Synaptotagmins I and IX function redundantly in regulated exocytosis but not endocytosis in PC12 cells

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Summary

Synaptotagmin I is considered to be a Ca^{2+} sensor for fast vesicle exocytosis. Because Ca^{2+}-dependent vesicle exocytosis persists in synaptotagmin I mutants, there must be additional Ca^{2+} sensors. Multiple synaptotagmin isoforms co-reside on vesicles, which suggests that other isoforms complement synaptotagmin I function. We found that full downregulation of synaptotagmins I and IX, which co-reside on vesicles in PC12 cells, completely abolished Ca^{2+}-dependent vesicle exocytosis. By contrast, Ca^{2+}-dependent exocytosis persisted in cells expressing only synaptotagmin I or only synaptotagmin IX, which indicated a redundancy in function for these isoforms. Although either isoform was sufficient to confer Ca^{2+} regulation on vesicle exocytosis, synaptotagmins I and IX conferred faster and slower release rates, respectively, indicating that individual isoforms impart distinct kinetic properties to vesicle exocytosis. The downregulation of synaptotagmin I but not synaptotagmin IX impaired compensatory vesicle endocytosis, which revealed a lack of isoform redundancy and functional specialization of synaptotagmin I for endocytic retrieval.

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Key words: Synaptotagmin, Exocytosis, Membrane fusion, Ca^{2+} sensor, Dense-core vesicles

Introduction

The release of neurotransmitters from neurons and peptide hormones from endocrine cells is mediated by the Ca^{2+}-triggered fusion of secretory vesicles with the plasma membrane. Ca^{2+}-triggered vesicle exocytosis exhibits different Ca^{2+} sensitivities and latencies depending upon cell- and vesicle-type (Burgoyne and Morgan, 1998; Martin, 2003). This implies that Ca^{2+} sensors for vesicle exocytosis respond to a range of intracellular Ca^{2+} levels with a range of kinetic characteristics. Synaptotagmin I (Syt I), an abundant synaptic vesicle (SV) protein, was proposed to be a Ca^{2+} sensor for SV exocytosis when its molecular characterization revealed two tandem cytoplasmic C2 domains (Perin et al., 1991; Brose et al., 1992). The tandem Syt I C2A and Syt I C2B domains bind three and two Ca^{2+} ions, respectively, with low affinities (Rizo and Sudhof, 1998) that are enhanced by interactions with phosphatidylserine (PS) (Brose et al., 1992; Bai et al., 2002). Syt I also exhibits Ca^{2+}-stimulated interactions with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin-1A and SNAP-25 and with heterotrimeric complexes containing VAMP-2 (see Chapman, 2002). Ca^{2+}-dependent interactions with PS and SNARE complexes are thought to mediate the regulatory role of Syt I in Ca^{2+}-dependent vesicle exocytosis (Augustine, 2001; Chapman, 2002; Sudhof, 2002).

There is considerable evidence that Syt I functions as a Ca^{2+} sensor for rapid synchronous SV exocytosis (Augustine, 2001; Chapman, 2002; Sudhof, 2002). The R233Q Syt I mutant, which exhibits reduced Ca^{2+}-dependent PS and SNAP-25 binding (Fernandez-Chacon et al., 2001; Wang et al., 2003), confers reduced Ca^{2+}-dependent transmitter release probabilities in neurons and neuroendocrine cells (Fernandez-Chacon et al., 2001; Sorensen et al., 2003). Conversely, neurons harboring Syt I mutants with increased Ca^{2+} affinity for PS binding exhibit enhanced Ca^{2+} sensitivity for release (Rhee et al., 2005). Characterized Syt I mutants in *Drosophila* also exhibit altered Ca^{2+} dependence for transmitter release (Yoshihara et al., 2003). Recent direct in vitro studies also showed that a Syt I C2A-C2B protein confers Ca^{2+} regulation on SNARE-dependent membrane fusion reconstituted in proteoliposomes (Tucker et al., 2004).

There is, however, uncertainty about the role of additional proteins functioning as Ca^{2+} sensors for vesicle exocytosis. In synapses, two kinetic components of Ca^{2+}-triggered SV exocytosis were characterized as rapid synchronous and delayed asynchronous release (Goda and Stevens, 1994). In Syt I mouse mutants and *Drosophila* mutants, the rapid synchronous component of Ca^{2+}-dependent release is decreased, whereas the delayed asynchronous component persists or is enhanced (Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004; Nicholson-Tomishima and Ryan, 2004; Maximov and Sudhof, 2005). Ca^{2+}-dependent dense-core vesicle (DCV) exocytosis also persists in chromaffin cells from Syt I mutant mice but with a reduced rapid phase (Voets et al., 2001). Loss of Syt I had no effect on Ca^{2+}-triggered DCV exocytosis in neuroendocrine PC12 cells (Shoji-Kasai et al.,

1992; Fukuda et al., 2002). Overall, deletion of Syt I does not impair and may enhance slower kinetic phases of Ca^{2+}-dependent secretion. These findings indicate either that Syt I functions as a kinetic facilitator of Ca^{2+}-dependent exocytosis mediated by other Ca^{2+} sensors, or that Ca^{2+} sensors other than Syt I mediate slower asynchronous modes of release (Geppert et al., 1994; Yoshihara and Littleton, 2002; Koh and Bellen, 2003).

Additional Syt isoforms may function as Ca^{2+} sensors for vesicle exocytosis. Genes encoding at least 16 Syt isoforms have been identified (Craxton, 2001; Fukuda, 2003) and individual Syt isoform proteins exhibit distinct Ca^{2+}-dependent PS- and SNARE-binding interactions in vitro (Shin et al., 2002; Tucker et al., 2003). Because different Syt isoforms sense different ranges of [Ca^{2+}] and exhibit distinct kinetic properties, multiple Syt isoforms co-resident on vesicles may confer distinct Ca^{2+}-sensing and kinetic properties to vesicle fusion. Although Syt isoform overexpression alters the properties of Ca^{2+}-dependent vesicle fusion (Fukuda et al., 2004; Wang et al., 2005), the functional role of multiple endogenous Syt proteins co-residing on vesicles has not been determined.

Previous studies yielded conflicting results as to which Syt isoforms (I, III, V, VII or IX) function in Ca^{2+}-triggered DCV exocytosis in PC12 cells (Shoji-Kasai et al., 1992; Sugita et al., 2002; Saegusa et al., 2002; Fukuda et al., 2002; Fukuda, 2004; Wang et al., 2005; Moore et al., 2006). In these studies, the inhibition or downregulation of individual Syt isoforms did not fully inhibit Ca^{2+}-triggered DCV exocytosis. This could indicate that multiple Syt isoforms operate in parallel or redundantly, or that Ca^{2+} sensors other than Syts are involved.

Syt I and Syt IX are the two most abundant vesicle Syt isoforms in PC12 cells (Fukuda et al., 2002; Zhang et al., 2002; Tucker et al., 2003). We found that the full downregulation of Syt I and Syt IX resulted in a complete loss of Ca^{2+}-dependent DCV exocytosis. By contrast, Ca^{2+}-dependent exocytosis fully persisted in cells expressing either Syt I or Syt IX alone. Expression of Syt I or Syt IX alone conferred faster and slower release kinetics, respectively. The data indicate that co-resident vesicle Syts function with redundancy but confer distinct Ca^{2+}-dependent kinetic properties to vesicle fusion. By contrast, Syt I but not Syt IX was found to function selectively in the compensatory endocytosis of DCVs.

Results

Syt I and Syt IX co-reside on DCVs in PC12 cells

PC12 cells lacking Syt I exhibit normal Ca^{2+}-dependent exocytosis (Shoji-Kasai et al., 1992; Fukuda et al., 2002). This indicates either that Syt I is not essential for Ca^{2+}-triggered DCV exocytosis in PC12 cells or that another Ca^{2+} sensor functions redundantly with Syt I. Syt IX is expressed in PC12 cells at levels comparable to Syt I (Fukuda et al., 2002; Zhang et al., 2002; Tucker et al., 2003). If Syt IX were to compensate for the loss of Syt I function in DCV exocytosis, it would need to co-reside with Syt I on DCVs. To address this possibility, quantitative coimmunolocalization studies were conducted in wild-type PC12 cells with Syt I- and Syt IX-specific antibodies. Confocal *Z* sections were deconvolved and colocalization was quantified after thresholding images by measuring overlap in each optical section through cells. Immunoreactive Syt I and Syt IX exhibited punctate vesicular distributions that strongly colocalized (Fig. 1A and supplementary material Movie1). Quantitative analysis, corrected for random overlap, revealed that 84 ± 4 % of Syt I colocalized with Syt IX and 67 ± 6 % of Syt IX colocalized with Syt I (Fig. 1B). Thus, a majority of the DCVs that contained Syt I also contained Syt IX and only a small percentage of DCVs contained only Syt I or only Syt IX.

