the plasma membrane but also in an undefined perinuclear region (Sadoul *et al.* 1995). We acknowledge, however, that we cannot exclude the possibility that the granular fraction might be contaminated by endosomes and that this accounts for the apparent presence of SNARE-proteins in the granular fraction.

Syntaxin 1 and SNAP-25 co-localize in clusters in primary β -cells

Both SNAP-25 and syntaxin 1 show a spot-like distribution along the plasma membrane (Fig. 1A,B, Fig. 2), suggesting that the two proteins are clustered. This would be in keeping with what has been reported in many other cell types (Lang *et al.* 2001, Ohara-Imaizumi *et al.* 2004a,b, Rickman *et al.* 2004). We estimated the degree of co-localizing SNAP-25/syntaxin 1 clusters by performing line-scans along (Fig. 2B) and perpendicular to the plasma membrane (Fig. 2C) using equatorial confocal sections. Thereby it could be ascertained that the proteins co-localized in at least two dimensions. Approximately one third of the clusters showed immunoreactivity for both SNAP-25 and syntaxin 1 ($34 \pm 2\%$; $n=9$). Using equation 1, the density of clusters containing both SNAP-25 and syntaxin 1 was determined to be 0.81 ± 0.04 clusters/ μm^2 ($n=9$). The surface area of a β -cell was calculated to be $526 \pm 25 \mu\text{m}^2$ ($n=31$; derived from measurements of membrane capacitance on single rat β -cells and a conversion factor of $9 \text{ fF}/\mu\text{m}^2$; Gentet *et al.* 2000). Using these values, the number of clusters in an entire β -cell was estimated to be 425 ± 22 ($n=9$).

The size of a cluster estimated from the immunostainings was $404 \pm 8 \text{ nm}$ ($n=48$). After correction for the PSF (equation 2), the size of the clusters was estimated to be $323 \pm 10 \text{ nm}$ ($n=48$; equation 3). All estimations were performed on immunostainings from rat β -cells since the best staining was achieved when using the mouse monoclonal antibody against SNAP-25, thus precluding the use of mouse β -cells for these experiments. However, the presence of both SNAP-25 and syntaxin 1 in mouse β -cells could be verified using polyclonal antibodies (data not shown).

Anti-SNAP-25 reduces Ca^{2+} -dependent exocytosis of insulin-containing granules

To investigate the involvement of SNAP-25 in exocytosis in primary mouse β -cells, we applied an antibody against SNAP-25 intracellularly by inclusion in the

Figure 1 Syntaxin 1 and SNAP-25 principally locate to the plasma membrane. (A) Immunoreactivity of SNAP-25 and insulin. Black-and-white illumination of two rat islet cells (left). Scale bar 5 μ m. Distribution of insulin binding (red) and SNAP-25 immunoreactivity (green) visualized by confocal immunocytochemistry (right) in the same cells as on the left. Note that of the two cells only one is insulin-positive, showing that SNAP-25 is also present in other islet cell types. (B) Same as in (A) but in different cells and using an antibody directed against syntaxin 1 (green). (C) Immunoblots of plasma membrane (P), cytosol (C) and granule (G) subcellular fractions obtained by fractionation of INS-1 cells. The cell homogenate (H) was used as a positive control. Identification of SNAP-25 (a), syntaxin 1 (b) and the plasma membrane fraction (c) using anti- Na^+/K^+ -ATPase.

pipette-filling solution. In an initial series of experiments, single β -cells were stimulated by a train of ten 500-ms depolarizations from -70 mV to 0 mV (Fig. 3). The total

increase in membrane capacitance evoked by a train was 169 ± 31 fF ($n=10$) under control conditions. This response was reduced to 47 ± 18 fF ($n=6$) when

Figure 2 Co-localization of SNAP-25 and syntaxin 1 in the plasma membrane. (A) Co-localization of SNAP-25 and syntaxin 1. Images appear as in Fig. 1A with the SNAP-25 binding in red and the syntaxin 1 immunoreactivity in green. Scale bar 2 μ m. (B) Results of line scans performed along the plasma membrane of the cell in (A). The results through the 3 marked clusters in (A) are indicated. The intensity of SNAP-25 and syntaxin 1 immunoreactivity is plotted against the distance along the membrane (d). Background level is indicated by the black dotted line. Note the perfect co-localization in clusters 1 and 2, the latter sitting next to non-colocalizing clusters. Cluster 3 contains both co-localizing and non co-localizing areas. (C) Line scans performed perpendicular to the plasma membrane crossing the clusters marked in (A).

anti-SNAP-25 was included in the pipette solution. Close inspection of the responses revealed that the inhibition was particularly strong for the first depolarization and no statistically significant inhibition was seen during pulses 2–10 (Fig. 3B). This was also reflected in the fact that the readily releasable pool (defined by the first two depolarizations) was reduced by 70% (Fig. 3C), whereas the suppression of mobilization (measured as the capacitance increase during the last 8 depolarizations of the train) was not statistically significant (Fig. 3D). The capacitance increase evoked by a second train (applied 1 min after the first) was reduced from 104 ± 21

fF ($n=5$) under control conditions to 31 ± 9 fF ($n=5$) in the presence of anti-SNAP-25. The observed decrease in the exocytotic capacity is unlikely to be the result of unspecific interactions because exocytosis measured in the presence of rabbit IgG was not reduced compared with control values (Fig. 3B–D).

