Synaptotagmins bind calcium to release insulin

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Gauthier BR, Wollheim CB. Synaptotagmins bind calcium to release insulin. Am J Physiol Endocrinol Metab 295: E1279–E1286, 2008. First published August 19, 2008; doi:10.1152/ajpendo.90568.2008.—Plasma insulin levels are determined mainly by the rate of exocytosis of the insulin-containing large dense core vesicles (LDCVs) of pancreatic islet β-cells. This process involves the recruitment of LDCVs to the plasma membrane, where they are docked by the assembly of multiprotein SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes. However, fusion of the two membranes will proceed only in the presence of Ca2+ ions, implicating a Ca2+ sensor protein. The synaptotagmin gene family, comprising 15 members, was proposed to act as such Ca2+ sensor in regulated exocytosis in neurons and neuroendocrine and endocrine cells. Herein, we review the physiological function of the various synaptotagmins with reference to their impact on insulin exocytosis. Cumulating evidence emphasizes the crucial role of synaptotagmin VII and IX as mediators of glucose-induced insulin secretion.

β-cells; islet; insulin secretion; β-cell lines; SNARE complex; large dense core vesicles; diabetes

THE ISLETS OF LANGERHANS play a central role in blood glucose homeostasis. Glycaemia is lowered by insulin, secreted from the islet β-cells (83), whereas the α-cell secretory product, glucagon raises blood glucose (27). The hormones are stored in secretory granules (LDCVs) which are recruited to the plasma membrane and secreted by regulated exocytosis (48, 62). By necessity, insulin secretion is tightly regulated to maintain blood glucose within a very narrow range thereby avoiding hypoglycaemia between meals and during exercise. Dysregulation of insulin secretion is causally related to the development of type 2 diabetes (76, 78). This may be manifested as hypo-secretion and failure to adapt to increased insulin demands in obesity, usually associated with resistance to the hormone, or as hyper-secretion initiating the disease (79). Under physiological conditions, insulin is released in response to increases of plasma concentrations of glucose, fatty acids and certain amino acids (54–56). Gastrointestinal hormones (GLP-1 and GIP) are secreted during food intake and potentiate insulin secretion (14) whereas neurotransmitters are either stimulatory (acetylcholine) or inhibitory (norepinephrine and epinephrine) (3). All of these as well as other factors control the secretory process either by the generation of intracellular messengers or through direct actions on the exocytotic process (41, 54, 83). This review will focus on the control of β-cell exocytosis, with special reference to recent advances in our understanding of how the calcium binding protein synaptotagmin is implicated in this process.

Glossary

cAMP-GEFII cAMP-regulated guanine nucleotide exchange factor II
Epac2 Exchange protein directly activated by cAMP 2
GAP GTPase-activating protein
GDI GDP dissociation inhibitor
GEP Guanine nucleotide exchange protein
GIP Gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide
GLP-1 Glucagon-like peptide-1
LDCV Large dense core vesicle
NSF N-ethylmaleimide-sensitive factor
Noc2 No C2 domain
PIF2 Phosphatidylinositol 4,5-bisphosphate
Rim Rab3-interacting molecule
SNAP Soluble NSF attachment protein
SNP Single nucleotide polymorphism
SNARE Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
t-SNARE Target membrane SNARE
v-SNARE Vesicle SNARE
SYT Synaptotagmin
VAMP-2 Vesicle-associated membrane protein-2

Spatiotemporal Aspects of Insulin Granule Secretion

Insulin secretion in response to glucose and leucine is biphasic: A rapid transient first phase is followed by a slowly increasing and sustained second phase (82). It is generally believed that the first phase corresponds at least in part to the membrane capacitance-defined readily releasable pool of insulin granules already docked to the membrane. This pool rep-
resides less than 1% of the 10,000 total insulin granules in a native β-cell. The second phase comprises granules from the reserve pool either already docked to the plasma membrane or recruited from the cell interior (62). However, recently, newly recruited insulin granules have also been shown to undergo exocytosis without obvious prior docking to the plasma membrane even during the first phase period (39). Irrespective of these two modalities, granule recruitment is accomplished through the action of conventional kinesin that together with myosin Va moves the granules along the microtubules to the membrane (74, 75). This process requires ATP and is favoured by glucose through provision of energy, but not by a simple increase in cytosolic Ca2+ (74, 80). Calcium on the other hand is implicated in reshaping the dense micro filamentous cell web (cortical actin) by stimulating the actin severing protein, gelsolin thereby facilitating the access of the granules to the plasma membrane (72).

