The first C₂ domain of synaptotagmin is required for exocytosis of insulin from pancreatic β -cells: action of synaptotagmin at low micromolar calcium

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The Ca²⁺- and phospholipid-binding protein synaptotagmin is involved in neuroexocytosis. Its precise role and Ca²⁺-affinity *in vivo* are unclear. We investigated its putative function in insulin secretion which is maximally stimulated by 10 μ M cytosolic free Ca²⁺. The well-characterized synaptotagmin isoforms I and II are present in pancreatic β -cell lines RINm5F, INS-1 and HIT-T15 as shown by Northern and Western blots. Subcellular fractionation and confocal microscopy revealed their presence mainly on insulin-containing secretory granules whereas only minor amounts were found on synaptic vesicle-like microvesicles. Antibodies or Fab-fragments directed against the Ca²⁺-dependent phospholipid binding site of the first C₂ domain of synaptotagmin I or II inhibited Ca²⁺-stimulated, but not GTP γ S-induced exocytosis from streptolysin-O-permeabilized INS-1 and HIT-T15 cells. Transient expression of wild-type synaptotagmin II did not alter exocytosis in HIT-T15 cells. However, mutations in the Ca²⁺-dependent phospholipid binding site of the first C₂ domain (Δ 180–183, D231S) again inhibited only Ca²⁺-, but not GTP γ S-evoked exocytosis. In contrast, mutations in the IP₄-binding sites of the second C₂ domain (Δ 325–341; K327,328,332Q) did not alter exocytosis. Synaptotagmin II mutated in both C₂ domains (Δ 180–183/K327,328,332Q) induced greater inhibition than mutant Δ 180–183, suggesting a discrete requirement for the second C₂ domain. Thus, synaptotagmin isoforms regulate exocytotic events occurring at low micromolar Ca²⁺.

Keywords: C₂ domain/cytosolic Ca²⁺/GTP/insulin exocytosis/synaptotagmin

Introduction

The secretion of insulin proceeds by exocytosis, i.e. fusion between secretory granules and plasma membranes with subsequent release of the granule content into the extracellular space (Wollheim *et al.*, 1996). Exposure of β -cells to secretagogues increases the cytosolic Ca²⁺ to micromolar levels, which in turn triggers insulin secretion

(Theler *et al.*, 1992; Ammala *et al.*, 1993). How Ca²⁺ triggers exocytosis in pancreatic β -cells is still not identified. In neuroexocytosis, a pivotal role for the Ca²⁺- and phospholipid-binding protein synaptotagmin has been demonstrated (Sudhof, 1995). Currently, 11 isoforms of synaptotagmin are known in rodents (Geppert *et al.*, 1991; Hilbush and Morgan, 1994; Mizuta *et al.*, 1994; Craxton and Goedert, 1995; Li *et al.*, 1995; Kwon *et al.*, 1996; Bability *et al.*, 1997). Antibody and peptide injections in PC12 cells provided early evidence for a role of synaptotagmin in exocytosis (Elferink *et al.*, 1993). The deletion mutation of one of these isoforms, i.e. synaptotagmin I, in transgenic mice or of *Drosophila* synaptotagmin, led to a major impairment of fast Ca²⁺-induced neuroexocytosis. Nonetheless, the precise role and Ca²⁺ affinity *in vivo* of synaptotagmin I are unknown (DiAntonio *et al.*, 1993; Broadie *et al.*, 1994; DiAntonio and Schwarz, 1994; Geppert *et al.*, 1994).

Synaptotagmin is an integral membrane protein of synaptic vesicles (Brose *et al.*, 1992) and of neurohormone-containing secretory vesicles (Walch Solimena *et al.*, 1993). It is composed of a short intravesicular N-terminal sequence, a single transmembrane region and a large cytosolic portion (Sudhof, 1995). The main features of the cytosolic part are two Ca²⁺-binding repeats, the C₂ domains, whose basic motif was first identified in protein kinase C and has subsequently been found in several other proteins. They mediate Ca²⁺-induced attachment to membrane lipids (Brose *et al.*, 1992). Several functions have been assigned to the C₂ domains in synaptotagmin. The first C₂ domain (C₂A) binds to phospholipids at ~5 μ M free Ca²⁺ (Brose *et al.*, 1992; Li *et al.*, 1995; Fukuda *et al.*, 1996) and to the membrane protein syntaxin 1 in the presence of several hundred micromolar Ca²⁺ (Li *et al.*, 1995). The latter Ca²⁺ requirement coincides with that for exocytosis in bipolar neurones (Heidelberger *et al.*, 1994). In addition to Ca²⁺-sensitive properties, the second C₂ domain (C₂B) binds inositol-1,3,4,5-tetrakisphosphate (IP₄) independent of Ca²⁺ (Fukuda *et al.*, 1994). The injection of IP₄ into a preterminal of the squid giant synapse inhibited neuroexocytosis. This effect can be prevented by concomitant injection of an antibody directed against the C₂B domain (Llinas *et al.*, 1994; Fukuda *et al.*, 1995b). Thus, an IP₄-binding site in the C₂B domain is seemingly required in neuroexocytosis and may mediate attachment to membrane lipids (Fukuda *et al.*, 1995b).