Anti-syntaxin 1 inhibits Ca^{2+} -dependent exocytosis

Next, the exocytotic response in the presence of an antibody against syntaxin 1 was investigated (Fig. 4A). Exocytosis was again initiated by a train of ten

Figure 3 Reduction of exocytosis by anti-SNAP-25 antibody. (A) Capacitance increase (ΔC_m) evoked by a train of ten 500 ms-depolarizations from -70 to 0 mV (V) in the absence and presence of polyclonal antibody against SNAP-25 (1:200; applied intracellularly via the recording pipette). (B) Mean increases in membrane capacitance evoked by the individual pulses ($\Delta C_{m,n} - \Delta C_{m,n-1}$) in the train under control conditions ($n=10$), in the presence of IgG ($n=7$) and in the presence of anti-SNAP-25 ($n=6$). (C) RRP determined from the same cells as in (B) using the capacitance increase during the first two depolarizations of the train by methods described previously (Voets *et al.* 1999). (D) Increases in membrane capacitance (ΔC_m) during the latter 8 depolarizations of the train as a measure of mobilization (mob). Data were taken from the same cells as in (B). The data are mean values $\pm$ S.E.M. $^{**}P < 0.01$, anti-SNAP25 vs control, and $^+P < 0.05$, anti-SNAP-25 vs IgG.

500 ms-depolarizing pulses as described above. In a series of 22 experiments, the capacitance increase evoked by ten depolarizations under control conditions was somewhat larger than in the previous series and

amounted to 431 ± 53 fF (Fig. 3B), illustrating the interexperimental variability and emphasizing the importance of carrying out separate control experiments for all experimental series. In the presence of anti-syntaxin 1, the exocytotic response was reduced by 90% to 35 ± 9 fF ($n=7$). To ensure that the antibody did not inhibit exocytosis simply by steric hindrance, we performed similar experiments in the presence of Fab-fragments (the antigen-binding part of the antibody) prepared from the anti-syntaxin 1 antibody (Fig. 4B). In accordance with the data obtained with the native antibody, the increase in membrane capacitance was almost abolished in the presence of the Fab-fragments (29 ± 13 fF; $n=5$), which are $5 \text{ nm} \times 5 \text{ nm}$ compared with about twice as much for the original antibodies, confirming the specificity of the anti-syntaxin 1 effect. The effect of the antibody was already pronounced during the first two depolarizations, and RRP was reduced by $>80\%$ in the presence of the antibody (Fig. 4D). The inhibition was equal throughout the train and the reduction of the exocytotic response during the latter depolarizations was equal to the reduction in the size of RRP (Fig. 4E). During a second train applied 2 min after the first, the capacitance increase was almost abolished in the presence of anti-syntaxin 1, and only amounted to 2 ± 1 fF ($n=6$) compared with 295 ± 72 fF ($n=8$; $P < 0.01$) under control conditions.

Kinetic parameters determined by SNAP-25 and syntaxin 1

The role of syntaxin 1 and SNAP-25 has previously only been examined by insulin release measurements, which have an inherently low temporal resolution (min; Sadoul *et al.* 1995, Martin *et al.* 1998, Nagamatsu *et al.* 1999). We subsequently investigated the importance of SNAP-25 and syntaxin 1 in rapid exocytosis. Membrane capacitance cannot be monitored during the depolarization and we therefore reconstructed the time course of secretion during the first 450 ms by applying progressively longer (5–450 ms) depolarizing pulses from -70 mV to 0 mV (Fig. 5). Under control conditions, as well as in the presence of anti-SNAP-25, significant increases in cell capacitance were elicited by depolarizations as short as 30 ms. The SNAP-25 antibody had no major impact on the response to depolarisations ≤ 100 ms. By contrast, pulses longer than 50 ms were required to elicit any exocytosis after inclusion of the syntaxin 1 antibody and the responses were significantly smaller than control values for all pulse durations. Whereas the increase in cell capacitance amounted to ~ 120 fF under control conditions, it plateaued at ~ 30 fF and ~ 20 fF in the presence of anti-SNAP-25 and anti-syntaxin 1 respectively.

