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Identification of synaptotagmin effectors via acute inhibition of secretion from cracked PC12 cells

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The synaptotagmins (syts) are a family of membrane proteins proposed to regulate membrane traffic in neuronal and nonneuronal cells. In neurons, the Ca^{2+}-sensing ability of syt I is critical for fusion of docked synaptic vesicles with the plasma membrane in response to stimulation. Several putative Ca^{2+}–syt effectors have been identified, but in most cases the functional significance of these interactions remains unknown. Here, we have used recombinant C2 domains derived from the cytoplasmic domains of syts I–XI to interfere with endogenous syt–effector interactions during Ca^{2+}-triggered exocytosis from cracked PC12 cells. Inhibition was closely correlated with syntaxin–SNAP-25 and phosphatidylinositol 4,5-bisphosphate (PIP_2)–binding activity. Moreover, we measured the expression levels of endogenous syts in PC12 cells; the major isoforms are I and IX, with trace levels of VII. As expected, if syts I and IX function as Ca^{2+} sensors, fragments from these isoforms blocked secretion. These data suggest that syts trigger fusion via their Ca^{2+}-regulated interactions with t-SNAREs and PIP_2, target molecules known to play critical roles in exocytosis.

Introduction

The Ca^{2+}-triggered fusion of secretory organelles with the plasma membrane mediates synaptic transmission and the release of hormones from neuroendocrine cells. The SNARE complex is thought to form the core of the membrane fusion complex (Söllner et al., 1993; Jahn and Südhof, 1999). This complex is composed of the t-SNAREs syntaxin and SNAP-25 and the v-SNARE synaptobrevin (also known as vesicle-associated membrane protein). Reconstitution experiments demonstrate that the SNARE complex can drive slow Ca^{2+}-independent membrane fusion in vitro (Weber et al., 1998). However, rapid release of neurotransmitters and hormones requires additional factors. Current evidence suggests that the integral membrane protein, synaptotagmin (syt),* may function as a Ca^{2+} sensor that triggers fast fusion events (Augustine, 2001).

Syts represent a gene family with at least 13 members that are widely distributed in neuronal and nonneuronal tissues (Perin et al., 1990; Craxton, 2001). Members of this family have the same overall structure consisting of an NH_2-terminal transmembrane domain and a cytoplasmic COOH-terminal domain. The cytoplasmic domain contains two regions called C2 domains (Perin et al., 1990). C2 domains are conserved motifs found in >120 genes in the human genome. The two C2 domains of syt, designated C2A (membrane proximal C2 domain) and C2B (membrane distal C2 domain) (Perin et al., 1990), function as Ca^{2+}-binding modules that mediate Ca^{2+}-dependent interactions with other molecules (Tucker and Chapman, 2002).

Syt I is the best characterized isoform and is a major constituent of synaptic vesicles and neuroendocrine secretory granules (Matthew et al., 1981; Perin et al., 1991; Chapman and Jahn, 1994). Detailed analysis of syt I mutants indicates that disruption of its Ca^{2+}-sensing properties impairs evoked exocytosis from nerve terminals (Fernández-Chácon et al., 2001; Littleton et al., 2001; Mackler et al., 2002; Robinson et al., 2002), but the mechanism by which Ca^{2+}–syt I triggers release is not known. In vitro studies indicate that at 10–200 μM Ca^{2+}, syt I binds anionic phospholipids (Brose et al., 1992; Bai et al., 2002) and the t-SNAREs, syntaxin, and SNAP-25 (Chapman et al., 1995; Schiavo et al., 1997; Gerona et al., 2000). Ca^{2+} also triggers the homo- and heterooligomerization of several syt isoforms (Fukuda and Mikoshiba, 2000; Wu et al., 2003). These Ca^{2+}-dependent interactions have been proposed to

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*Abbreviations used in this paper: LDCV, large dense core vesicle; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; syt, synaptotagmin.

Key words: synaptotagmin; SNARE; membrane fusion; C2 domain; exocytosis

couple Ca^{2+} influx to fusion (Augustine, 2001; Tucker and Chapman, 2002).