Endocrine exocytosis, such as that of insulin in the pancreatic β -cells, is maximally stimulated by 7–10 μ M free Ca²⁺ with an EC₅₀ of 1.6 μ M (Vallar *et al.*, 1987; Ullrich *et al.*, 1990; Bokvist *et al.*, 1995; Proks *et al.*, 1996). β -Cells therefore provide a good model to test the action of synaptotagmin domains *in situ* at low micromolar Ca²⁺. Although the occurrence of several synaptotagmin isoforms has been described in pancreatic β -cells at the

Fig. 1. RNA blot analysis of synaptotagmin I and II mRNAs in insulin-secreting β -cell lines. 30 μ g of total RNA from whole rat brain or indicated cell lines and primary islet cells were denatured and electrophoresed as described in the text. Upper panel, expression of synaptotagmin I RNA; Lower panel, expression of synaptotagmin II RNA. For autoradiography, the nylon membrane was exposed to X-ray film with an intensifying screen at -80°C for 3 days. Arrowheads indicate the position of 18S and 28S RNA.

RNA level, their subcellular localization is unresolved (Mizuta *et al.*, 1994; Wheeler *et al.*, 1996). This is of crucial importance as insulin-secreting cells contain not only insulin-containing secretory granules, but also small synaptic vesicle-like microvesicles (SLMVs) containing GABA (Reetz *et al.*, 1991). We have therefore investigated the subcellular distribution of two well-characterized isoforms of synaptotagmin, i.e. synaptotagmin I and II, and characterized their putative function. Our findings demonstrate that synaptotagmin I and II are mainly localized on secretory granules. Domains essential for Ca^{2+} -mediated phospholipid binding in the C_2A domain of synaptotagmin I and II are required for endocrine exocytosis of insulins operating at low micromolar Ca^{2+} .

Results

We first investigated the expression and subcellular distribution of synaptotagmin I and II. RNA blot analysis for synaptotagmin I and II revealed the presence of a 4.8 kb transcript for the synaptotagmin I in rat brain RNA and RNA from the insulin-secreting cells RINm5F, HIT-T15 and INS-1 as well as primary islet cells (Figure 1, upper panel). Similarly, a 7 kb band was detected in blots probed for synaptotagmin II (Figure 1, lower panel) and, in addition, a minor transcript migrating at ~ 9 kb was apparent. The abundance of mRNAs for synaptotagmin I and II was less in insulin-secreting cells as compared with rat.

Western blot demonstrated the presence of the corresponding proteins in crude membrane preparations (Figure 2). Monoclonal antibodies directed against the C_2A domain (mab 41.1) or against the intravesicular N-terminus of synaptotagmin I (mab 604) both detected a band of ~ 60 kDa in the three cell lines. Furthermore, two other antibodies recognizing synaptotagmin I and II (mab 48

Fig. 2. Immunoblot analysis of synaptotagmin I and II in insulin-secreting β -cell lines. (A) Crude membranes (30 μ g) from rat brain (1) or the indicated insulin-secreting cell lines RINm5F (2), HIT-T15 (3) or INS-1 (4) were separated on 12% SDS-PAGE, electrotransferred to PVDF and probed with antibodies against the first C_2 -domain (C_2A) of synaptotagmin I (mab 41.1) or of synaptotagmin I and II (anti-sytI/II), against synaptotagmin I and II (mab 48) or against the intravesicular N-terminus of synaptotagmin I (mab 604) or synaptotagmin II (mab 8G2b). (B) Crude membranes (5 μ g) from rat brain (5), 200 μ g of Triton X-100 extract from crude HIT-T15 membranes (6) and 20 or 50 μ g of enriched HIT-T15 insulin granules (7 and 8) were separated by 12% SDS-PAGE, electrotransferred to PVDF and probed with antibody against synaptotagmin II (mab 8G2b).

and anti-sytI/II) gave positive signals. In contrast, a monoclonal antibody directed against the N-terminus of synaptotagmin II (mab 8G2b) recognized a 60 kDa protein only in RINm5F and INS-1 cells, derived from a rat insulinoma, but not in the hamster HIT-T15 cells. Inter-species variations in the synaptotagmin II sequence can be excluded as mab 8G2b strongly reacted with a 60 kDa band in immunoblots from hamster brain (data not shown). We therefore prepared secretory granule membranes and detergent extracts of crude membranes from HIT-T15 cells. In those preparations synaptotagmin II is clearly detectable. This isoform is therefore also expressed in this cell line, albeit at lower levels than in the other insulin-secreting cells.

Insulin-secreting cells contain two types of exocytotic vesicles: small synaptic-like microvesicles, SLMVs (Reetz *et al.*, 1991) and insulin-containing granules. Exocytotic vesicle proteins may be localized on both of them (Regazzi *et al.*, 1995). Therefore, subcellular fractionation was performed on INS-1 cells using a continuous sucrose gradient to distinguish between the two vesicle types. Using this method, insulin-containing granules migrated at ~ 1.4 M sucrose (Figure 3, lower panel) as indicated by the distribution of immunoreactive insulin in fractions 12–15. The distribution of the SLMVs is indicated by the vesicle protein synaptophysin, which is recovered at

Fig. 3. Subcellular distribution of synaptotagmin I and II in INS-1 cells. INS-1 cells were homogenized by nitrogen cavitation and centrifuged through a continuous sucrose gradient. Lower panel: distribution of synaptophysin (SVP38, ●) and insulin (○) as measured by immunoblots with subsequent densitometry or by RIA. Upper panel: distribution of synaptotagmin I (SYT I; mab 41.1) and synaptotagmin I and II (anti-sytI/II).