The size of IRP and the time constant of capacitance increase (τ) under the different experimental conditions

Figure 4 Anti-syntaxin 1 inhibits exocytosis in β -cells. (A) As in Fig. 3A but in the absence or presence of an antibody against syntaxin 1 (1:100). (B) Same as in (A) but in the presence and absence of Fab-fragments of the antibody used in (A) (Fab-anti-syntaxin 1; 14 μ g/ml). (C) Summary of mean capacitance increases evoked by the individual pulses ($\Delta C_{m,n} - \Delta C_{m,n-1}$) in the train under control conditions ($n=22$), in the presence of anti-syntaxin 1 ($n=7$) and in the presence of Fab-fragments ($n=5$). (D) Estimate of RRP as in Fig. 3C under control conditions (C), in the presence of anti-syntaxin 1 (aS) or in the presence of Fab-anti-syntaxin 1 (Fab). (E) As in Fig. 3D. Data were taken from the same cells as in (B). The data are mean values $\pm$ S.E.M. $**P < 0.01$, $***P < 0.001$, anti-syntaxin 1 vs control, and $^+P < 0.05$, $^{++}P < 0.01$, Fab-anti-syntaxin 1 vs control.

can be obtained by solving equation 5. The results are summarized in Table 1. It is apparent that IRP was markedly reduced in the presence of both anti-SNAP-25 and anti-syntaxin 1 but that the effect of the latter was more pronounced. The effect on IRP was paralleled by changes in τ indicating that the rate of exocytosis was slowed by a factor of 2 (anti-SNAP 25) or 3 (anti-syntaxin). We finally estimated the maximum rates of exocytosis by calculating the time derivative ($\delta C/\delta t$) of the solution to equation 5. Under control conditions the peak rate was 740 ± 153 fF/s ($n=14$). This was decreased to 368 ± 99 fF/s ($n=5$; $P < 0.05$ vs control) and 294 ± 76 fF/s ($n=6$; $P < 0.05$ vs control) in the presence of the SNAP-25 and the syntaxin 1 antibodies respectively.

Changed Ca^{2+} -channel properties in the presence of anti-SNAP-25 and anti-syntaxin 1

To examine whether the effects on exocytosis of the antibodies were due to a reduction in Ca^{2+} -influx, depolarization-evoked Ca^{2+} -currents were measured in the absence and presence of anti-SNAP-25 and anti-syntaxin 1 respectively. The currents were elicited by 50-ms depolarizations from -70 mV to voltages between -40 and $+50$ mV. Figure 6A shows the

Ca^{2+} -current during a depolarization to zero mV under control conditions, in the presence of anti-SNAP-25 and in the presence of anti-syntaxin 1. The mean peak Ca^{2+} -current amplitude elicited by this pulse (Fig. 6B) under control conditions amounted to -69 ± 6 pA ($n=20$). In the presence of the SNAP-25 antibody, the peak amplitude was slightly reduced by 15% ($n=7$), whereas the syntaxin 1 antibody significantly reduced the current amplitude by 38% ($n=9$; $P < 0.05$). To estimate the activation properties of the current, equation 4 was approximated to the data points. Under control conditions, the values of G , k , V_r and V_h (see Materials and methods section) averaged 1.5 ± 0.1 nS, 8.7 ± 0.4 mV, 64 ± 3 mV and -11 ± 2 mV respectively. The SNAP-25 antibody reduced the whole-cell conductance G to 1.2 ± 0.1 ($n=7$; $P < 0.05$) and increased k to 10.5 ± 0.7 mV ($n=7$; $P < 0.02$). V_r and V_h were not affected by the presence of the antibody (not shown). In the presence of the syntaxin 1 antibody, G was reduced to 0.9 ± 0.1 nS ($n=9$; $P < 0.01$). The slope coefficient was similar to that in the presence of anti-SNAP-25 and amounted to 10 ± 1 mV ($n=9$; $P < 0.05$ vs control).

We next investigated whether it was possible to further reduce the Ca^{2+} -current using isradipine to block

Figure 5 Blockade of the SNARE-complex affects release kinetics. (A) Voltage-dependent currents (I) and changes in membrane capacitance (ΔC_m) evoked by a single depolarization (V) from -70 mV to 0 mV during either 30 ms, 150 ms or 450 ms under control conditions (a), in the presence of anti-syntaxin 1 (b) and in the presence of anti-SNAP-25 (c). (B) Mean capacitance increase (ΔC_m) as a function of the length of the depolarization (t) in control cells ($n=14$) and in cells where anti-SNAP-25 ($n=5$) or anti-syntaxin 1 ($n=6$) have been added by inclusion through the pipette solution. A solution of equation 5 have been approximated to the data points. Data are mean values $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$, anti-syntaxin 1 vs control and † $P<0.05$, †† $P<0.01$ and ††† $P<0.001$, anti-SNAP-25 vs control.