Presenting the Players of Exocytosis

The main trigger to exocytosis is an increase in cytosolic calcium [Ca2+]c. In the β-cell, glucose metabolism promotes ATP generation by the mitochondria (80), resulting in the closure of ATP-sensitive K+ channels (KATP) membrane depolarization and opening of voltage-dependent Ca2+ channels (6, 62). Calcium acts as ligand for Ca2+-binding proteins such as calmodulin and synaptotagmins (SYTs). In addition, calmodulin-dependent protein kinase and calcineurin, a Ca2+-activated phosphatase, have also been implicated in the regulation of β-cell exocytosis (16, 61). cAMP generated by glucose (40) as well as by GLP-1 and GIP (14) potentiates the actions of Ca2+ on distal events in insulin secretion. Its actions are mediated via cAMP-dependent protein kinase and by allosteric activation of Epac2/cAMP-GEFII located at the site of exocytosis (58, 66).

As in neurotransmitter exocytosis, formation of a SNARE complex appears to be necessary for docking of the insulin-containing secretory granule to the plasma membrane and the subsequent fusion of the two membranes (Fig. 1A). The interaction of the main secretory granule v-SNARE VAMP-2/synaptobrevin 2, with the two plasma membrane t-SNAREs syntaxin-1 and SNAP-25, generates the SNARE fusion complex (Fig. 1B). Several SNARE complexes may participate in the fusion of a single vesicle, leading to the formation of the exocytotic pore and melting of the lipid bilayer (Fig. 1C) (66). A rise in [Ca2+]c is required for multiple steps in the exocytotic process, including fusion of the granule and plasma membranes. The soluble SNAP proteins constitute the receptors on the SNARE complex for NSF, an ATPase that catalyzes the disassembly of the extremely stable complex (Fig. 1, B and C). Of the many accessory proteins that have been implicated in the regulation of SNARE complex function, complexin binding to the complex in stoichiometry appears essential for normal exocytosis (for review see Ref. 81), including that of insulin (1). SNAPIN, which binds to SNAP-25, is also a putative determinant of NSF-mediated fusion (25). The role of Munc18 has been more extensively studied. It dissociates from syntaxin-1 to allow formation of the SNARE complex. It has also recently been demonstrated that insulin-containing secretory granule docking involves the binding of granuphilin to Munc18, resulting in a preliminary, not yet fusion-competent state (71). While Munc18 is required for insulin exocytosis, granuphilin acting as a clamp restraining the process is in fact dispensable. Granuphilin-null mice have increased exocytosis and are hypoglycemic (39). Tomosin is also a ligand for Munc18 and appears to act after the granule docking step. Its overexpression or suppression has, however, yielded contrasting results in terms of insulin secretion (10, 86).
SYNAPTOTAGMINS AND INSULIN EXOCYTOSIS

Non-Islet Biological Actions Of SYT Isoforms

A wealth of investigations has defined SYT I and SYT II as the most important calcium sensors of rapid neurotransmitter release (for review see Ref. 67). Indeed, disruption of the synaptotagmin 1 gene in the mouse selectively abolishes the initial rapid phase of exocytosis for example in hippocampal synapses (25) and in chromaffin cells (30). Similarly, Ca\(^{2+}\)-triggered neurotransmitter release was impaired in striatal neurons as well as in neuromuscular junctions of SYT II-deficient mice (59). These two SYT isoforms also participate in endocytosis (SYT I) (38) and intracellular trafficking of endosomes (SYT II) (60). It was reported that endocytosis is regulated by SYT I in PC12 cells through the distal part of the cytoplasmic tail as well as by the AP2-binding C\(_{2}\)B domain (Fig. 2) (60). Calcium-mediated lysosomal insertion into the plasma membrane has been shown to be the fundamental process of wound healing in many cell types. This involves SYT VII, which spans the lysosomal membrane where it interacts with the \(v\)-SNARE vamp-7. At the plasma membrane, SYT VII forms complexes with the t-SNAREs syntaxin 4 and SNAP-23 (4). The phenotype of SYT VII-null mice is characterized by impaired wound healing, even leading to autoimmune muscle
inflammatory disease (8). SYT VII also seems to participate in phagosome-lysosome fusion that is essential for intracellular bacterial killing (63). The same isoform also regulates bone formation and resorption, as illustrated by osteopenia in SYT VII-null mice (87). Another exocytotic process, the insulin-stimulated translocation of the glucose transporter GLUT4 to the plasma membrane in skeletal muscle and adipocytes, is also defective in SYT VII-deficient mice (46). Apart from a report on the localization of SYT I on the secretory granules of parotid gland cells, no information is available on the function of SYTs in the regulation of Ca^{2+}-induced secretion in exocrine cells (43). This differs from endocrine secretion in that several cell systems exhibit SYT-dependent exocytosis. In neuroendocrine chromaffin cells, both SYT I and SYT VII have been implicated in LDCV exocytosis (30, 65). Additionally, SYT IX and VII, but not SYT I, mediate Ca^{2+} in exocytosis in chromaffin-derived PC12 cells (17, 19). Whether SYT V also localizes to LDCVs in PC12 cells and participates in exocytosis remains unclear (64). This research field is evolving rapidly, and the involvement of given SYTs in these and other biological processes awaits further studies.