~0.8 M sucrose in fractions 5 and 6 (Figure 3, middle panel). The distribution of synaptotagmin I and II was tested with mab 41.1, directed against synaptotagmin I, and with anti-sytI/II, which recognizes synaptotagmin I and II. As indicated in Figure 3 (upper panel), immunoreactivity for synaptotagmin I and II was mainly concentrated in the fractions 12–15 containing secretory granules. In contrast, only a small amount of immunoreactivity was found in the synaptophysin-reactive fractions 5 and 6 containing SLMVs. In addition, some synaptotagmin immunoreactivity sedimented in a region previously identified as containing plasma (Lang *et al.*, 1995).

The subcellular distribution of synaptotagmin in INS-1 cells was further examined by histocytochemistry and confocal microscopy (Figure 4). The upper panel demonstrates that insulin and synaptophysin—markers for secretory granules and synaptic-like microvesicles, respectively—do not co-localize. As already observed in subcellular fractionation, synaptotagmin does stain synaptophysin-positive structures (Figure 4). A different situation was encountered in the co-staining for insulin (green) and synaptotagmin (red) (Figure 4B). Synaptotagmin was found in a granular pattern and outlining the cell surface. Most of the immunoreactivity for insulin co-localizes with synaptotagmin, as indicated by the yellow stain. A similar co-localization between synaptotagmin and insulin, but not with synaptophysin, was observed in HIT-T15 cells (data not shown). Immunocytochemistry on pancreatic islets extended previous observations for synaptotagmin I (Jacobsson *et al.*, 1994) to synaptotagmin II. Immunoreactivities for both isoforms were absent from primary β -cells but were found on non-insulin endocrine islet cells (data not shown).

The above results suggested that synaptotagmin I and II are concentrated on insulin-containing secretory granules. We have therefore studied whether they could play a functional role in exocytosis, the final step of insulin secretion. To this end we initially used two antibodies which bind to defined regions of synaptotagmin. Affinity-purified anti-sytI/II binds to the first C₂ domain (C₂A) of synaptotagmin and inhibits Ca²⁺-dependent interaction of synaptotagmin II with phospholipid vesicles (Fukuda *et al.*, 1995a; Mikoshiba *et al.*, 1995). The monoclonal antibody mab 41.1 has been shown to require a short stretch of nine amino acids in the C₂A domain of synaptotagmin I for binding (Chapman and Jahn, 1994) and these amino acids are required for Ca²⁺-dependent interaction with phospholipid vesicles (Chapman and Jahn, 1994; Fukuda *et al.*, 1996). In the upper panel of Figure 5, the isoform specificity of these two antibodies is illustrated from experiments with recombinant fusion proteins. Both antibodies interacted with the first C₂ domain (sytl-C₂A), but not with the second C₂ domain of synaptotagmin I (sytl-C₂B). Anti-sytI/II reacted equally well with synaptotagmin II, but none of the two antibodies recognized synaptotagmin III, synaptotagmin IV or GST alone, the tag of the fusion protein. To test the functional activity of the antibodies, INS-1 or HIT-T15 cells were permeabilized with streptolysin-O (SL-O) and subsequently preincubated at 0.1 μ M Ca²⁺ with the indicated concentration of affinity-purified IgG (anti-sytI/II) or Fab-fragments of mab 41.1 (Fab 41.1) as shown in Figure 5 (lower panel). After aspiration of antibody solutions, cells were exposed to basal levels of free Ca²⁺ (0.1 μ M), or maximally stimulatory levels of Ca²⁺ (10 μ M) as previously demonstrated for β -cells and derived cell lines

Fig. 4. Immunofluorescence of synaptotagmin in insulin-secreting INS-1 cells. INS-1 cells were fixed, permeabilized and subsequently incubated with anti-synaptophysin (1:400), anti-sytI/II (1:10 000) or anti-insulin (1:400). After processing with fluorescent second antibodies, pictures were taken by confocal microscopy at the mid-cellular level. (A) Anti-synaptophysin (green) and anti-insulin (red); (B) anti-insulin (green) and anti-synaptotagmin I/II (red); (C) anti-synaptophysin (green) and anti-synaptotagmin I/II (red). Left panels: immunofluorescence; right panels: phase contrast. Bars equal 5 μ m.

(Vallar *et al.*, 1987; Ullrich *et al.*, 1990; Ammala *et al.*, 1993; Bokvist *et al.*, 1995). Alternatively, cells were stimulated with GTP γ S. This stable GTP analogue induces Ca^{2+} -independent exocytosis (Vallar *et al.*, 1987; Jonas

et al., 1994; Kiraly-Borri *et al.*, 1996). The difference between insulin release at basal Ca^{2+} conditions and at stimulatory conditions (10 μ M Ca^{2+} , 100 μ M GTP γ S) in the absence of antibodies was normalized to 100%. In

both cell lines, HIT-T15 and INS-1, the Fab-fragment and the affinity-purified IgG inhibited Ca^{2+} -evoked insulin release by ~60%, whereas the GTP γ S-induced release remained unchanged (Figure 5, lower panel). In contrast to the inhibitory action of native Fab 41.1 or anti-sytI/II IgG, preheated preparations were inactive (Table I). Furthermore, control IgG or Fab did not alter insulin release, and a monoclonal antibody against the intravesicular N-terminus, which is located inside the secretory granule, did not affect hormone levels. A possible effect of Fab 41.1 or anti-sytI/II IgG on the cell surface could be excluded as Fab fragments or IgG were inactive in

cells permeabilized by *Staphylococcus aureus* α -toxin (Table I). This toxin produces only small pores in the plasma membrane which do not allow the entry of molecules larger than 0.5–2 kDa. The inefficacy of the specific antibodies in α -toxin-permeabilized cells also excludes any contribution by antibody buffers to the inhibitory effect observed in SL-O-permeabilized cells.