Table 1 Summary of the effects by anti-SNAP-25 and anti-syntaxin 1 on the kinetics of β -cell exocytosis. The values for τ and IRP were derived from approximating equation 5 to the data points in Fig. 5B. Data are mean values $\pm$ S.E.M. of the indicated number of cells tested (n)

Condition	τ (ms)	IRP (fF)	n
Control	16 ± 4	114 ± 23	14
Anti-SNAP-25	$36 \pm 12^*$	$42 \pm 5^{**}$	5
Anti-syntaxin 1	$51 \pm 7^{***}$	$27 \pm 7^{**}$	6

Statistical significances refer to comparisons within the same column: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs control.

the L-type Ca^{2+} -channel (Fig. 6C). In this series of six experiments, the peak Ca^{2+} -current, elicited by a 100 ms-pulse from -70 mV to 0 mV, measured in the presence of anti-syntaxin 1 alone amounted to -24 ± 4 pA. Application of $2 \mu\text{M}$ isradipine to the same cell significantly ($\sim 50\%$) reduced the mean peak current to -12 ± 4 pA ($P<0.01$). This is similar to the inhibition produced by isradipine in the absence of the antibody and it therefore appears that intracellular application of the syntaxin 1 antibody blocks L-type and non-L-type Ca^{2+} -channels to the same extent.

We evaluated the relationship between Ca^{2+} -entry and exocytosis under control conditions and in the presence of the anti-syntaxin 1 or anti-SNAP-25 antibodies (Fig. 6D). Under control conditions, there was a linear relationship between the integrated Ca^{2+} -current (Q_{Ca}) and the exocytotic response (capacitance increase; ΔC_m) between 1 and 8 pC. The slope of the relationship provides an estimate of the Ca^{2+} sensitivity of exocytosis and it amounted to 14 ± 3 fF/pC ($n=11$) under control conditions. In the presence of anti-syntaxin 1, Q_{Ca} was reduced but the slope of the ΔC_m - Q_{Ca} relationship was only marginally reduced for $1 \text{ pC} < Q_{\text{Ca}} < 4 \text{ pC}$ and amounted to 10 ± 2 fF/pC ($n=4$). By contrast, inclusion of the anti-SNAP-25 antibody in the pipette solution led to a reduction in the Ca^{2+} -sensitivity and the slope of the curve fell to 4 ± 1 fF/pC ($n=4$; $P<0.05$). Thus, it appears that whereas the effect of the anti-syntaxin-1 antibody on exocytosis can be almost entirely attributed to the reduction of Ca^{2+} -influx, the anti-SNAP-25 antibody interferes with exocytosis by a mechanism exerted subsequent to Ca^{2+} -influx and possibly via a direct effect on the exocytotic machinery.

It has been suggested that there exists a close connection between the α_{1c} Ca^{2+} -channel and the SNARE-proteins in the pancreatic β -cells (Wiser *et al.* 1999, Barg *et al.* 2001, 2002). In Fig. 6E, immunostaining of the SNARE-complex using the SNAP-25 antibody was performed in INS-1 cells transfected with EGFP-N1/ α_{1c} -DHPi. This plasmid contains the

Figure 6 Effects of the antibodies on the Ca^{2+} -current. (A) Inward Ca^{2+} -currents were evoked by 50 ms voltage-clamp depolarizations from -70 mV to 0 mV in the presence and absence of anti-SNAP-25 or anti-syntaxin 1. The traces shown were obtained by averaging the responses in 5 different cells in each group. (B) Peak current (I)–voltage (V) relationship of Ca^{2+} -currents recorded in the absence and presence of anti-SNAP-25 and syntaxin 1. The peak-current (I) was measured and plotted against the voltage (V). The Boltzmann equation (equation 4) was fitted to the data points. Data are mean values $\pm$ S.E.M. of 20 (control), 7 (anti-SNAP-25) and 9 (anti-syntaxin 1) experiments. * $P < 0.05$ and ** $P < 0.01$, anti-syntaxin vs control. (C) Ca^{2+} -currents evoked by 100 ms-depolarization from -70 mV to 0 mV in the continuous presence of anti-syntaxin 1 (applied intracellularly) and 2 min after addition of 2 μ M isradipine in the extracellular medium. (D) Relationship between Ca^{2+} -entry (integrated Ca^{2+} -current; Q_{Ca}) and exocytosis (ΔC_m) under control conditions and in the presence of anti-syntaxin 1 and anti-SNAP-25 antibodies. Data are mean values $\pm$ S.E.M. of the ΔC_m plotted against Q_{Ca} of 4–11 experiments. (E) SNAP-25 binding (green; a) in INS-1 cells overexpressing EGFP-N1/ $\alpha 1c$ -DHPi (red; b). Note co-localization (yellow; c) between SNAP-25 and the Ca^{2+} -channels subunit at some spots along the plasma membrane in the INS-1 cell that has been successfully transfected (within the dotted square and highlighted in d). Scale bar 5 μ m.