A line scan analysis was used to measure the Syt I and Syt IX immunofluorescence on DCVs (Fig. 1C,D). The narrower peaks of fluorescence (Fig. 1D, *) with half-widths (~290 nm) similar to those of 100-nm fluorescent beads (~220 nm) probably correspond to individual PC12-cell DCVs, which were estimated to be 74-158 nm in diameter (Schubert et al., 1980; Wagner, 1985). These individual DCVs (*) exhibited both Syt I and Syt IX immunofluorescence (Fig. 1D). Together with the high degree of colocalization, these data indicate that Syt I and Syt IX co-reside on DCVs and could function redundantly in Ca^{2+}-triggered DCV exocytosis.

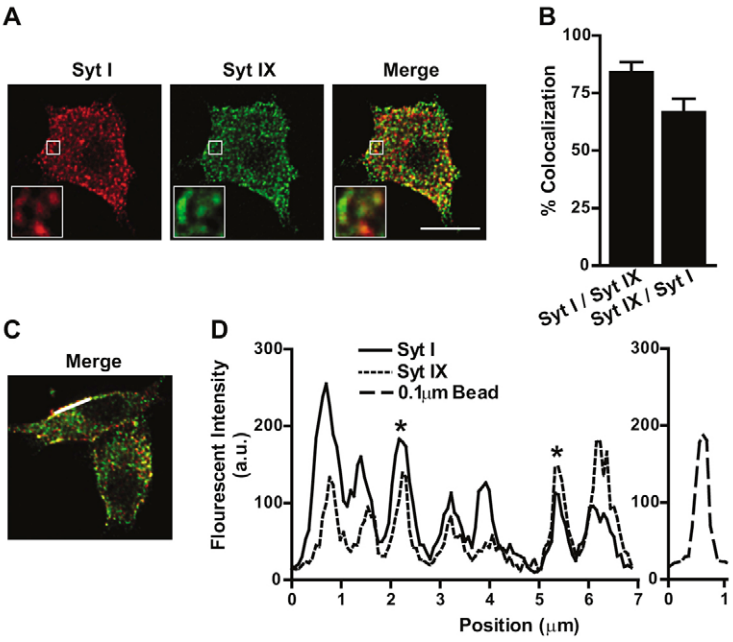

Fig. 1. Syt I and Syt IX colocalize on DCVs in PC12 cells. (A) PC12 cells were fixed and stained with Syt I- specific (red channel) and Syt IX-specific (green channel) antibodies and coimmunolocalization was determined (yellow). 3D reconstruction of deconvolved confocal sections is shown in supplementary material Movie 1. Bar, 5 μm; square insets are 1 μm×1 μm. (B) Syt I and Syt IX colocalization was quantified in deconvolved confocal *Z*-sections of 12 randomly selected PC12 cells. Bar graphs give the total percent overlap (corrected for random overlap) for Syt I with Syt IX and Syt IX with Syt I as the mean ± s.d. (C) Representative deconvolved *Z*-section of two PC12 cells showing colocalization (yellow) of Syt I (red channel) and Syt IX (green channel). A 7-μm line was drawn through the edge of one PC12 cell for line-scan analysis. (D) Fluorescence intensity, as arbitrary fluorescence units of Syt I and Syt IX along the 7-μm line shown in C. *, individual DCVs. Fluorescence intensity of a 0.1-μm bead is shown for size comparison.

The downregulation of Syt I and Syt IX abolishes Ca^{2+}-triggered DCV exocytosis

We determined whether Syt I and Syt IX function in Ca^{2+}-triggered DCV exocytosis by downregulating Syt isoforms using RNA interference. The high degree of sequence relatedness of Syt I and Syt IX enabled the design of short hairpin RNA (shRNA) constructs that target homologous regions of both (Fig. 2A). The designed Syt I/IX-shRNA plasmid fully downregulated Syt I and Syt IX in transient transfections (not shown). In addition, we isolated stably transfected PC12 cell lines in which Syt I and IX were not detected by western blot analysis (Fig. 2B). Syt VII and Syt IV, which are present at <3% and <1% of the level of Syt I in wild-type PC12 cells (Fukuda et al., 2002; Tucker et al., 2003), were completely unaffected by expression of the Syt I/IX-shRNA plasmid (Fig. 2B). Other exocytosis-related proteins, such as SNAP-25 (Fig. 2B), Rab 3 or syntaxin 1A (not shown) were expressed at wild-type levels in the cell lines lacking Syt I and Syt IX (Syt I/IX-null cell lines). As described below, we have characterized the secretory phenotype of the Syt I/IX-null cell lines. It should be noted that, in each case, we obtained the same results with several independent Syt I/IX-null cell lines or by employing transient Syt I/IX-shRNA plasmid expression. Moreover, re-expression of Syts in the Syt I/IX-null cells fully reversed any detected loss-of-function (see below). Thus, all of the secretory phenotypes described below can be attributed to Syt I and Syt IX downregulation rather than to secondary changes in the cell lines or off-target effects of the shRNA.

Because we found that loading of DCVs with catecholamine is altered by Syt downregulation (see Discussion), we used a human growth hormone (hGH) secretion assay to assess Ca^{2+}-dependent DCV exocytosis. hGH expressed by plasmid transfection in PC12 cells is properly targeted to DCVs (Schweitzer and Paddock, 1990). hGH secretion from cells in basal or depolarizing high $[K^+]$ medium was determined and the difference between the two values taken as Ca^{2+}-dependent hGH release. Compared to wild-type PC12 cells, Syt I/IX-null cells exhibited a complete loss (93.2% decrease) of Ca^{2+}-dependent hGH secretion (Fig. 2C). The Syt I/IX-null cells were incapable of secreting hGH in response to Ca^{2+} influx over the entire stimulation period (Fig. 2D). The same results were obtained using three independent Syt I/IX-null cell lines and in transient transfection assays with Syt I/IX-shRNA. This is the first example in which elimination of Syt isoforms resulted in a full loss of Ca^{2+}-dependent vesicle exocytosis. The results indicate that Syt I or Syt IX or both are essential for Ca^{2+}-dependent DCV exocytosis in PC12 cells and comprise the sole Ca^{2+} sensors for regulated DCV exocytosis in these cells.

When compared with wild-type cells, the Syt I/IX-null cells were found to exhibit higher levels of hGH secretion under