These results suggest a specific role for the C₂A domain of synaptotagmin I in Ca^{2+} -stimulated exocytosis from insulin-secreting cells. As the functional antibody anti-sytI/II reacted with both isoforms—that is, synaptotagmin I and II—we used a separate approach to evaluate further the role of synaptotagmin II in the secretion of insulin. In HIT-T15 cells we transiently expressed wild-type synaptotagmin II or synaptotagmin II mutated in the C₂ domains. To determine the subcellular localization of transiently expressed synaptotagmin II, we analysed its distribution by confocal microscopy. Thus, we took advantage of the finding that only low endogenous levels of synaptotagmin II are present in HIT-T15 cells (see also Figure 2). Therefore, a monoclonal antibody could be used that is directed against the N-terminus (Nishiki *et al.*, 1996) which is highly isoform-specific (Li *et al.*, 1995). At the dilution used, the antibody did not react with endogenous synaptotagmin II (data not shown). Transiently expressed synaptotagmin II again co-localized largely with secretory granules as indicated by the co-distribution of insulin (Figure 6). In addition, synaptotagmin immunoreactivity was observed on the cell border (Figure 6) similar to endogenous synaptotagmin in INS-1 cells (see Figure 4). To determine whether this localization corresponds indeed to the plasma membrane, we used double staining with anti-synaptotagmin II and an antibody against syntaxin, a known plasma membrane protein. As syntaxin and synaptotagmin are differentially distributed, the rim-like stain observed with anti-synaptotagmin II corresponds probably not to localization at the plasma membrane, but to a subplasmalemmal region (Figure 6). All mutants used in this study exhibited a similar subcellular distribution (data not shown). To determine the levels of overexpressed protein, cells were co-transfected with a plasmid encoding the green fluorescent protein (GFP). This allows subsequent purification of the small population of the transiently transfected cells (~8–13% of all cells) by fluorescence-activated cell sorting. As shown in the upper

Fig. 5. Effect of functional anti-synaptotagmin antibodies on insulin release from permeabilized INS-1 and HIT-T15 cells. Upper panel: reactivity of antibodies against recombinant proteins. Recombinant GST (GST), first (sytI-C₂A) or second C₂ domain (sytI-C₂B) or both C₂ domains of synaptotagmin II, III and IV (100 ng/lane) were separated by SDS-PAGE, immunoblotted and incubated with mab 41.1 (left part) or anti-sytII/II (right part). Lower panel: insulin-secreting cells (INS-1, ■, □; HIT-T15, ●, ○) were permeabilized with streptolysin-O, preincubated at 0.1 μM Ca^{2+} with the indicated concentrations of Fab-fragments of mab 41.1 (Fab 41.1) or affinity-purified polyclonal IgG against the C₂A domain of synaptotagmin II (anti-sytII/II). Subsequently, supernatants were aspirated and replaced by antibody-free solution containing 0.1 μM , 10 μM Ca^{2+} or 100 μM GTP γ S. After 7 min, supernatants were sampled and prepared for the determination of released insulin by radioimmunoassay. The Ca^{2+} - or GTP γ S-induced stimulation of insulin release in the absence of IgG or Fab was normalized to 100%. For fold-increase upon stimulation see Table I. $n = 6$ –21 from at least three separate experiments for each point; *, $2P < 0.05$.

Table I. Effect of IgG or Fab-fragments on exocytosis in SL-O- or α -toxin-permeabilized cells

Preincubation	SL-O permeabilization			α -toxin permeabilization		
	0.1 μM Ca^{2+}	GTP γ S	10 μM Ca^{2+}	0.1 μM Ca^{2+}	GTP γ S	10 μM Ca^{2+}
Control	100 $\pm$ 23	256 $\pm$ 45	375 $\pm$ 28	100 $\pm$ 5	375 $\pm$ 12	621 $\pm$ 43
Anti-sytI/II	112 $\pm$ 7	248 $\pm$ 41	231 $\pm$ 17*	98 $\pm$ 12	405 $\pm$ 56	604 $\pm$ 56
Anti-sytI/II heated	93 $\pm$ 30	n.d.	398 $\pm$ 21	—	—	—
IgG	105 $\pm$ 12	289 $\pm$ 58	345 $\pm$ 9	114 $\pm$ 17	371 $\pm$ 4	658 $\pm$ 13
Fab 41.1	97 $\pm$ 28	234 $\pm$ 66	217 $\pm$ 28*	103 $\pm$ 11	352 $\pm$ 31	599 $\pm$ 25
Fab 41.1 heated	114 $\pm$ 6	n.d.	405 $\pm$ 13	—	—	—
Fab	89 $\pm$ 17	265 $\pm$ 39	402 $\pm$ 5	105 $\pm$ 9	368 $\pm$ 8	683 $\pm$ 38
Mab604.4	132 $\pm$ 31	276 $\pm$ 7	398 $\pm$ 31	121 $\pm$ 21	389 $\pm$ 24	595 $\pm$ 37

Cells were permeabilized with streptolysin-O or with α -toxin and pretreated with IgG (anti-sytIIC2A, mab 604.4, non-specific IgG, 40 $\mu\text{g}/\text{ml}$) or Fab-fragments (Fab 41.1, non-specific Fab, 40 $\mu\text{g}/\text{ml}$) as in Figure 6. Heat inactivation of antibodies was performed for 20 min at 60°C. Cells were subsequently exposed to 0.1 μM Ca^{2+} , 0.1 μM Ca^{2+} with 100 μM GTP γ S (GTP γ S) or 10 μM Ca^{2+} for 7 min and insulin release determined in the supernatants. Values are expressed as percent of controls (0.1 μM Ca^{2+} in the absence of IgG or Fab) and means $\pm$ SEM are given for $n = 6$ –9 from three separate experiments; *, $2P < 0.05$; n.d., not determined.