α_{1c} -subunit of the L-type Ca^{2+} -channel together with EGFP. It is clear that SNAP-25 immunoreactivity co-localizes with the α_{1c} subunit. Although this only shows co-localization of an overexpressed construct of α_{1c} with SNAP-25, this observation nevertheless reinforces the notion that the SNARE-complex and the α_{1c} -subunit of the Ca^{2+} -channel are capable of physical association.

Discussion

SNAP-25 and syntaxin 1 are key regulators of both synaptic transmission and regulated secretion (Lin & Scheller 2000, Bruns & Jahn 2002, Gerber & Sudhof 2002). Here we have examined the participation of these proteins in the exocytotic process of the insulin-secreting pancreatic β -cell by combining confocal immunocytochemistry with high-resolution capacitance measurements with intracellular application of antibodies against SNAP-25 and syntaxin 1. We present evidence that SNARE-proteins are required for a rapid exocytotic response, that they determine the kinetic properties of the release and, at least in the case of syntaxin-1, determine the Ca^{2+} -current amplitude.

SNAP-25 reduces rapid exocytosis

In the presence of the SNAP-25 antibody both IRP (Fig. 5B, Table 1) and RRP (Fig. 3B,C) are significantly reduced and the time constant of exocytosis (τ) is significantly increased (Table 1). These strong effects contrast with the weaker effect on mobilization of new granules into the RRP (Fig. 3D). This argues that SNAP-25 controls the speed of rapid exocytosis in pancreatic β -cells, a finding which is in accordance with the observation that mutation of SNAP-25 selectively interferes with rapid exocytosis in archetypal endocrine adrenal chromaffin cells (Xu *et al.* 1999, Sorensen *et al.* 2002). In the latter cells, the influence of SNAP-25 mutations on rapid exocytosis was interpreted in terms of the SNARE-complex formation being intimately linked to exocytosis and that mutations in the complex therefore culminate in a changed Ca^{2+} -dependence of exocytosis (Sorensen *et al.* 2002). The latter interpretation is supported by the present finding that the apparent Ca^{2+} -sensitivity of exocytosis was reduced by 70% in the presence of the anti-SNAP-25 antibody. A close association between Ca^{2+} -channel and exocytosis is also suggested by the finding that the SNAP-25 antibody not only reduces the exocytotic response (Figs 3 and 5), but also changes the activation properties of the Ca^{2+} -current (Fig. 6) indicating that SNAP-25 is indeed closely associated to the Ca^{2+} -channel and is necessary for proper channel function.

Interference with syntaxin 1 results in impaired exocytosis

It has been suggested that the N-terminal of syntaxin 1 has a specific role in Ca^{2+} -dependent exocytosis (Fernandez *et al.* 1998), since the 120-residue N-terminal domain is conserved in plasma membrane syntaxins and the C2A domain of the Ca^{2+} -sensor protein synaptotagmin has a binding site in this part of the protein. Our data indicate a strong reduction in IRP, RRP and mobilization after interference with the N-terminal part of syntaxin 1 (Figs 4 and 5). The fact that inhibition is equally strong at all levels of exocytosis suggests that syntaxin 1 is required for fusion of the LDCVs in the β -cell.

The reduction might, at least in part, be due to the reduced Ca^{2+} -influx observed in the presence of syntaxin 1 (Fig. 6A,B) since exocytosis in the pancreatic β -cells is strongly dependent on Ca^{2+} -influx (Barg *et al.* 2001). Indeed, our analysis of the Ca^{2+} -sensitivity of exocytosis indicates that the anti-syntaxin 1 antibody reduced the exocytotic responses in β -cells by reduction of the Ca^{2+} -current. The fact that the anti-SNAP-25 and anti-syntaxin 1 antibodies apparently inhibit exocytosis by distinct mechanisms reinforces the conclusion reached on the basis of the Fab-fragments (Fig. 4B-E) that the observed effects cannot be attributed to steric hindrance, but do indeed reflect the specific interaction with their respective SNARE-proteins. We considered that the 40% reduction in the peak amplitude by the syntaxin antibody (Fig. 6A,B) might be due to the selective inhibition of L-type Ca^{2+} -currents. However, when the L-type channel blocker isradipine was applied together with the syntaxin 1 antibody, the Ca^{2+} -current was reduced by 75% compared with control (Fig. 6B,C), which is more than the $\sim 50\%$ normally observed in the absence of the antibody (Schulla *et al.* 2003). This suggests that syntaxin 1 interacts promiscuously with the different types of Ca^{2+} -channels present in the β -cell and, if anything, affects non-L-type Ca^{2+} -channels stronger than L-type Ca^{2+} -channels. If this is the case, the reduction of late exocytosis during the trains may in part be attributed to the reduction of R-type Ca^{2+} -currents with resultant interference with granule mobilization, the latter process being strongly dependent on the function of these channels (Jing *et al.* 2005).