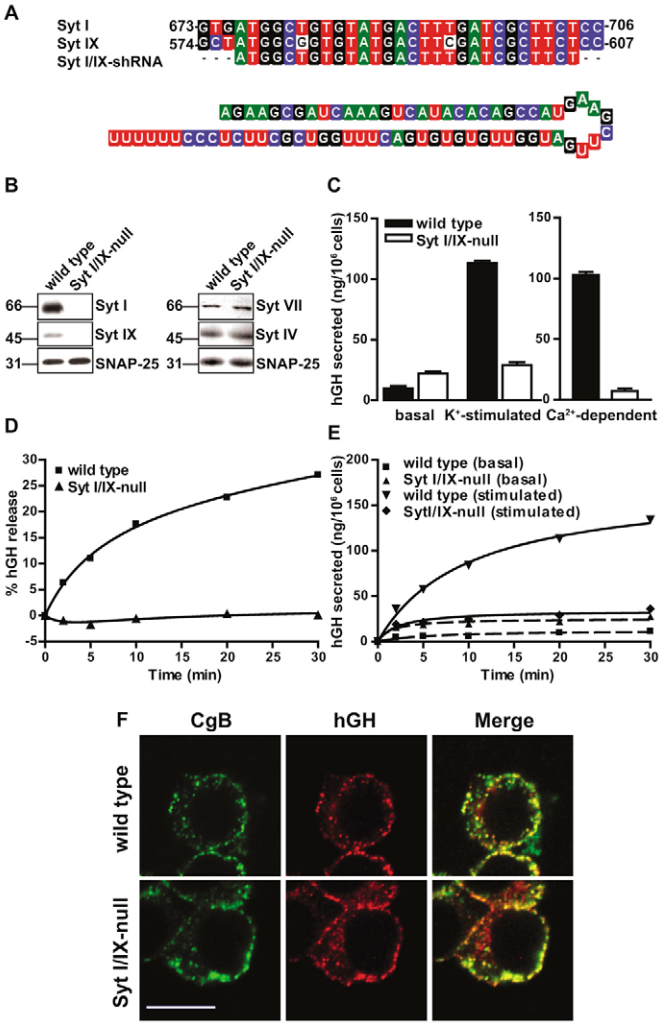

Fig. 2. shRNA knockdown of Syt I and IX abolishes regulated exocytosis. (A) Nucleotide sequence alignment of Syt I and Syt IX in the part of the ORF targeted by shRNA. Predicted structure of the shRNA transcript (Syt I/IX). (B) Clones stably expressing pSHAG-Syt I/IX (Syt I/IX-null) were isolated and an immunoblot of a representative clone is shown using Syt-I-specific and Syt-IX-specific antibodies. The Syt I/IX-null cells were further characterized with Syt-IV-specific and Syt-VII-specific antibodies. SNAP-25 staining was used as a loading control for both immunoblots. (C) Wild-type and Syt I/IX-null cells were transfected with a plasmid encoding hGH, and intact cell secretion assays were conducted. Ca^{2+}-dependent hGH secretion was determined as the amount of hGH secreted in 20 minutes at 37°C, stimulated by depolarization medium (K^+-stimulated) minus the amount of hGH secreted in basal medium (basal). Values represent the mean of triplicate determinations , the standard diviation (s.d.) is indicated. Ca^{2+}-dependent DCV exocytosis was similarly abolished in three Syt I/IX-null cell lines and in transient transfections with the Syt I/IX-shRNA plasmid. (D) hGH secretion assays in intact cells were conducted at the indicated time points. Ca^{2+}-dependent hGH secretion (K^+-stimulated minus basal) was plotted as percent hGH release (total hGH secreted divided by total hGH secreted plus total hGH remaining in the cells ×100). The results shown are representative of three independent experiments and similar results were obtained for three Syt I/IX-null cell lines. (E) Time course for basal and K^+-stimulated hGH release of wild-type and Syt I/IX-null cells. In Syt I/IX-null cells, release of hGH was the same in the absence or presence of a stimulus, and was significantly reduced compared with hGH release in K^+-stimulated wild-type cells. Results are representative of three independent experiments and were similar for three Syt I/IX-null cell lines. (F) Immunocytochemistry of CgB (DCV marker) and transiently transfected hGH in wild-type and Syt I/IX-null cells. hGH is targeted to DCVs in wild-type and Syt I/IX-null cells. The cellular distribution of DCVs was unaffected by the downregulation of Syt I and Syt IX. Bar, 10 μm.

basal unstimulated conditions (Fig. 2E; also Fig. 2C). Whereas elevated basal hGH secretion in Syt I/IX-null cells could indicate that Syts function as fusion clamps to inhibit DCV exocytosis at resting Ca^{2+} levels, other assays for DCV exocytosis did not reveal increased basal DCV exocytosis in Syt I/IX-null cells (see below). Elevated basal hGH secretion and the elimination of Ca^{2+}-dependent hGH secretion in Syt I/IX-null cells could alternatively result from the mis-sorting of hGH to the constitutive or constitutive-like secretory pathway (Arvan and Castle, 1998). To assess hGH sorting and DCV biogenesis, we determined the distribution of hGH as well as that of the DCV protein chromogranin B (CgB). Expressed hGH was found to colocalize with CgB in both wild-type and Syt I/IX-null cells (Fig. 2F). Moreover, the distributions of these proteins were very similar in both wild-type and Syt I/IX-null cells. Thus, hGH targeting to DCVs and DCV biogenesis were not affected by the downregulation of Syt I and Syt IX.

Loss of regulated DCV exocytosis occurs at a late step downstream from Ca^{2+} entry

Syt proteins interact with Ca^{2+} channels, which was suggested to mediate tethering of vesicles in close proximity to sites of Ca^{2+} entry to enhance exocytosis (Charvin et al., 1997; Kim and Catterall, 1997). To determine whether the elimination of regulated DCV exocytosis in Syt I/IX-null PC12 cells was due to altered vesicle-Ca^{2+} channel coupling, we used permeable PC12 cells in which Ca^{2+} addition directly activates DCV exocytosis (Grishanin et al., 2004). hGH-expressing PC12 cells were permeabilized and the cell ghosts were incubated at 30° for 5 minutes in the presence of ATP and brain cytosol without (basal) or with 10 μM Ca^{2+} (Ca^{2+}-stimulated). Basal release of hGH was indistinguishable in wild-type and Syt I/IX-null cells (Fig. 3A). Ca^{2+} stimulated the release of hGH about fourfold in wild-type cells but did not stimulate release in Syt I/IX-null cells (Fig. 3A). Thus, similar to the results for intact cells, permeable Syt I/IX-null cells exhibited virtually no Ca^{2+}-triggered hGH release (Fig. 3A). Because the Ca^{2+} triggering of DCV exocytosis in permeable cells by-passes Ca^{2+} channels, loss of DCV exocytosis in the Syt I/IX-null cells cannot be attributed to altered vesicle-Ca^{2+} channel coupling. We conclude that Syt I/IX function is required downstream from Ca^{2+} entry.

Because it was possible that the downregulation of Syt I and Syt IX alters the Ca^{2+} dependence of DCV exocytosis, we also used the permeable cell assay to address this issue. The optimal Ca^{2+} concentration for stimulated hGH release from permeable wild-type cells was found to be 10^{-6} M (Fig. 3B). By contrast, permeable Syt I/IX-null cells failed to exhibit Ca^{2+}-stimulated hGH release over the full range of Ca^{2+} concentrations (Fig. 3B). Thus, the Ca^{2+} dependence of DCV exocytosis was not shifted in Syt I/IX-null cells but was instead entirely eliminated.

Syt I and Syt IX function in DCV exocytosis at a stage beyond DCV docking

It has been suggested that Syt proteins function in vesicle docking reactions at the plasma membrane (Schiavo et al., 1997; Reist et al., 1998; Chieregatti et al., 2004). Defects in DCV docking as a result of Syt downregulation would impair Ca^{2+}-dependent exocytosis. To determine whether DCV docking is affected in Syt I/IX-null cells, we employed a fluorescent DCV cargo protein and total internal reflection fluorescence (TIRF) microscopy. ANF-EGFP, the fusion protein of preproatrial natriuretic factor (ANF) and enhanced green fluorescent protein (EGFP) was well expressed and sorted to DCVs in wild-type and Syt I/IX-null PC12 cells. The evanescent field generated by TIRF illuminated ANF-EGFP-containing DCVs in cell footprints representing regions of the plasma membrane that adhere to the coverslip (Fig. 4A). As previously reported (Steyer et al., 1997), the majority of DCVs in the footprints of live cells were relatively immobile, corresponding to a plasma membrane-docked subset of DCVs. The overall behavior of DCVs was similar in wild-type and Syt I/IX-null cells (see supplementary material Movie 2 and Movie 3). To determine whether there are differences in docking, DCVs in each footprint (n=30 for both wild-type and Syt I/IX-null cells) were counted and divided by the footprint area to obtain vesicle density. Syt I/IX-null cells contained 7.5±1.1 DCVs/μm^2, a result that was not different to wild-type cells, which contained 7.4±1.5 DCVs/μm^2 (Fig. 4B). These data eliminate the possible role of Syt I and Syt IX in DCV docking in PC12 cells. Moreover, they confirm that DCV biogenesis and transport to the plasma membrane do not require Syt I or