Fig. 6. Localization of transiently expressed synaptotagmin II in HIT-T15 cells. Upper panel: immunostaining of transiently transfected HIT-T15 cells for synaptotagmin II (red) and insulin (green). Lower panel: immunostaining for transiently expressed synaptotagmin II (red) and for syntaxin 1 (green). The monoclonal antibody against synaptotagmin II was used at a dilution (1:800) which did not stain endogenous synaptotagmin II. A similar distribution was found for the different synaptotagmin mutants used in this study (data not shown). Bars equal 5 μ m.

panel of Figure 7, again using a monoclonal antibody specific for synaptotagmin II (Nishiki *et al.*, 1996), wild-type protein and its different mutants were expressed at comparable levels.

To study the role of synaptotagmin II in secretion and exocytosis, the GFP-encoding plasmid was replaced by a construct expressing human preproinsulin, and human insulin C-peptide release was measured from hamster HIT-T15 cells (Figure 7, lower panel). This approach reliably reflects release from co-transfected cells as published previously (Lang *et al.*, 1995). Wild-type synaptotagmin II (wt) did not alter basal insulin release from intact cells or hormone release induced by stimulation due to depolarization of membrane potential by KCl leading to Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Wollheim and Sharp, 1981; Wollheim *et al.*, 1996). Deletion mutation $\Delta 180$ –183 and the point mutation D231S in the C_2A domain of synaptotagmin II have been shown to inhibit Ca^{2+} -dependent phospholipid binding (Fukuda *et al.*, 1996). Here they significantly reduced stimulated insulin secretion by some 50% and 35%, respectively (Figure 7, lower panel). Two mutations in the C_2B domain, i.e. $\Delta 325$ –341 or the exchange of K326,327,332, to Q (KQ), abolishes the binding of IP_4 and other inositol high-polyphosphates to synaptotagmin II (Fukuda *et al.*, 1995a), a process which is thought to

mimic binding of the C_2B domain of synaptotagmin to membranes (Llinas *et al.*, 1994; Mochida *et al.*, 1997). In our hands these mutants did not alter the secretion of insulin. However, a double mutant (DM) containing $\Delta 180$ –183 and KQ mutations of the C_2A and C_2B domains inhibited the release to a much larger extent than any mutation in the C_2A domain alone (Figure 7). To study whether the observed effects of synaptotagmin II mutants on secretion occur at the level of exocytosis, or occur indirectly, we also measured their effect in SL-O-permeabilized cells. As shown in Figure 8, under this condition the effects of all tested constructs were comparable with those observed in intact cells. Similar to the effect of the functional antibodies, again only the Ca^{2+} -, but not the GTP γ S-stimulated exocytosis, was affected.

Discussion

Exocytosis from pancreatic β -cells shares a number of features with exocytosis in neurones and neuroendocrine cells. We and others have previously shown that the release of insulin involves essential components of the general docking and fusion machinery. The process is ATP-dependent (Vallar *et al.*, 1987; Lang *et al.*, 1995) and requires the SNARE proteins synaptobrevin/VAMP (Regazzi *et al.*, 1995, 1996), SNAP-25 (Sadoul *et al.*,

Fig. 7. Effect of transiently expressed mutants of synaptotagmin II on insulin release from intact insulin-secreting HIT-T15 cells. Upper panel: HIT-T15 cells transiently expressing synaptotagmin II wild-type or mutants were purified by fluorescence-activated cell sorting using co-transfected green fluorescent protein. Control cells and fluorescent cells were solubilized in SDS-sample buffer and subjected to Western blot analysis (6×10^4 cells/lane) using an antibody specific for synaptotagmin II (mab 8G2b). Lower panel: HIT-T15 cells were co-transfected with plasmid encoding for human preproinsulin and control plasmid (pcDNA3, CON) or pcDNA3 containing the inserts for synaptotagmin II wild-type (WT), mutants in the first C_2 domain ($\Delta 180-183$; D231S), in the second C_2 domain ($\Delta 325-341$; KQ, where K327,328 and 332 were exchanged for Q) or in both domains (double mutant DM carrying the mutations $\Delta 180-183$ and the exchange of K327,328,332 to Q). Secretion experiments were performed 48–72 h later using Krebs–Ringer buffer with 3.4 mM KCl (BASAL) or with 50 mM KCl (50 mM KCl). Subsequently, the amount of human insulin C-peptide in the supernatants was measured reflecting the secretion from co-transfected cells only. $n = 12-24$ from at least three separate experiments for each point; *, $2P < 0.05$.