Of the SYT isoforms that modulate insulin secretion in islets and in derived cell lines, only SYT isoforms with putative biological function in insulin-secreting cells will be considered in the following sections (Table 1).

**SYT I and SYT II**

SYT I and II were initially located in nerve endings within the rat islet and to somatostatin-secreting δ-cells in the islet periphery (37). In addition, rat insulinoma RINm5F cells were also stained positive. The authors employed a non-isoform-specific antibody probably reacting mainly with SYT I. The exclusive expression of SYT I in δ-cells of the islets has since been established (7). Clonal β-cell lines, including rat insulinoma INS-1 cells, promiscuously express SYT I and II. Both isoforms were shown to be implicated in Ca^{2+} but not GTPγS- (activator of G proteins) induced insulin secretion in hamster HIT-T15 and INS-1 cells (42). These results were later corroborated by RNA interference directed against SYT I in INS-1 cells. In addition, silencing of SYT I markedly reduced the fast component of endocytosis as assessed by patch-clamp recordings (84). These findings suggest that SYT I can regulate as well exocytosis as endocytosis of LDCVs reminiscent of its actions in synaptic vesicle exocytosis and recycling in nerve endings. There are several publications concurring with the absence of SYT I and II in native primary β-cells (7, 23, 42).

**SYT III**

The expression and function of SYT III in pancreatic β-cells has been controversial. Three independent studies localized SYT III to secretory granules of primary β-cells and clonal Min6, βTC3, RINm5F, and HIT-T15 cells (7, 23, 51). Functional evidence for its implication in Ca^{2+}-stimulated insulin secretion was obtained in permeabilized preparations of the cell lines or by measuring capacitance by the patch clamp technique. A fourth study confirmed SYT III expression in Min6, HIT-T15, and RINm5F cells (29). However, the isoform was not present in primary rat β-cells or in INS-1 cells but rather in islet δ-cells. Furthermore, rather than targeting to granules (7, 23, 51), transient transfections of primary β-cells caused plasma membrane localization of SYT III (29). Consistent with the lack of SYT III expression in native β-cells, a recombinant C2AB domain peptide did not compete with endogenous SYTs to inhibit calcium-induced exocytosis in permeabilized cells (29). Further studies should elucidate the putative role of SYT III in somatostatin secretion.

**SYT IV**

Two independent studies have found that the SYT IV transcript is expressed in clonal β-cell lines as well as in islet cells.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Islet Cell Type</th>
<th>Cell Line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYT I δ</td>
<td>RINm5F, INS-1, HIT-T15 and βTC3</td>
<td>(37)</td>
<td></td>
</tr>
<tr>
<td>SYT II</td>
<td>RINm5F, INS-1, HIT-T15 and βTC3</td>
<td>(23, 42)</td>
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<tr>
<td>SYT III β δ</td>
<td>RINm5F, HIT-T15, βTC3 and Min6</td>
<td>(7, 23, 51)</td>
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<tr>
<td>SYT IV</td>
<td>RINm5F, INS-1, HIT-T15, βTC3 and Min6</td>
<td>(23, 29)</td>
<td></td>
</tr>
<tr>
<td>SYT V α</td>
<td>RINm5F, INS-1, HIT-T15, βTC3 and Min6</td>
<td>(33, 64)</td>
<td></td>
</tr>
<tr>
<td>SYT VI</td>
<td>RINm5F, INS-1, HIT-T15 and Min6</td>
<td>(29, 33)</td>
<td></td>
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<tr>
<td>SYT VII</td>
<td>RINm5F, INS-1, HIT-T15 and Min6</td>
<td>(23, 29)</td>
<td></td>
</tr>
<tr>
<td>SYT VIII</td>
<td>RINm5F, INS-1, INS-1E, HIT-T15 and Min6</td>
<td>(24, 29, 53)</td>
<td></td>
</tr>
<tr>
<td>SYT IX β</td>
<td>RINm5F, INS-1, HIT-T15, βTC3 and Min6</td>
<td>(23, 52)</td>
<td></td>
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<tr>
<td>SYT X–XII ND</td>
<td>INS-1E</td>
<td>(23, 33)*</td>
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<tr>
<td>SYT XIII–XV ND</td>
<td>ND</td>
<td>(15)</td>
<td></td>
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</table>