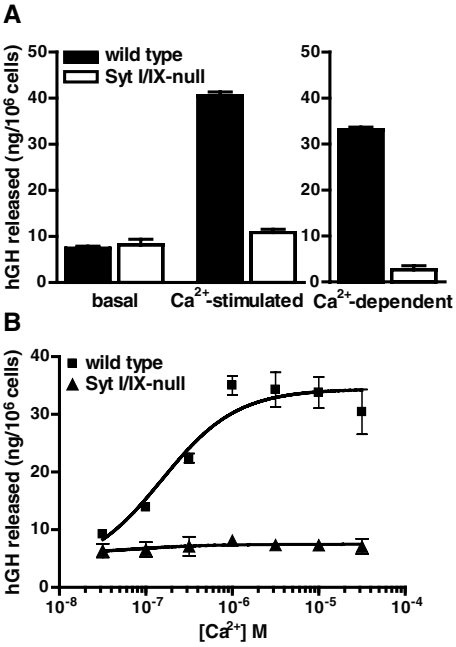

Fig. 3. Loss of regulated exocytosis occurs at a late step downstream from Ca^{2+} entry. (A) Wild-type and clones stably expressing pSHAG-Syt I/IX (Syt I/IX-null) were transfected with a plasmid expressing hGH and permeable cell secretion assays were conducted (see Materials and Methods) in the absence (basal) or presence (Ca^{2+}-stimulated) of 10 μM free Ca^{2+}. Ca^{2+}-dependent hGH secretion was determined as the amount of hGH secreted in 5 minutes at 30°C in Ca^{2+}-stimulated minus basal conditions. Values represent the mean ± s.d. of triplicate determinations. (B) Ca^{2+} dose-response for exocytosis in wild-type and Syt I/IX-null cells. Secretion assays in permeable cells were conducted at the indicated Ca^{2+} concentrations in wild-type and Syt I/IX-null cells. hGH released was determined and plotted as the mean ± s.d. of triplicate determinations.

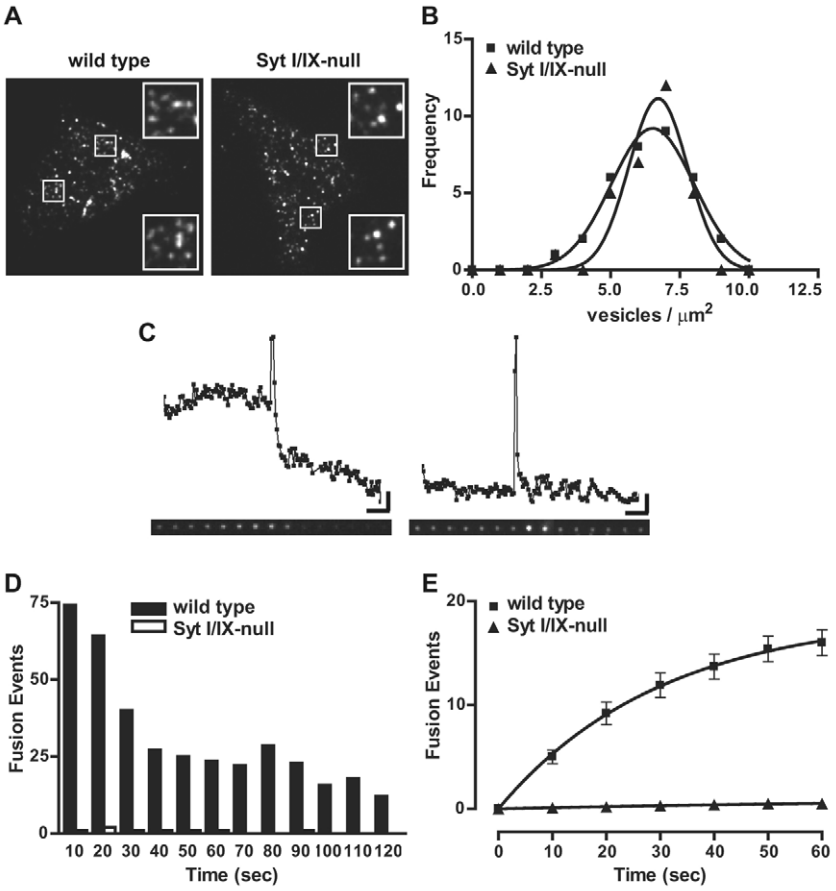

Fig. 4. TIRF analysis of ANF-EGFP secretion in wild-type cells and cells lacking Syt I and Syt IX (Syt I/IX-null cells). (A) Representative footprints of wild-type and Syt I/IX-null cells. TIRF images were acquired of live cells resting in basal medium. Square insets are 1 μm^2. (B) DCVs in each footprint were counted and divided by the total footprint area. The frequency distribution of DCVs for wild-type and Syt I/IX-null cells was similar. The average number of DCVs per μm^2 (± s.d.) was 7.4±1.5 for wild-type and 7.5±1.1 for Syt I/IX-null cells (n=30). (C) Single ANF-EGFP secretion events are shown as sequential images at 4 Hz (bottom) with fluorescence intensity plots (top). The event shown on the left corresponds to full fusion with release of ANF-EGFP whereas the event on the right corresponds to transient fusion event with incomplete ANF-EGFP release; x=2.5 seconds and y=10 arbitrary units. (D) Wild-type and Syt I/IX-null cells expressing ANF-EGFP were stimulated with depolarization medium and images were acquired at 0.25-second intervals. Fusion events were counted manually as a flash (or puff) of fluorescence in wild-type cells or Syt I/IX-null cells and the sum of fusion events in 10-second intervals was plotted (n=21). The number of fusion events was drastically reduced in the Syt I/IX-null cells. (E) Same data as in C plotted as the sum of the average number of fusion events per cell over time (mean ± s.d.).

Syt IX. We conclude that the essential role for Syt I and Syt IX in Ca^{2+}-triggered DCV exocytosis is for a step that follows DCV docking.

To further analyze Syt function in DCV exocytosis, individual exocytic events of ANF-EGFP secretion were monitored by TIRF microscopy. Fusion events occurred rarely in wild-type cells resting in basal medium (see supplementary material Movie 2). The probability of fusion in wild-type cells increased dramatically upon incubation in depolarizing high [K^+] buffer (see supplementary material Movie 4). Two examples of depolarization-evoked fusion events recorded at 4 Hz are shown in Fig. 4C. In the first example (Fig. 4C, left), ANF-EGFP fluorescence increased abruptly due to loss of DCV acidification and movement of ANF-EGFP deeper into the evanescent field (Taraska et al., 2003) and subsequently decreased due to diffusion of the secreted ANF-EGFP. This event represents full DCV fusion. In the second example (Fig. 4C, right), ANF-EGFP fluorescence increased transiently and decreased to pre-peak values. Events of this type, in which the ANF-EGFP is not fully released from DCVs, have been termed cavicapture to indicate vesicle resealing before full fusion occurs (Taraska and Almers, 2004). Both types of events appeared as flashes of fluorescence that occurred within 1-2 seconds and represent DCV fusion events.

Depolarization-evoked fusion events were counted and summed up in 10-second intervals for wild-type and Syt I/IX-null cells (Fig. 4D, n=21 for each cell type). In wild-type cells, 50% of the fusion events occurred within ~30 seconds of stimulation. In Syt I/IX-null cells, fusion events occurred only rarely at all time points (Fig. 4D and supplementary material Movie 5). The time course of averaged (n=21 cells) cumulative fusion events indicated 0.5±0.1 events for Syt I/IX-null cells in 60 seconds compared with 17.0±2.5 events for wild-type PC12 cells (Fig. 4E). These data confirm that elimination of Syt I and Syt IX virtually abolishes Ca^{2+}-triggered DCV exocytosis in PC12 cells. Spontaneous DCV exocytosis was not observed in Syt I/IX-null cells indicating that a role for Syts as fusion clamps is unlikely.