SNAP-25 and syntaxin 1 form clusters at the plasma membrane

Although the precise stoichiometry is unknown, it seems likely that several SNARE-complexes aggregate at the individual release sites (Lang *et al.* 2001). Clustering of SNARE-proteins in the plasma membrane has previously been demonstrated in the insulin-secreting cell line MIN6 (Ohara-Imaizumi *et al.* 2004a) and in primary rat β -cells (Ohara-Imaizumi *et al.* 2004b) as well as in

other cell types (Lang *et al.* 2001, Rickman *et al.* 2004). These syntaxin 1/SNAP-25 complexes have been demonstrated to represent sites of membrane fusion in MIN-6 cells and rat β -cells using total internal reflection fluorescence (TIRF) microscopy (Ohara-Imaizumi *et al.* 2004a,b). Interestingly, the number of clusters was reduced in β -cells from the diabetic GK rat. Here we verify the presence of clusters at the plasma membrane (Fig. 2). Our estimate of the diameter of a syntaxin/SNAP-25 cluster (320 nm) confirmed earlier observations (Ohara-Imaizumi *et al.* 2004a,b). The fact that two-thirds of the plasma membrane SNAP-25 and syntaxin 1 clusters do not co-localize suggests that the proteins may have functions in addition to exocytotic membrane fusion. Indeed, it has been reported that SNAP-25 controls voltage-dependent Kv2.1 Ca^{2+} -channels in insulin-secreting cells (MacDonald *et al.* 2002).

Pathophysiological relevance of SNAP-25 and syntaxin 1 in insulin secretion

Are these observations of pathophysiological relevance? It has been demonstrated in GK rats, a non-obese model of type-2 diabetes, that expression of both syntaxin 1 and SNAP-25 is reduced and partly responsible for impaired insulin secretion (Nagamatsu *et al.* 1999, Zhang *et al.* 2002). Our data indicate that interference with the proteins results in a reduction of both the rapid capacitive component, suggested to correspond to the first phase of insulin release (Eliasson *et al.* 1997), and the slower and more sustained capacitance increase reflecting the second phase, as well as the influx of Ca^{2+} through the L-type Ca^{2+} -channels necessary for initiating fusion of the insulin-containing LDCVs. Lack of the SNARE-proteins, or inhibition or impairment of their normal function would lead to reduced Ca^{2+} -influx and a decrease in or even absence of the first phase of insulin release. It is therefore important to consider SNAP-25 and syntaxin 1 in the aetiology of type 2 diabetes, a disease associated with the complete loss of the rapid first phase and substantial reduction in the second phase secretion.

Acknowledgements

We thank Kristina Borglid and Britt-Marie Nilsson for expert technical assistance. This work was supported by the Swedish Research Council (grant no. 13147 and 8647), The European Foundation for the Study of Diabetes (EFSD-Novo Nordisk), the Albert Pahlssons Foundation, the Swedish Diabetes Association, the Novo Nordisk Foundation, the Crafoordska Foundation, the Royal Physiographic Society in Lund, the Fredrik and Ingrid Thuring's Foundation, the Swedish Society for Medical Research (SSMF), The Diabetes programme at