SYT V is the isoform initially reported by Li et al. (45); SYT IX represents the isoform characterized by either Craxton and Goedert (13) or Hudson and Birnbaum (31). ND, not determined. *Referred to as SYT V in the references.
SYT V and SYT IX

Two different SYT V sequences were reported simultaneously. The one cloned by Li et al. (45) should be referred to as SYT V, whereas the other sequence, published by Craxton and Goedert (13) as well as Hudson and Birnbaum (31), has been given the name SYT IX. Both SYT V and SYT IX were shown to be expressed in INS-1E cells and to localize to the secretory granules. RNA interference experiments of either SYT V or SYT IX showed inhibition of insulin secretion stimulated by glucose, whereas Ca$^{2+}$-independent secretion was not attenuated (33). Not surprisingly, the expression profile was different in primary islet cells: SYT V is restricted to glucagon-producing $\beta$-cells (33, 64) whereas SYT IX is predominantly expressed in $\beta$-cells (32, 33). Immunogold labeling confirmed the secretory granule localization of SYT IX in primary $\beta$-cells. Adenoviral-mediated siRNA repression of SYT IX resulted in selective inhibition of glucose- or tolbutamide-stimulated insulin secretion in rat islets (32). Interestingly, even though SYT IX protein was suppressed by more than 80%, calcium-mediated exocytosis was inhibited by only 50%, suggesting that other SYTs may be implicated in insulin secretion (32). It was demonstrated that, in contrast to SYT I, only the $C_2\alpha$ domain of SYT IX is implicated in Ca$^{2+}$-induced insulin exocytosis in clonal $\beta$-cells. Rather, the $C_2\beta$ domain appears to function in endocytosis (26). Although a SYT IX-null mouse has been reported, no information is yet available on islet physiology of these animals (85). Regarding the putative function of SYT V in $\alpha$-cells, future experiments should elucidate whether this isoform acts as the principal Ca$^{2+}$ sensor in glucagon secretion.

SYT VI

SYT VI is a noncanonical isoform suggested to be expressed in the endoplasmatic reticulum, Golgi complex, cytosol, and potentially at the plasma membrane of PC12 cells (20). Although transcript levels were detected in several $\beta$-cell lines as well as primary islets in one study (29), another study was negative in this regard (23). The presence of SYT VI protein therefore needs to be investigated in islet cells. However, it is unlikely that SYT VI is a main player in insulin exocytosis, as its recombinant $C_2\beta$ domain did not attenuate Ca$^{2+}$-stimulated insulin secretion (29).