Syt I or Syt IX individually mediate DCV exocytosis

In contrast to our finding that downregulation of Syt I and Syt IX eliminates Ca^{2+}-dependent DCV exocytosis, it was previously reported that regulated DCV exocytosis persists in PC12 cells lacking Syt I (Shoji-Kasai et al., 1992; Fukuda et al., 2002). In the original Syt-I-deficient PC12 cell clone (Shoji-Kasai et al., 1992), Syt IX levels were upregulated (Fukuda et al., 2002). The persistence of Ca^{2+}-dependent DCV exocytosis in the Syt I-deficient cells would indicate either that Syt IX compensates for the loss of Syt I function or that Syt IX alone mediates the Ca^{2+} regulation of DCV exocytosis. To distinguish these alternatives, we developed methods for the selective downregulation of either Syt I or Syt IX. In characterizing stable cell lines targeted with the Syt I/IX shRNA, we identified cell lines in which Syt I expression was abolished but Syt IX was expressed at wild-type (but not elevated) levels (Fig. 5A). In addition, we generated a shRNA plasmid that selectively downregulated Syt IX but not Syt I

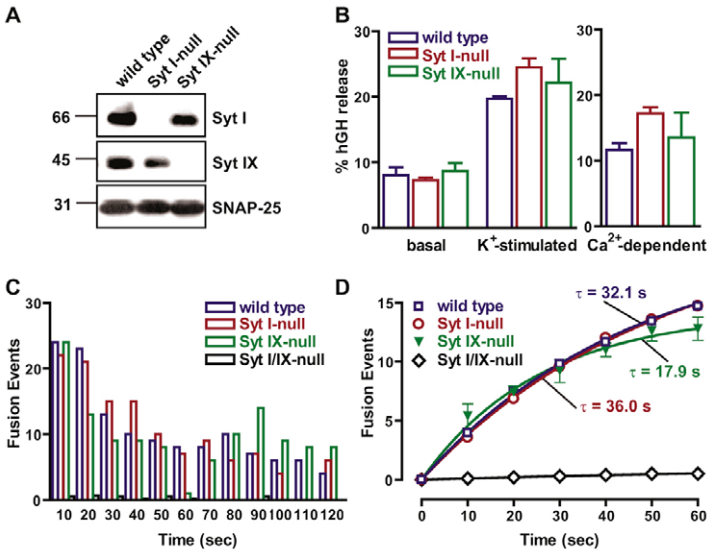

Fig. 5. Downregulation of Syt I or Syt IX alone has no effect on stimulated exocytosis. (A) During isolation of stable cell lines lacking Syt I and Syt IX, clones were isolated in which Syt I was fully downregulated but Syt IX was expressed at wild-type levels (Syt I-null cells). pSHAG-Syt IX downregulated Syt IX without affecting Syt I expression (Syt IX-null cells). Immunoblot analysis was used to analyze Syt I and Syt IX. (B) Wild-type, Syt I-null and Syt IX-null cells were transfected with a plasmid expressing hGH and intact cell secretion assays were conducted. Ca^{2+}-dependent hGH release was unaffected by the downregulation of either Syt I or Syt IX. (C) Wild-type, Syt I-null and Syt IX-null cells, transfected with a plasmid expressing ANF-EGFP, were stimulated in depolarization medium and TIRF images were acquired at 0.25-second intervals. The data were analyzed as in Fig. 4 and plotted as the sum of the events for ten wild-type, ten Syt I-null, ten Syt IX-null and ten Syt I/IX-null cells. DCV exocytosis occurred in the Syt I-null or Syt IX-null cells. (D) The sum of the average number of fusion events per cell over time (mean ± s.d.) for wild-type (n=30), Syt I-null (n=15) and Syt IX-null cells (n=10). τ values for fusion in wild-type, Syt I-null and Syt IX-null cells were 32.1 seconds, 36.0 seconds and 17.9 seconds, respectively. Differences between Syt IX-null and wild-type or Syt I-null were significant (P<0.001).

(Fig. 5A). When Syt I and Syt IX were independently downregulated, basal and depolarization-evoked hGH secretion were found to be indistinguishable from those in wild-type cells (Fig. 5B). The Ca^{2+}-dependent release of hGH was not affected by the selective loss of either Syt I or Syt IX (Fig. 5B). Similarly, the depolarization-evoked increase of fusion probabilities measured as ANF-EGFP exocytic events in either Syt I- or Syt IX-null cells was not markedly different from wild-type cells (Fig. 5C). These results show that regulated DCV fusion persists in the absence of either Syt I or Syt IX but not when both isoforms are absent. Thus, Syt I or Syt IX is each sufficient to confer Ca^{2+}-regulation on DCV exocytosis in PC12 cells. This clearly indicates that Syt I and Syt IX function redundantly for DCV exocytosis in wild-type PC12 cells.

Although Ca^{2+}-dependent DCV exocytosis was similar in cells expressing either Syt I or Syt IX or both (Fig. 5C), a more detailed analysis revealed significant differences in the time courses of depolarization-evoked fusion events (Fig. 5D). The time courses of averaged cumulative fusion events fitted well to an exponential function that provided time constants (τ) of 32.1±6.3 seconds, 36.0±7.9 seconds and 17.9±5.1 seconds for wild-type, Syt I-null and Syt IX-null cells, respectively (Fig. 5D). Although either Syt I or Syt IX alone could mediate Ca^{2+}-triggered DCV exocytosis, Syt I acted as a kinetically faster isoform, whereas Syt IX functioned as a kinetically slower isoform. The kinetics of secretion in wild-type cells resembled that of Syt I-null cells.

Re-expression of Syts in Syt I/IX-null PC12 cells rescues regulated DCV exocytosis

To assess whether deficits in DCV exocytosis in Syt I/IX-null cells are a result of shRNA-mediated downregulation and not due to off-target effects, we re-expressed Syt I or Syt IX. Silent mutations were introduced into Syt I or Syt IX expression constructs to by-pass the Syt I/IX shRNA. Syt I or Syt IX were expressed in the Syt I/IX-null cells at a level comparable with that of Syt I or Syt IX in wild-type cells (Fig. 6A). Syt I or Syt IX re-expression was found to rescue Ca^{2+}-triggered DCV exocytosis in the Syt I/IX-null cells (Fig. 6B). Depolarizing K^+ buffer elicited a similar number of ANF-EGFP exocytic events over the 120-second stimulation period in wild-type and Syt I or Syt IX re-expressing Syt I/IX-null cells in contrast to very few events in the Syt I/IX-null cells (Fig. 6B). Analysis of the time courses for averaged cumulative fusion events (Fig. 6C) revealed that the depolarization-evoked fusion probability was significantly faster for Syt I-expressing Syt I/IX-null cells (τ=15.6±1.9 seconds; n=20) than for wild-type cells (τ=31.2±9.1 seconds; n=40) or for Syt IX-expressing Syt I/IX-null cells (τ=31.9±11.2 seconds; n=12). These results were consistent with the previous finding of more rapid kinetics for Syt IX-null cells and indicated that Syt I operates as a kinetically faster isoform than Syt IX.

Syt I and IX do not function redundantly in endocytosis

Syt I has previously been implicated in clathrin-dependent endocytosis (Zhang et al., 1994; Jorgensen et al., 1995; von Poser et al., 2000). The endocytic retrieval of SVs in nerve terminals of Syt-I-deficient mice is slowed but not abolished (Nicholson-Tomishima and Ryan, 2004). The partial loss of compensatory endocytosis could be due to Syt isoform redundancy because the multiple Syt isoforms present on SVs were each found to bind the clathrin adapter AP-2 (Li et al., 1995). Because Syt I and IX exhibit overlap in function for DCV exocytosis in PC12 cells, we determined whether there was functional redundancy of Syt isoforms for compensatory DCV endocytosis. The stimulated uptake of Texas-Red-conjugated transferrin (Tf) by cells in depolarizing high [K^+] buffers was used to measure compensatory endocytosis (Fig. 7). Stimulated but not basal Tf uptake was strongly reduced in Syt I/IX-null cells. This was expected because DCV exocytosis does not occur and there would be no compensatory endocytosis (Fig. 7A,B). Downregulation of Syt IX, which does not compromise DCV exocytosis, did not affect Tf