Lund University (DPLU), the Göran Gustafsson Foundation for Research in the Natural Sciences and Medicine, the Swedish Strategic Foundation (SSF) and The Wellcome Trust. P R is a Royal Society Wolfson Research Fellow. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Asfari M, Janjic D, Meda P, Li G, Halban PA & Wollheim CB 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* **130** 167–178.
- Barg S, Renström E, Berggren PO, Bertorello A, Bokvist K, Braun M, Eliasson L, Holmes WE, Köhler M, Rorsman P *et al.* 1999 The stimulatory action of tolbutamide on Ca^{2+} -dependent exocytosis in pancreatic beta cells is mediated by a 65-kDa mdr-like P-glycoprotein. *PNAS* **96** 5539–5544.
- Barg S, Ma X, Eliasson L, Galvanovskis J, Göpel SO, Obermüller S, Platzer J, Renström E, Trus M, Atlas D *et al.* 2001 Fast exocytosis with few Ca^{2+} channels in insulin-secreting mouse pancreatic B cells. *Biophysical Journal* **81** 3308–3323.
- Barg S, Eliasson L, Renström E & Rorsman P 2002 A subset of 50 secretory granules in close contact with L-type Ca^{2+} channels accounts for first-phase insulin secretion in mouse beta-cells. *Diabetes* **51 Suppl 1** S74–S82.
- Bruns D & Jahn R 2002 Molecular determinants of exocytosis. *Pflügers Archives* **443** 333–338.
- Dotta F, Previti M, Neerman-Arbez M, Dionisi S, Cucinotta D, Lenti L, Di Mario U & Halban PA 1998 The GM2–1 ganglioside islet autoantigen in insulin-dependent diabetes mellitus is expressed in secretory granules and is not beta-cell specific. *Endocrinology* **139** 316–319.
- Eliasson L, Renström E, Ding WG, Proks P & Rorsman P 1997 Rapid ATP-dependent priming of secretory granules precedes Ca^{2+} -induced exocytosis in mouse pancreatic B-cells. *Journal of Physiology* **503** 399–412.
- Eliasson L, Ma X, Renström E, Barg S, Berggren PO, Galvanovskis J, Gromada J, Jing X, Lundquist I, Salehi A *et al.* 2003 SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic B-cells. *Journal of General Physiology* **121** 181–197.
- Fernandez I, Ubach J, Dulubova I, Zhang X, Sudhof TC & Rizo J 1998 Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell* **94** 841–849.
- Gentet IJ, Stuart GJ & Clements JD 2000 Direct measurement of specific membrane capacitance in neurons. *Biophysical Journal* **79** 314–320.
- Gerber SH & Sudhof TC 2002 Molecular determinants of regulated exocytosis. *Diabetes* **51 Suppl 1** S3–S11.
- Gonelle-Gispert C, Halban PA, Niemann H, Palmer M, Catsicas S & Sadoul K 1999 SNAP-25a and -25b isoforms are both expressed in insulin-secreting cells and can function in insulin secretion. *Biochemical Journal* **339** 159–165.
- Haynes LP, Morgan A & Burgoyne RD 1999 nSec-1 (munc-18) interacts with both primed and unprimed syntaxin 1A and associates in a dimeric complex on adrenal chromaffin granules. *Biochemical Journal* **342** 707–714.
- Jacobsson G, Bean AJ, Scheller RH, Juntti-Berggren L, Deeney JT, Berggren PO & Meister B 1994 Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *PNAS* **91** 12487–12491.
- Ji J, Yang SN, Huang X, Li X, Sheu L, Diamant N, Berggren PO & Gaisano HY 2002 Modulation of L-type Ca^{2+} channels by distinct domains within SNAP-25. *Diabetes* **51** 1425–1436.