SYT VII

We and others (21, 23, 29) have reported the expression of SYT VII in insulin-producing cell lines as well as in primary islets. Experiments in permeabilized primary $\beta$-cells showed that introduction of a recombinant peptide containing the $C_2$ domains of SYT VII inhibited Ca$^{2+}$-stimulated insulin release (29). Furthermore, overexpression of SYT VII in intact RINm5F cells caused amplification of carbachol-induced insulin secretion (23). Interestingly, SYT VII was found to produce several alternative splice variants derived mainly from within the linker domain (between the transmembrane and $C_2\alpha$ domains); multiple variants were expressed in various mouse, rat, and human tissues, including the pancreas (21, 69). More recently, a detailed analysis has revealed that only three splice variants, $\alpha$, $\beta$, and $\delta$, are specifically expressed in purified primary rat $\beta$-cells and INS-1E cells. Moreover, the protein was localized to insulin granules (24). This contrasts with another study, in which SYT VII was detected on endosomes rather than LDCVs of MIN6 and INS-1E cells (53). Spliced variant specific RNA interference and electrophysiological studies performed in INS-1E cells revealed that SYT VII$\alpha$ is indispensable for glucose-induced hormone secretion. Interestingly, suppression of SYT VII$\beta$ increased the hormone secretion, suggesting that this variant may interfere with one of the several steps in the exocytotic pathway. Preliminary data indicate that SYT VII$\beta$ may be implicated in endocytosis. It is noteworthy that the combined repression of SYT VII$\alpha$ and $\beta$ completely abolished glucose-induced hormone secretion (24). This strongly suggests that SYT VII is as important as SYT IX in glucose-stimulated exocytosis (33). Consistent with this notion, SYT VII and SYT IX were found to heterodimerize in PC12 cells upon Ca$^{2+}$ stimulation (19), indicating that the presence of both isoforms may be mandatory for exocytosis. Corroborating in vitro studies, SYT VII-null mutant mice exhibited impaired glucose tolerance and insulin secretion in vivo as well as attenuated secretion from isolated islets (28, 46). Mutant mice exhibited normal islet architecture. Furthermore, the islets had unaltered insulin content and displayed normal calcium responses to glucose, suggesting a defect in exocytosis beyond the Ca$^{2+}$ signal (28). Taken together, the in vivo and in vitro investigations clearly establish SYT VII as a crucial player in the regulation of insulin secretion.

SYT VIII

SYTV III is an atypical isoform that lacks calcium binding properties (52, 68). It is expressed in islets and insulin secreting cell lines (29, 52). SYT VIII is mainly cytosolic and only a minor proportion was localized to membrane containing structures in Min6 insulinoma cells like in PC12 cells. Consistent with the lack of calcium binding, SYT VIII did not translocate during calcium stimulated exocytosis (52), making its involvement in insulin secretion unlikely. The latter conclusion seems at variance with earlier findings in permeabilized $\beta$-cells demonstrating that recombinant SYT VIII $C_2\beta$ domain inhibited calcium-induced insulin exocytosis (29). However, this result merely indicates interference with the exocytotic process, which may suggest interaction with other functional synaptotagmin isoforms or other proteins of the secretory machinery.

Synaptotagmins and Their Putative Implication in Diabetes

To date, no mutations or polymorphisms in the synaptotagmin gene family have been directly associated with type 2 diabetes. However, the SYT IX transcript was shown to be decreased by $\sim$75% in postmortem islets of type 2 diabetic patients compared with islets isolated from nondiabetic specimens (57). Similarly, the same isoform was also reduced by $\sim$20% in intact rat islets cultured in the presence of 30 mM glucose for 48 h, so-called glucotoxicity conditions. The latter was also accompanied by a decrease in glucose-induced insulin secretion (73). In contrast, no clear-cut changes in SYT isoforms were reported for INS-1E cells after culture at 30 mM glucose for 48 h, so-called glucotoxicity conditions. The latter was also accompanied by a decrease in glucose-induced insulin secretion (73). In contrast, no clear-cut changes in SYT isoforms were reported for INS-1E cells after culture at 30 mM glucose for 48 h, so-called glucotoxicity conditions.
glucose (15). Irrespective of whether reduction in SYT IX is primary or secondary to high glucose, exocytosis in type 2 diabetic islets is impaired, leading to inefficient insulin release and thus aggravating the hyperglycemic state. Identification of SYT VII and SYT IX as the major Ca\(^{2+}\)-sensors implicated in insulin secretion should direct future genetic studies on identifying SNPs in these genes that may correlate with the disease.

Conclusion

More than a decade of investigations focusing on the expression and function of synaptotagmin isoforms in islet cells and derived cell lines has defined the clear participation of SYT VII and SYT IX in Ca\(^{2+}\)-regulated insulin exocytosis. The cross talk between these two synaptotagmins may determine the overall rate of this process (24, 32). A lesson to be learned from these studies is the imperative necessity to use native islet preparations rather than clonal cell lines, which tend to display mixed endocrine cellular phenotypes. Thus, the presence of SYT I in many clonal β-cell lines, not expressed in primary β-cells but detected in δ-cells, probably reflects the somatostatin-cell phenotype of the insulinomas. As new synaptotagmins are emerging (SYT X–XV), their possible involvement in Ca\(^{2+}\)-mediated insulin secretion should be investigated in future studies.

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