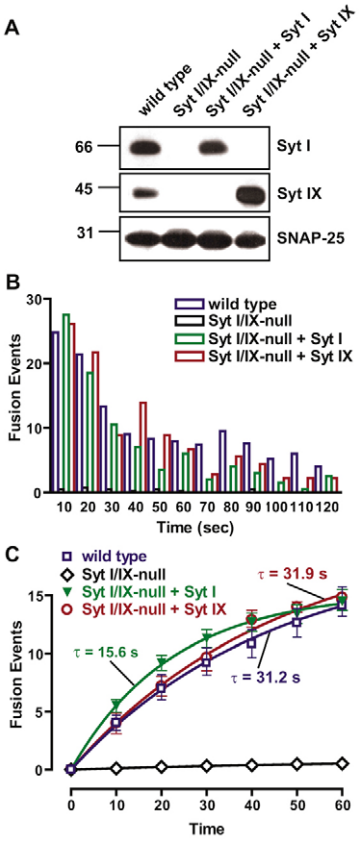

Fig. 6. Rescue of function by re-expression of Syt I or Syt IX. (A) Immunoblot analysis of wild-type cells, cells that lack Syt I and Syt IX (Syt I/IX-null) and Syt I/IX-null cells that express the pcDNA3-Syt I or pcDNA3-Syt IX rescue vector. (B) Wild-type and Syt I/IX-null cells were transfected with a plasmid expressing ANF-EGFP or Syt I/IX-null cells were co-transfected with a plasmid expressing ANF-EGFP and the pcDNA3-Syt I or pcDNA3-Syt IX rescue vector. Cells were stimulated in depolarization medium and images were acquired at 0.25-second intervals. The data were analyzed as in Fig. 4D and plotted as the sum of the events for ten wild-type, ten Syt I/IX-null, ten Syt I/IX-null + Syt I and ten Syt I/IX-null + Syt IX cells. Re-expression of either Syt I or Syt IX restored Ca^{2+}-dependent DCV exocytosis in the Syt I/IX-null cells. (C) The sum of the average number of fusion events per cell over time (mean ± s.d.) for wild-type (n=40), Syt I/IX-null (n=10), Syt I/IX-null + Syt I (n=20) and Syt IX-null + Syt IX (n=12). τ values for fusion in wild-type (31.2±9.1 seconds) and Syt I/IX-null + Syt I (15.6±1.9 seconds) cells were significantly different (P<0.001).

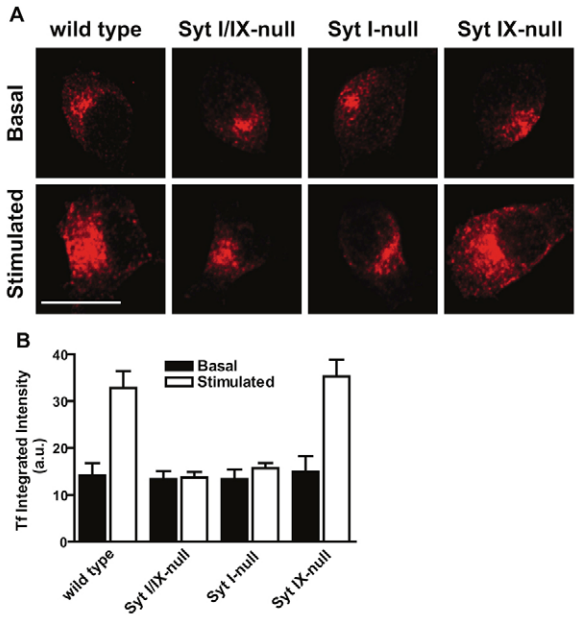

Fig. 7. Syt I but not Syt IX downregulation inhibits compensatory endocytosis. (A) Wild-type, Syt I-null, Syt IX-null and Syt I/IX-null cells expressing the human Tf receptor were incubated with Texas-Red-conjugated Tf in basal or depolarization medium for 20 minutes. Representative images for the uptake of Tf into the four indicated cells types resting in basal or depolarization medium are shown. Bar, 10 μm. (B) Z-stacks of confocal images were added up to give Tf integrated intensity per cell. The mean Tf integrated intensity ± s.d. is plotted for wild-type, Syt I/IX-null, Syt I-null and Syt IX-null cells (n=15 cells for each cell type) in basal or depolarization medium.

uptake. By contrast, downregulation of Syt I, which also does not compromise DCV exocytosis, resulted in an extensive (68.2%) decrease in stimulated but not basal Tf uptake (Fig. 7A,B). These results reveal a selective role for Syt I but not Syt IX for endocytic retrieval and indicate that Syt isoforms do not function redundantly in the compensatory endocytosis that follows exocytosis.

Discussion

Functional redundancy of multiple Syt isoforms

Genes encoding at least 16 Syt isoforms have been identified to date (Craxton, 2001; Fukuda, 2003). In many instances, multiple Syt proteins co-reside on secretory vesicles (Marqueze et al., 2000; Tucker and Chapman, 2002; Sudhof, 2002) but the functional significance of co-residence has not been previously determined. A major conclusion of the current work is that the two major Syt isoforms that co-reside on DCVs in PC12 cells exhibit redundancy in function. We found that the concomitant and full downregulation of Syt I and Syt IX abolished Ca^{2+}-triggered DCV exocytosis, whereas the downregulation of either Syt I or Syt IX alone did not diminish exocytosis. Our results explain the long-standing observation that Syt-I-deficient PC12 cells exhibit normal Ca^{2+}-dependent exocytosis (Shoji-Kasai et al., 1992) because co-resident Syt IX compensates for the loss of Syt I function.

Our results differ from a previous report (Fukuda, 2004), which concluded that Syt IX but not Syt I mediates Ca^{2+} regulation of DCV exocytosis in PC12 cells. This conclusion was based on partial downregulation of Syt IX and partial inhibition of Ca^{2+}-triggered DCV exocytosis. However, the rescue of shRNA inhibition by Syt re-expression, an important control to eliminate off-target effects of shRNA, was not demonstrated. By contrast, in the present studies we achieved complete downregulation of Syt I or Syt IX or of both simultaneously. The downregulation of Syt I and Syt IX together but not individually resulted in complete inhibition of Ca^{2+}-triggered DCV exocytosis. Importantly, re-expression of either Syt I or Syt IX alone in the null background resulted in the complete restoration of Ca^{2+}-dependent DCV exocytosis. These results eliminate concerns about shRNA off-target effects and provide compelling evidence that Syt I or Syt IX

can fully function as Ca^{2+} sensors for DCV exocytosis. Related studies in pancreatic β-cell lines showed that the partial downregulation of Syt I (Xiong et al., 2006) or Syt IX (Iezzi et al., 2004) resulted in partial inhibition of glucose-stimulated insulin secretion. However, Syt II, Syt III, Syt V, Syt VII and Syt VIII have also been implicated in regulated insulin secretion (Lang et al., 1997; Gao et al., 2000; Gut et al., 2001; Iezzi et al., 2004; Iezzi et al., 2005; Xiong et al., 2006). Whether multiple Syt isoforms operate in parallel or redundantly has not been addressed. In light of the present study, the systematic elimination of multiple Syts in pancreatic β-cells could resolve the issue as to whether multiple isoforms function in insulin secretion.

That Syt I and Syt IX are the functionally important Ca^{2+} sensors for regulated DCV exocytosis in PC12 cells is somewhat surprising because of the described biochemical properties of these Syt isoforms. Based on its in vitro properties, it has been argued that Syt I is highly specialized for a role as a low-affinity Ca^{2+} sensor that mediates rapid vesicle exocytosis in response to high [Ca^{2+}] concentrations (Sudhof, 2002; Shin et al., 2002). DCV exocytosis in PC12 cells is, by contrast, triggered by relatively low (~1-10 μM) Ca^{2+} concentrations and is kinetically slow (Grishanin et al., 2002; Martin, 2003). In vitro Syt I exhibits rapid Ca^{2+}-dependent binding to PS and SNAREs but at high (10-200 μM) [Ca^{2+}] concentrations (Chapman, 2002; Sudhof, 2002; Bai et al., 2004). Syt IX is a closely-related isoform (Marqueze et al., 2000; Craxton, 2004) that exhibits Ca^{2+}-dependent PS- and SNARE-binding properties similar to those of Syt I (Tucker et al., 2003; Rickman et al., 2004) (see also Shin et al., 2004). In spite of apparent low-affinity Ca^{2+}-dependent interactions in vitro, endogenous Syt I and Syt IX in PC12 cells can be crosslinked to SNAP-25 at 1-10 μM Ca^{2+}, which demonstrates that Syt isoforms can exhibit higher Ca^{2+} sensitivity in situ in cells than in vitro (Zhang et al., 2002). The Ca^{2+}-dependent Syt-effector interactions in situ probably differ from those observed in vitro because of the increase of Ca^{2+} affinity for Syts in the presence of anionic phospholipids (Brose et al., 1992). The current work, establishing functional roles for Syt I and Syt IX in Ca^{2+}-dependent DCV exocytosis in PC12 cells, indicates that classification of Syt isoforms into low-affinity and high-affinity Ca^{2+}-binding classes based on in vitro characteristics (Sudhof, 2002; Shin et al., 2002) cannot predict cellular function.