Whereas syt I regulates exocytosis from neurons and neuroendocrine cells, the function of other members of the syt family are less clear. It has become apparent recently that the Ca^{2+} sensitivity of release can differ by orders of magnitude between distinct cell types (Heidelberger et al., 1994; Bollmann et al., 2000; Schneggenburger and Neher, 2000). These findings are consistent with the idea that syts with distinct Ca^{2+} sensitivities might determine the Ca^{2+} requirements for fusion (Li et al., 1995; Gerona et al., 2000; Sugita et al., 2002). Interestingly, some syt isoforms are weak Ca^{2+} sensors, and up-regulation of one of these isoforms, syt IV, reduces secretion (Littleton et al., 1999; Wang et al., 2001; but see also Robinson et al., 2002).

Multiple syt isoforms can operate in parallel in at least some cell types. For example, PC12 cells express high levels of syts I and IX, and antibodies directed against either isoform partially interfere with release (Elferink et al., 1993; Fukuda et al., 2002a). However, PC12 cells lacking syt I exhibit robust Ca^{2+}-dependent exocytosis (Shoji-Kasai et al., 1992); thus, syts I and IX appear to function in a redundant manner. Finally, in mice, *Caenorhabditis elegans*, and *Drosophila*, some degree of regulated exocytosis persists in syt I–null mutants (DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994; Jorgensen et al., 1995), suggesting that additional syt isoforms, or other Ca^{2+}-sensing proteins, also regulate release.

Some of the first evidence that syt I plays a role in secretion came from inhibition experiments using recombinant fragments and peptides corresponding to regions of syt I (Bommert et al., 1993; Elferink et al., 1993). To understand how syt triggers release, we used a panel of recombinant C2 domains derived from several syt isoforms to acutely inhibit secretion from cracked PC12 cells. We then screened the biochemical properties of these C2 domains and found that inhibition of exocytosis was closely related to the ability of C2 domains to bind three molecules essential for fusion: phosphatidylinositol 4,5-bisphosphate (PIP_2), syntaxin, and SNAP-25. These findings suggest that syts regulate exocytosis via direct interactions with both target membrane phospholipids and components of the fusion complex.

Results

Inhibition of Ca^{2+}-triggered catecholamine release from PC12 cells by C2A domains derived from syt isoforms I–XI

We reasoned that C2 domains derived from multiple syt isoforms would inhibit secretion from cracked PC12 cells based on their ability to disrupt endogenous syt–effector interactions that are critical for large dense core vesicle (LDCV) fusion with the plasma membrane (Fig. 1 A). Since many syt isoforms are likely to function in the same manner, inhibitory C2 domains would be expected to interact with a common set of effectors, thus providing insight into the mechanism of syt function during Ca^{2+}-triggered fusion. To address this, we generated C2A domains from syts I–XI and measured their ability to inhibit catecholamine release from cracked PC12 cells using a recently developed real-time voltammetry assay (Earles et al., 2001). C2A domains were chosen because of their relative ease of bacterial expression compared with the C2B or tandem C2A-C2B domains of the syt family. Furthermore, isolated C2 domains engage fewer effectors than the tandem C2 domains, simplifying screens for common effectors. We then correlated the ability of the C2 domains to inhibit release with their ability to engage a set of well-characterized syt-binding partners.