1995), syntaxin (Martin *et al.*, 1995, 1996) and α -SNAP (Király-Borri *et al.*, 1996). Insulin release is maximal at low micromolar free Ca^{2+} (Vallar *et al.*, 1987; Ullrich *et al.*, 1990; Bokvist *et al.*, 1995; Proks *et al.*, 1996). Therefore, insulin-secreting cells provide a suitable system to test the Ca^{2+} -dependency of putative Ca^{2+} -sensing proteins *in vivo*.

Here, we demonstrate that the neuronal Ca^{2+} -sensing proteins synaptotagmin I and II (Geppert *et al.*, 1991, 1994), are expressed in pancreatic β -cell lines: they are mainly localized on secretory granules and are implicated in the exocytosis of insulin. As already suggested by the first report on synaptotagmin I (Matthew *et al.*, 1981), expression is low as compared with neuronal or neuroendocrine cells and may explain previously reported negative findings (Jacobsson *et al.*, 1994; Mizuta *et al.*, 1994; Wheeler *et al.*, 1996). This scarcity may reflect the lower number of insulin-containing granules in β -cells (Dean, 1973; Asfari *et al.*, 1992) as compared with exocytotic vesicles in chromaffin cells (Burgoyne *et al.*, 1982). The subcellular distribution of synaptotagmin I and II is remarkable. The pancreatic β -cells contain two types of exocytotic vesicles, i.e. the insulin granule and SLMVs. The latter are characterized by the presence of synapto-

Fig. 8. Effect of transiently expressed mutants of synaptotagmin II on exocytosis of insulin from permeabilized HIT-T15 cells. Cells were transfected with the same constructs as in Figure 6. Cells were permeabilized 48–72 h later with recombinant streptolysin-O. Cells were subsequently exposed for 7 min to 0.1 μM Ca^{2+} , 100 μM $GTP\gamma S$ or 10 μM Ca^{2+} and human insulin C-peptide measured from the supernatants. $n = 10-27$ from at least three separate experiments for each point; *, $2P < 0.05$.

physin and thought to release neurotransmitters (Reetz *et al.*, 1991). This differential distribution of synaptotagmin I and II on insulin-containing granules cannot be explained by different numbers of vesicles or different membrane surfaces. Indeed, the synaptic vesicle protein synaptobrevin/VAMP-2 is distributed equally between the two vesicle types (Regazzi *et al.*, 1995). The location of synaptotagmin I on peptide hormone vesicles had been previously established in neuroendocrine cells, although its relative distribution was not compared with that on other vesicle types (Walch Solimena *et al.*, 1993). Our findings suggest either the presence of other synaptotagmin isoforms on SMLVs—for example synaptotagmin III (Mizuta *et al.*, 1994)—or the possibility that only a fraction of these synaptophysin-positive microvesicles undergoes Ca^{2+} -dependent exocytosis. In addition to their vesicular localization, synaptotagmin I and II were also distributed on the plasma membrane, as reported for chromaffin cells (Matthew *et al.*, 1981) and neurones (Takahashi *et al.*, 1991). In neurones, the appearance of synaptotagmin on the cellular surface is linked to stimulation of exocytosis (Matteoli *et al.*, 1992), which is followed by rapid endocytosis. As the secretory rate is lower in pancreatic β -cells and exo- and endocytosis are less tightly coupled when compared with neurones (Bokvist *et al.*, 1995; Proks *et al.*, 1996), the presence of synaptotagmin on plasma membranes most probably reflects a slower turnover of the protein, thereby leading to its prolonged persistence.

The function of synaptotagmin I has been extensively studied in neuroexocytosis (Sudhof, 1995), which requires levels of free Ca^{2+} of one to two orders of magnitude higher than those observed during the exocytosis of insulin (Heidelberger *et al.*, 1994; Heinemann *et al.*, 1994). Mice and *Drosophila* exhibit a lethal phenotype after mutation of synaptotagmin I or *Drosophila* syt gene and selective impairment of Ca^{2+} -dependent neurotransmitter release

(DiAntonio *et al.*, 1993; Broadie *et al.*, 1994; DiAntonio and Schwarz, 1994; Geppert *et al.*, 1994). In neuroendocrine PC12 cells the injection of Fab-fragments directed against synaptotagmin I inhibits regulated exocytosis (Elferink *et al.*, 1993). In conjunction with the known biochemical characteristics of synaptotagmin I (Chapman and Jahn, 1994; Li *et al.*, 1995; Sudhof, 1995; Sugita *et al.*, 1996), these reports demonstrate that this protein may serve as an exocytotic Ca^{2+} -sensor. Our study has addressed the role of Ca^{2+} -dependent and -independent phospholipid binding to C_2A or C_2B domains in endocrine exocytosis by the use of functional antibodies and mutants of synaptotagmin II. Both antibodies have previously been shown to inhibit Ca^{2+} -dependent phospholipid binding and neuroexocytosis from squid giant synapse (Mikoshiba *et al.*, 1995) and chromaffin cells (Ohara Imaizumi *et al.*, 1997) or to interact with an epitope on synaptotagmin required for this function (Chapman and Jahn, 1994). The inhibition observed here was specific for the antibodies and selective for Ca^{2+} -dependent exocytosis not affecting stimulation with $\text{GTP}\gamma\text{S}$. Similar results were obtained with mutations of synaptotagmin II C_2A domain deficient in Ca^{2+} -dependent phospholipid binding ($\Delta 181\text{--}183$, D231S) (Fukuda *et al.*, 1996), which produced a dominant negative phenotype on overexpression in HIT-T15 cells. The epitope recognized by Fab 41.1 and the deletion mutation SytII $\Delta 181\text{--}183$ probably do not interfere with binding of synaptotagmin to syntaxin. Fab 41.1 binding to synaptotagmin I requires the amino acids 177–185 in synaptotagmin I (Chapman and Jahn, 1994), corresponding to amino acids 178–186 in synaptotagmin II (Geppert *et al.*, 1991). The deletion mutant synaptotagmin I $\Delta 177\text{--}185$ no longer binds the antibody, whereas Ca^{2+} -dependent binding to syntaxin is not altered (Chapman *et al.*, 1995). Our functional data thus clearly demonstrate that phospholipid binding by the C_2A domain previously found in biochemical assays is required for Ca^{2+} -stimulated endocrine exocytosis. This provides an indication for the role of synaptotagmin II. The Ca^{2+} -dependency of this phospholipid-binding step as characterized with recombinant or native proteins coincides well with the concentration range of free Ca^{2+} occurring in endocrine exocytosis, i.e. between 1 and 10 μM (Vallar *et al.*, 1987; Ullrich *et al.*, 1990; Bokvist *et al.*, 1995; Proks *et al.*, 1996).