Steps in DCV fusion regulated by Syt

Multiple roles have been proposed for Syts. Our studies showed that the loss-of-function in Syt I/IX-null PC12 cells corresponded to a late post-docking Ca^{2+}-triggering step in DCV exocytosis. Studies in permeable cells revealed that the direct triggering of DCV exocytosis by Ca^{2+} was ineffective in Syt I/IX-null PC12 cells, which indicates that defects in a proposed Syt–Ca^{2+}-channel coupling (Charvin et al., 1997; Kim and Catterall, 1997) were not responsible for the loss of Ca^{2+}-dependent DCV exocytosis. A potential involvement of Syt I and Syt IX in docking vesicles at the plasma membrane, as suggested for various Syts in other studies (Reist et al., 1998; Chieregatti et al., 2004), was eliminated by direct determination of the number of docked DCVs in wild-type and cells lacking SytI and Syt IX by TIRF microscopy. Overall, these studies indicated that the biogenesis, pool size, distribution and docking of DCVs were unaffected by the complete downregulation of Syt I and Syt IX. The possible role of Syt I or Syt IX as fusion clamps that prevent vesicle exocytosis at resting cytosolic Ca^{2+} concentrations, as initially suggested for Syt I in *Drosophila* (DiAntonio and Schwarz, 1994; Littleton et al., 1994) (see also Marek and Davis, 2002), was eliminated in studies of ANF-EGFP secretion, which indicated that an enhanced frequency of spontaneous DCV exocytic events could not be detected in Syt I/IX-null PC12 cells. We did observe enhanced hGH release in unstimulated Syt I/IX-null cells but attribute this to increased mis-sorting of hGH to a constitutive secretory pathway, which may indicate other membrane trafficking defects in Syt I/IX-null cells and other roles for Syts (see below).

Syt I has also been shown to function in the compensatory endocytosis of SVs (Zhang et al., 1994; Jorgensen et al., 1995; von Poser et al., 2000; Littleton et al., 2001; Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004), a specialized form of clathrin-dependent endocytosis. Syt I is proposed to promote the nucleation of clathrin coats on exocytosed vesicles by recruiting AP-2 to the patch of vesicle membrane for endocytosis (Zhang et al., 1994; von Poser et al., 2000; Haucke et al., 2000). Our results indicate that Syt I is required for the compensatory clathrin-mediated endocytosis of DCVs, as shown by the strong inhibition of evoked Tf uptake in Syt I-null PC12 cells. By contrast, compensatory endocytosis was not affected by Syt IX downregulation. This indicates that Syt I is specialized for clathrin-dependent endocytosis and that Syt IX cannot compensate for the loss of Syt I function. Because Syt I and Syt IX are closely related isoforms with conserved K326 and K327 (Syt I) residues that mediate AP-2 and phosphatidylinositol (4,5)-bisphosphate binding (Chapman et al., 1998), the basis for the specialization of Syt I in endocytosis is unclear. Studies indicating that Syt I oligomerization is required for AP-2 binding (Grass et al., 2004) and that Syt I oligomerizes, more readily than Syt IX (Fukuda and Mikoshiba, 2000), may provide an explanation. Also, Ca^{2+} coordination within the C2B domain of Syt I contributes to the control of endocytic rate (Poskanzer et al., 2006) and differences in the intrinsic Ca^{2+}-sensing abilities of the C2B domain of Syt I and Syt IX (Shin et al., 2004) could account for different roles in endocytosis. Finally, it should be noted that Syt I-null PC12 cells exhibit additional properties that may be attributed to altered clathrin-dependent endocytosis. Norepinephrine uptake by Syt I-null cells is strongly impaired because of the mis-sorting of the plasma membrane norepinephrine transporter to intracellular membranes (X. Zhang, M. Cyr, T. Martin, M. Caron, Society for Neurosciences, Abstract 337.1, 2003). Indeed, recent studies demonstrated a strong reduction in the catecholamine content of DCVs in Syt I-deficient PC12 cells (Moore et al., 2006).

Distinct kinetic properties of individual Syt isoforms

Although Syt I or Syt IX were found to be sufficient to mediate Ca^{2+}-triggered DCV exocytosis in PC12 cells, each isoform exhibited distinct kinetic properties. Our results taken together with those of Chapman and co-workers (Hui et al., 2005) indicate that dissociation rates of Ca^{2+} are a key property for Syt isoform function in exocytosis. Syt I exhibits high off-rates for dissociation of Syt–Ca^{2+}–phospholipid complexes upon

Ca^{2+} removal, whereas Syt IX exhibits slower off-rates (Hui et al., 2005). These characteristics would enable Syt I to function as a rapid and reversible regulator of exocytosis in synchrony with Ca^{2+} rises, whereas Syt IX responses would be longer-lived and sustained beyond the dissipation of a Ca^{2+} rise. These features could account for multiphasic secretory responses to Ca^{2+} elevations. Consistent with this, we observed significant kinetic differences for Ca^{2+}-dependent DCV exocytosis in PC12 cells supported by Syt I or by Syt IX alone. Responses to Syt I alone exhibited τ values of ~15-18 seconds, whereas responses to Syt IX alone exhibited τ values of ~31-35 seconds. Although the overall kinetics of Ca^{2+}-triggered DCV exocytosis in PC12 cells are relatively slow (Martin, 2003), our results clearly indicate that Syt isoform properties shape kinetic responses to Ca^{2+} in DCV exocytosis. Wild-type cells exhibited kinetic responses to Ca^{2+} that were similar to those observed for cells expressing Syt IX alone. Thus, the kinetics of secretion in wild-type cells were conferred by the properties of the slower co-resident vesicle Syt. In its absence, the kinetics of secretion were dictated by Syt I, the faster of the two isoforms.

Syt I is the best candidate for a presynaptic molecule that serves as a Ca^{2+} sensor for rapid synchronous SV release (see Introduction). However, Ca^{2+}-dependent transmitter release is not eliminated by the loss of Syt I, raising the possibility that Syt I functions to kinetically facilitate and synchronize vesicle exocytosis rather than to mediate its Ca^{2+} triggering (Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004). Alternatively, Ca^{2+} sensors of unknown identity have been proposed to function in parallel with Syt I (Burgoyne and Morgan, 1998). Our work represents the first time that Ca^{2+}-dependent vesicle exocytosis is fully abolished by the elimination of Syts; and we conclude that co-resident Syts are entirely sufficient to confer Ca^{2+} regulation to vesicle exocytosis. It is anticipated that the systematic elimination of multiple vesicle co-resident Syts in other experimental systems will reveal that various Syt isoforms function in parallel – as Ca^{2+} sensors that confer distinct kinetic characteristics to vesicle exocytosis.