- Jing X, Li DQ, Olofsson CS, Salehi A, Surve VV, Caballero J, Ivarsson R, Lundquist I, Pereverzev A, Schneider T *et al.* 2005 CaV2.3 calcium channels control second-phase insulin release. *Journal of Clinical Investigation* **115** 146–154.
- Kang Y, Huang X, Pasyk EA, Ji J, Holz GG, Wheeler MB, Tsushima RG & Gaisano HY 2002 Syntaxin-3 and syntaxin-1A inhibit L-type calcium channel activity, insulin biosynthesis and exocytosis in beta-cell lines. *Diabetologia* **45** 231–241.
- Lang J 1999 Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. *European Journal of Biochemistry* **259** 3–17.
- Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C & Jahn R 2001 SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO Journal* **20** 2202–2213.
- Lin RC & Scheller RH 2000 Mechanisms of synaptic vesicle exocytosis. *Annual Reviews in Cell Developmental Biology* **16** 19–49.
- Liu G, Dilmac N, Hilliard N & Hockerman GH 2003 Cav 1.3 is preferentially coupled to glucose-stimulated insulin secretion in the pancreatic beta-cell line INS-1. *Journal of Pharmacology and Experimental Therapeutics* **305** 271–278.
- MacDonald PE, Wang G, Tsuk S, Dodo C, Kang Y, Tang L, Wheeler MB, Catral MS, Lakey JR, Salapatek AM *et al.* 2002 Synaptosome-associated protein of 25 kilodaltons modulates Kv2.1 voltage-dependent K⁺ channels in neuroendocrine islet beta-cells through an interaction with the channel N terminus. *Molecular Endocrinology* **16** 2452–2461.
- Martin F, Moya F, Gutierrez LM, Reig JA & Soria B 1995 Role of syntaxin in mouse pancreatic beta cells. *Diabetologia* **38** 860–863.
- Martin F, Salinas E, Barahona F, Vazquez J, Soria B & Reig JA 1998 Engineered peptides corresponding to segments of the H3 domain of syntaxin inhibit insulin release both in intact and permeabilized mouse pancreatic beta cells. *Biochemical and Biophysical Research Communications* **248** 83–86.
- Nagamatsu S, Nakamichi Y, Yamamura C, Matsushima S, Watanabe T, Ozawa S, Furukawa H & Ishida H 1999 Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion. *Diabetes* **48** 2367–2373.
- Ohara-Imaizumi M, Nakamichi Y, Ozawa S, Katsuta H, Ishida H & Nagamatsu S 2001 Mastoparan stimulates GABA release from MIN6 cells: relationship between SNARE proteins and mastoparan action. *Biochemical and Biophysical Research Communications* **289** 1025–1030.
- Ohara-Imaizumi M, Nishiwaki C, Kikuta T, Kumakura K, Nakamichi Y & Nagamatsu S 2004a Site of docking and fusion of insulin secretory granules in live MIN6 beta cells analyzed by TAT-conjugated anti-syntaxin 1 antibody and total internal reflection fluorescence microscopy. *Journal of Biological Chemistry* **279** 8403–8408.
- Ohara-Imaizumi M, Nishiwaki C, Nakamichi Y, Kikuta T, Nagai S & Nagamatsu S 2004b Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy. *Diabetologia* **47** 2200–2207.
- Oho C, Seino S & Takahashi M 1995 Expression and complex formation of soluble N-ethyl-maleimide-sensitive factor attachment protein (SNAP) receptors in clonal rat endocrine cells. *Neuroscience Letters* **186** 208–210.
- Rickman C, Meunier FA, Binz T & Davletov B 2004 High affinity interaction of syntaxin and SNAP-25 on the plasma membrane is abolished by botulinum toxin E. *Journal of Biological Chemistry* **279** 644–651.
- Rorsman P & Renström E 2003 Insulin granule dynamics in pancreatic beta cells. *Diabetologia* **46** 1029–1045.
- Rorsman P, Eliasson L, Renström E, Gromada J, Barg S & Göpel S 2000 The cell physiology of biphasic insulin secretion. *News Physiology Science* **15** 72–77.
- Sadoul K, Lang J, Montecucco C, Weller U, Regazzi R, Catsicas S, Wollheim CB & Halban PA 1995 SNAP-25 is expressed in islets of Langerhans and is involved in insulin release. *Journal of Cell Biology* **128** 1019–1028.
- Schulla V, Renström E, Feil R, Feil S, Franklin I, Gjinovci A, Jing XJ, Laux D, Lundquist I, Magnuson MA *et al.* 2003 Impaired insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca²⁺ channel null mice. *EMBO Journal* **22** 3844–3854.
- Sørensen JB, Matti U, Wei SH, Nehring RB, Voets T, Ashery U, Binz T, Neher E & Rettig J 2002 The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. *PNAS* **99** 1627–1632.
- Tagaya M, Toyonaga S, Takahashi M, Yamamoto A, Fujiwara T, Akagawa K, Moriyama Y & Mizushima S 1995 Syntaxin 1 (HPC-1) is associated with chromaffin granules. *Journal of Biological Chemistry* **270** 15930–15933.
- Tagaya M, Genma T, Yamamoto A, Kozaki S & Mizushima S 1996 SNAP-25 is present on chromaffin granules and acts as a SNAP receptor. *FEBS Letters* **394** 83–86.
- Voets T, Neher E & Moser T 1999 Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. *Neuron* **23** 607–615.
- Walch-Solimena C, Blasi J, Edelmann L, Chapman ER, von Mollard GF & Jahn R 1995 The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *Journal of Cell Biology* **128** 637–645.
- Wheeler MB, Sheu L, Ghai M, Bouquillon A, Grondin G, Weller U, Beaudoin AR, Bennett MK, Trimble WS & Gaisano HY 1996 Characterization of SNARE protein expression in beta cell lines and pancreatic islets. *Endocrinology* **137** 1340–1348.
- Williams MA 1977 Quantitative methods in biology. In *Practical Methods in Electron Microscopy*, vol. 6, part II, pp 5–84. Ed. AM Glaupert. Amsterdam: Elsevier/North Holland.
- Wilson T 1995 The role of pinhole in confocal imaging system. In *Handbook of Biological Microscopy*, edn 2, pp 167–182. Ed JB Pawley. New York: Plenum Press.
- Wiser O, Trus M, Hernandez A, Renström E, Barg S, Rorsman P & Atlas D 1999 The voltage sensitive Lc-type Ca²⁺ channel is functionally coupled to the exocytotic machinery. *PNAS* **96** 248–253.
- Xu T, Binz T, Niemann H & Neher E 1998 Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity. *Nature Neuroscience* **1** 192–200.
- Xu T, Rammner B, Margittai M, Artalejo AR, Neher E & Jahn R 1999 Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. *Cell* **99** 713–722.
- Yang SN, Larsson O, Brannström R, Bertorello AM, Leibiger B, Leibiger IB, Moede T, Köhler M, Meister B & Berggren PO 1999 Syntaxin 1 interacts with the L(D) subtype of voltage-gated Ca²⁺ channels in pancreatic beta cells. *PNAS* **96** 10164–10169.
- Zhang W, Khan A, Östenson CG, Berggren PO, Efendic S & Meister B 2002 Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats. *Biochemical and Biophysical Research Communications* **291** 1038–1044.

Received in final form 25 January 2006

Accepted 17 February 2006