It is still unclear whether all the biochemically characterized activities of synaptotagmin participate in exocytosis. A sequential model has been proposed (Chapman *et al.*, 1996) to account for the described high and low Ca^{2+} affinities on the one hand (phospholipid binding, oligomerization and syntaxin binding) and the Ca^{2+} requirements of exocytosis as in retinal bipolar neurones on the other (Heidelberger *et al.*, 1994). It has been suggested that synaptotagmin binding to syntaxin may serve for colocalization of fusion sites with Ca^{2+} entry (Leveque *et al.*, 1994). The Ca^{2+} levels described for effective interaction with syntaxin (0.1–1 mM) do not occur in pancreatic β cells and derived cell lines (Theler *et al.*, 1992; Bokvist *et al.*, 1995). Therefore, either still unknown additional factors may drive this interaction in the β -cell, or the characteristics of syntaxin–synaptotagmin interaction as observed *in vitro* may not be relevant in endocrine exocytosis. Indeed, electrophysiological studies performed with secretion assays suggest that secretory granules are

less tightly coupled to Ca^{2+} entry sites in β -cells than in neuronal systems (Bokvist *et al.*, 1995). Our results suggest that two synaptotagmin isoforms, synaptotagmin I and II, fulfil rather similar roles in insulin exocytosis in terms of Ca^{2+} -dependent phospholipid binding by the C_2A domain.

In contrast to the role of the C_2A domain, we did not find clear evidence for the function of the IP_4 -binding site of the C_2B domain in endocrine exocytosis. As demonstrated by genetic analysis in *Drosophila*, the C_2B domain is necessary for correct function of synaptotagmin (DiAntonio and Schwarz, 1994). In addition, a role for the IP_4 -binding site in C_2B has previously been indicated by the injection of peptides (Bommert *et al.*, 1993) or inositol high-polyphosphates (Llinas *et al.*, 1994; Fukuda *et al.*, 1995b) into squid giant presynaptic terminals. However, more detailed investigation suggested that the main function of the IP_4 -binding site in the C_2B domain involves recycling via endocytosis as shown in superior cervical ganglion cells (Mochida *et al.*, 1997). Our negative results are therefore not surprising in view of the differences between synaptic vesicle and secretory granule biogenesis (Bauerfeind and Huttner, 1993). It should also be pointed out that our biochemical assay of exocytosis does not discern rapid kinetic changes.

Exocytosis of insulin can be triggered reversibly by either Ca^{2+} or in a Ca^{2+} -independent fashion by GTP and its more stable analogue $\text{GTP}\gamma\text{S}$ (Vallar *et al.*, 1987). Both stimuli are temperature- and ATP-dependent (Vallar *et al.*, 1987; Lang *et al.*, 1995), but differ in their kinetics and requirements for cytosolic factors (Jonas *et al.*, 1994; Kiraly-Borri *et al.*, 1996; Proks *et al.*, 1996). The selective alteration of Ca^{2+} -triggered exocytosis found here underscores the specificity of the methods employed and adds to the previously described differences between the two stimuli. As activation of a GTP-binding protein is required for Ca^{2+} -stimulated exocytosis of insulin (Vallar *et al.*, 1987; Proks *et al.*, 1996), the GTPase may intervene between the Ca^{2+} targets, such as synaptotagmin, and membrane fusion itself. However, more detailed studies are clearly required to elucidate the exact organization of the two intertwining stimulatory pathways in exocytosis.

In conclusion, our data demonstrate that synaptotagmin function is implicated in endocrine exocytosis, a process being maximal at 7–10 μM Ca^{2+} . Furthermore, this involves the well-known capacity of synaptotagmin to bind phospholipids. This characterizes synaptotagmin as a Ca^{2+} sensor in regulated endocrine exocytosis from the pancreatic β -cells.

Materials and methods

Materials

Recombinant streptolysin-O (SL-O) was produced in *Escherichia coli*. The cDNAs encoding for the C_2A or the C_2B domain of synaptotagmin I (Elferink *et al.*, 1993) were kindly provided by Dr R.Scheller (Stanford, USA). The cloning of synaptotagmin II and the construction of the mutants used have been described previously (Fukuda *et al.*, 1995a). The following monoclonal antibodies were generously donated to us: mab 604.1 and mab or Fab 41.1 (Dr R.Jahn, New Haven, CT), mab 8G2b (Dr M.Takahashi, Tokyo, Japan) and mab 48 (Dr L.Reichardt, San Francisco, CA).