Materials and Methods

Constructs

The plasmid pCMV-GH encoding human GH was generously provided by H. Herweijer (Mirus Corp., Madison, WI), human Tf receptor by F. Maxfield (Weill Medical College of Cornell University, New York, NY), and ANF-EGFP by E. Levitan (University of Pittsburgh School of Medicine, Pittsburgh, PA). Oligonucleotides encoding shRNAs were ligated into pSHAG-1 vector (generously provided by G. Hannon, Cold Spring Harbor, NY) via *Bam*HI and *Bse*RI sites. The construct designated pSHAG-Syt I/IX targets an mRNA sequence corresponding to Syt I coding sequence nucleotides 676-704 and Syt IX coding sequence nucleotides 577-605. [Syt IX corresponds to the rat isoform reported by Craxton and Goedert (Craxton and Goedert, 1995) previously referred to as Syt V.] This construct contains two Syt IX mismatches in this coding sequence. The oligonucleotide sequence was 5′-AGAAGCGATCAAAGTCATACACAGCCATGAAGCTTGATGATGGTTGTGTGTGACTTTGGTCGCTTCTCCCTTTTTT-3′ (sense) and 5′-GATCAAAAAAGGGAGAAGCGACCAAAGTCACACACAACCATCAAGCTTCATGGCTGTGTATGACTTTGATCGCTTCTCG-3′ (antisense). The construct designated pSHAG-Syt IX targets an mRNA sequence that corresponds to Syt IX coding sequence nucleotides 232-260 and is specific for Syt IX. The oligonucleotide sequence was 5′-GCTGAACCTTATCTATGTAACTCCGGCCGAAGCTTGGGCCGGAGTTGCATAGGTAAGGTTCGGCCTGTTTTTT-3′ (sense) and 5′-GATCAAAAAACAGGCCGAACCTTACCTATGCAACTCCGGCCCAAGCTTCGGCCGGAGTTACATAGATAAGGTTCAGCCG-3′ (antisense). The specificity of each construct was verified by a BLAST search of the public databases. The open reading frames of Syt I and Syt IX were reverse transcribed and PCR amplified from rat PC12 cells, then ligated into pcDNA3.1 (Invitrogen) via *Bam*HI and *Eco*RI sites. Primers used for RT-PCR for Syt I were 5′-ATGGTGAGTGCCAGTCATCCTGAG-3′ and 5′-GGCTTCGTTTTCCCTTTACTTCTTGA-3′ and for Syt IX were 5′-CCATGTTTCCAGAACCCCCGACCCC-3′ and 5′-TCCCAACTTTTCCACCTCCCC-3′. The QuickChange® site-directed mutagenesis method (Stratagene) was used to generate the following Syt I and Syt IX silent mutants: pcDNA3-Syt I sm, sense primer 5′-GGTGATGGCTGTCTATGATTTTGACCGCTTTTCCAAGCACG-3′ and pcDNA3-Syt IX sm, sense primer 5′-GGTCATGGCGGTCTACGATTTCGACCGGTTTTCCCGCAACGATGC-3′.

Antibodies

Antibodies used were anti-Syt I monoclonal (clone 604.1; Synaptic Systems); anti-Syt IX polyclonal (generously provided by E. Chapman, University of Wisconsin, Madison, WI); anti-Syt IX polyclonal (generously provided by M. Fukuda, RIKEN Brain Science Institute, Wako, Japan); Syt IV polyclonal (Immuno-Biological Laboratories, Inc); anti-Syt VII polyclonal (Santa Cruz Biotechnology, Inc.); anti-Syt VII polyclonal (Synaptic Systems); anti-SNAP-25 monoclonal (Sternberger); anti-hGH polyclonal (ICN Pharmaceuticals); and anti-CgB monoclonal (generously provided by W. Huttner, Max Plank Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Anti-Syt I C2AB polyclonal was generated using a Syt I C2AB fusion protein.

Cell culture, transfection and immunoblot analysis

PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% horse serum and 5% calf serum. Transfections were done by electroporation using an Electroporator II (Invitrogen) set at 1000 μF and 330 V. PC12 cells (2.5×10^7) suspended in 0.5 ml cytomix (25 mM HEPES, 120 mM KCl, 10 mM KH_2PO_4, 0.15 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM EGTA, pH 7.6) were transfected with 10-100 μg plasmid DNA. For selection of stable clones, PC12 cells were transfected with 30 μg pSHAG-Syt I/IX and 30 μg pcDNA3.1 that were linearized prior to transfection. At 24 hours, growth medium was changed to fresh medium containing 500 μg/ml G418. G418 concentration was reduced to 250 μg/ml when cell colonies were evident. Protein expression levels were determined from total cell lysates prepared in 1 mM PMSF and 1% Triton X-100 and clarified by centrifugation at 16,000 ***g*** for 5 minutes. For immunoblot analysis, 10 μg of total protein (100 μg of total protein for detection of Syt IV and Syt VII), determined by BCA (Pierce Chemical Co.), was loaded per lane for gel electrophoresis. Immunoblot analysis was conducted by standard methods.

Ca^{2+}-dependent hGH secretion

Wild-type PC12 cells, and PC12 cells lacking Syt I (Syt I-null cells) or Syt I and Syt IX (Syt I/IX-null cells) were transfected with 10 μg pCMV-GH. For Syt IX downregulation, PC12 cells were transfected with 40 μg pSHAG-Syt IX and 10 μg pCMV-GH. After 48 hours, cells were incubated in 5 μg/ml brefeldin A for 30 minutes to reduce constitutive hGH secretion. To assay intact cells, cells were incubated in basal medium (15 mM HEPES pH 7.4, 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 0.5 mM ascorbic acid, 0.1% BSA) or depolarization medium (basal medium adjusted to 95 mM NaCl and 56 mM KCl) plus brefeldin A for indicated times at 37°C. Medium was removed and cells were solubilized in 0.1% SDS containing complete protease inhibitor cocktail (Roche). To assay permeable cells, cells were broken by a single pass through a stainless steel ball homogenizer for preparation of a cell-ghost fraction that contains docked DCVs (Grishanin et al., 2004). The secretion assays in permeable cells were conducted in KGlu buffer (120 mM potassium glutamate, 20 mM HEPES, 20 mM potassium acetate, 2 mM EGTA, pH 7.2) containing approximately 10^6 permeable cells, 2 mM ATP, 2 mM $MgCl_2$, 1 mg/ml rat brain cytosol and free Ca^{2+} adjusted to indicated values. Supernatants and solubilized cell pellets were collected following centrifugation at 16,000 ***g*** for 10 minutes. hGH in medium, supernatants and solubilized cells was determined using the hGH radioisotopic assay (Nichols Institute Diagnostics). The percentage hGH released was calculated from the hGH in medium or supernatant divided by the total hGH obtained from solubilized cells plus medium or supernatant.

Immunocytochemistry and Tf-uptake assay

For immunocytochemistry, cells were plated on poly-L-lysine- coated and collagen-coated coverslips. After 48 hours, cells were washed with PBS, fixed with 4% formaldehyde (w/v), permeabilized with PBS containing 0.3% Triton X-100 and blocked in 10% fetal bovine serum (FBS) in PBS. Primary and secondary antibodies were diluted in FBS blocking solution. Following extensive washing with PBS, coverslips were mounted on slides with Mowiol® 4-88 Reagent (Calbiochem). For transferrin uptake, transfected cells were incubated in basal medium or depolarization medium containing 50 μg/ml Texas-Red-conjugated transferrin (Molecular Probes, Inc.) for 20 minutes at 37°C, washed in PBS and fixed. Cells were imaged on a Nikon C1 laser scanning confocal microscope with a 60× oil immersion objective with NA 1.4. *Z*-series images were obtained by 250-nm sectioning with oversampling. The resulting *Z*-stacks were deconvolved using Autodeblur/autovisualize software (AutoQuant Imaging Inc.). Colocalization was quantified by thresholding all images and measuring the overlap between channels for each optical section using Metamorph software (Universal Imaging Corp.)

Random overlap was determined and subtracted from the total percent overlap by multiplying percent thresholded area for Syt I with that of Syt IX. Colocalization for each cell was determined for each *Z*-section through the entire cell to ensure the *Z*-position within the cell did not influence the analysis. For analysis of Tf-uptake assays, *Z*-stacks of entire cells were summed to give Tf integrated intensity for the whole cell.

TIRF microscopy

Cells were transiently transfected with ANF-EGFP and plated on 35-mm dishes with a glass bottom (MatTek Corp.) that had been coated with poly-L-lysine and collagen. After 48 hours, cells were imaged on a Nikon TIRF Microscope Evanescent Wave Imaging System used with a TE2000-U Inverted Microscope (Nikon) and an Apo TIRF 100X, NA 1.45 (Nikon) objective lens. EGFP fluorescence was excited with the 488-nm laser line of an argon ion laser. Cells were imaged in basal medium and stimulated in depolarization medium (same as hGH assay). Images were acquired at 250-msecond intervals with a CoolSNAP-ES Digital Monochrome CCD camera system (Photometrics) controlled by Metamorph software (Universal Imaging Corp.). All data analysis was done using Metamorph software.

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