Cell culture, transient co-transfection and FACS

Insulin-secreting cell lines (HIT-T15 and INS-1E) were cultured as described previously (Asfari *et al.*, 1992; Lang *et al.*, 1995). INS-1E

cells were cultured on microwells coated with fibroblast matrix (ELDAN-Tech, Jerusalem, Israel) to ensure persistent cell attachment during the permeabilization with streptolysin-O. For transient co-transfection all constructs have been subcloned into pCDNA3 (Invitrogen, San Diego, CA). HIT-T15 cells were transiently co-transfected as published (Lang *et al.*, 1995) except for the use of 1 μ l of Transfectam and 5 μ g of plasmid per well (2.5 μ g pHINS encoding for human insulin, 1.25 μ g pCDNA3 and 1.25 μ g of synaptotagmin-encoding plasmids). For fluorescence-activated cell sorting, pHINS was replaced by green fluorescent protein (GFP65T) kindly provided by Dr Cormack (San Francisco, CA). Two days after transfection, cells were detached by trypsinization, resuspended in medium/2% FCS and purified by fluorescence-activated cell sorting (Facstar+).

Cell permeabilization

Preparation of intracellular buffers and permeabilization with streptolysin-O were as described (Lang *et al.*, 1995, 1997). IgGs or Fabs were added to the permeabilized cells only during 9 min preincubation in intracellular buffer at 0.1 μ M free Ca^{2+} . Subsequently this solution was exchanged for intracellular buffer containing 0.1 μ M free Ca^{2+} , 10 μ M free Ca^{2+} or 0.1 μ M free Ca^{2+} and 100 μ M $\text{GTP}\gamma\text{S}$ (Boehringer, Rotkreuz, Switzerland). After 7 min of stimulation, supernatants were removed, centrifuged and their content of insulin or human C-peptide determined by radioimmunoassay (Lang *et al.*, 1995, 1997) or ELISA (DAKO, Zug, Switzerland), respectively.

Northern blots

Total RNA was extracted using guanidine thiocyanate (Gibco, Basel, Switzerland). For RNA transfer blots, 30 μ g of total RNA were denatured with formaldehyde, separated by electrophoresis in a 1% agarose gel and transferred to nylon membranes. The blots were probed with *HindIII*-*KpnI* or *BamHI*-*KpnI* fragments of synaptotagmin I and II, respectively, encompassing the nucleotides encoding the N-terminus up to the first half of the C_2A domain. Hybridizations were carried out in 1 M NaCl/1% SDS/30% dextran overnight at 55°C and subsequently washed in 3 $\times$ SSC/1% SDS for 30 min at 50–60°C with several changes of washing solution before autoradiography for 72 h at –80°C.

Subcellular fractionation of INS1-E cells and immunoblotting

Small synaptic-like microvesicles (SLMV) and insulin-containing secretory granules were separated as described previously (Lang *et al.*, 1995; Regazzi *et al.*, 1995) according to the method of Reetz *et al.* (1991). Crude membrane preparations from insulin-secreting cell lines were obtained by sonication of cells in homogenization buffer (5 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EGTA, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin) and subsequent centrifugation at 140 000 g for 60 min. SDS-PAGE was performed as described (Lang *et al.*, 1995) and primary antibodies were used at the following dilutions: affinity-purified polyclonal anti-synaptotagmin I/II C_2A 1 ng/ml (Fukuda *et al.*, 1995a), anti-synaptotagmin I C_2A mab41.1. 0.3 μ g/ml (Chapman and Jahn, 1994), anti-synaptotagmin N-terminus mab 604.4 1:2000 (Chapman and Jahn, 1994), anti-synaptotagmin I mab 48 1:6000 (Matthew *et al.*, 1981), anti-synaptotagmin II N-terminus mab 8G2b 1:8000 (Nishiki *et al.*, 1996), and anti-synaptophysin 1:5000 (Boehringer, Rotkreuz, Switzerland). Binding of horseradish peroxidase-coupled second antibodies were revealed by the use of enhanced chemiluminescence (Pierce, Oud Beijerland, The Netherlands).

Immunofluorescence

Cells grown on coverslips were washed twice with phosphate-buffered saline (PBS), fixed for 10 min in PBS–4% paraformaldehyde, washed twice again for 5 min in PBS and permeabilized for 30 min with PBS containing 0.1% Saponin (Sigma, Buchs, Switzerland) and 5% BSA. Primary antibodies were added for 1 h, cells were washed three times for 5 min with PBS–1% BSA, and incubated with secondary antibodies in PBS–1% BSA for 1 h. After three washes with PBS for 5 min cells were mounted on glass slides using Vectastain (Vector Lab., Burlingame, USA). Confocal microscopy was performed with a Zeiss Axiophot fluorescence microscope and an LSM confocal scanner (Zurich, Switzerland). Primary antibodies were diluted as indicated in the figure legends. Fluorophore-labelled secondary antibodies (Pierce) were used at a dilution of 1:100.

Statistical analysis

Results are presented as mean $\pm$ SEM from experiments performed independently on at least three different cell preparations, unless stated otherwise. Statistical analysis was performed by Student's two-tailed *t*-test for unpaired data (2P).

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