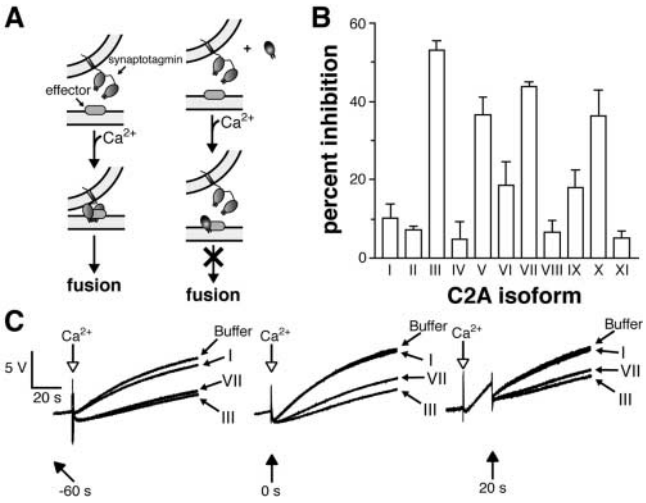


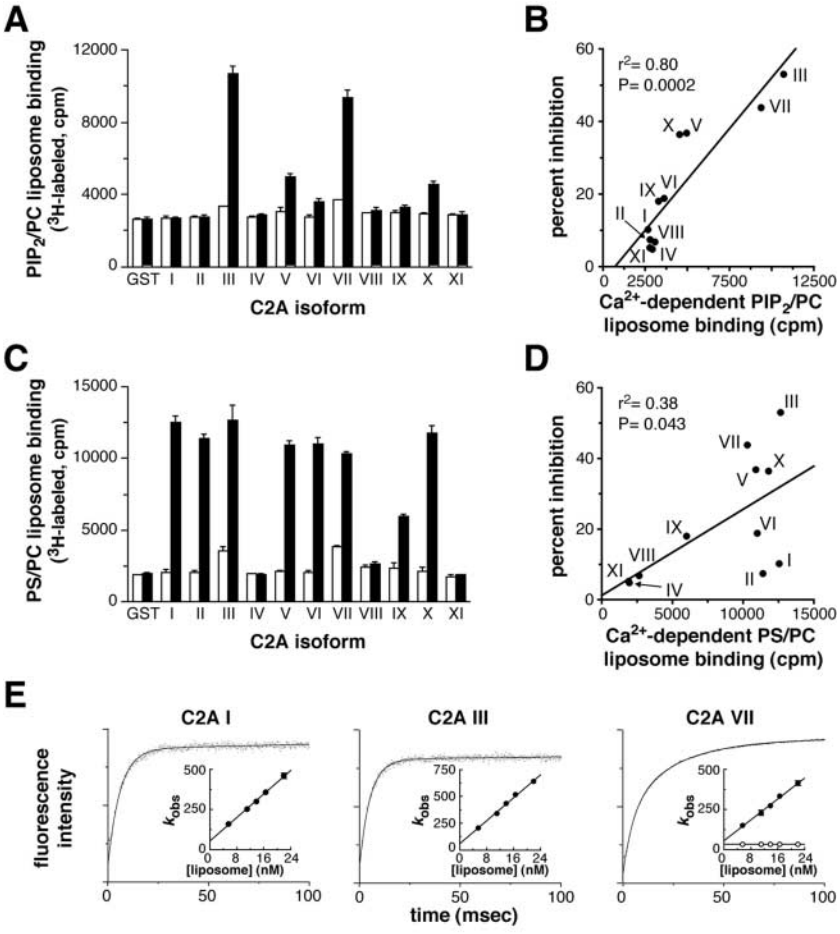
Figure 1. **Screening the C2A domains of syt I–IX for inhibition of exocytosis.** (A) A model of syt function during exocytosis; Ca^{2+}–syt triggers release via interactions with effector molecules. Addition of exogenous C2 domains derived from different syt isoforms competes with endogenous syt for effector interactions, thereby inhibiting fusion. (B) C2A domains derived from syts I–XI exhibit different abilities to disrupt catecholamine release from PC12 cells. The indicated C2A domain (10 μM) was added to the reaction chamber 1 min before release was triggered with 100 μM $CaCl_2$. Control samples lacking recombinant protein (buffer alone) were analyzed in parallel and used to calculate the percentage of inhibition. We note that some experiments were performed at the lowest [Ca^{2+}] at which we could reliably measure secretion; in these experiments, the C2A domains of syt III and VII exhibited the same inhibitory activity that was observed at 100 μM Ca^{2+} (unpublished data). (C) Inhibition of catecholamine release by C2A-III and C2A-VII occurs at all times during the release process. C2A-I, -III, and -VII (10 μM) were added at the indicated time (relative to Ca^{2+} addition). For each timing experiment, release profiles were superimposed along with a control trace (buffer only).

The C2A domains derived from syts I–XI exhibited distinct abilities to interfere with exocytosis: III, V, VII, and X were the most effective inhibitors (>35% at 10 μM; Fig. 1 B). This is in agreement with previous reports showing that C2A-III and C2A-VII inhibit release of ^{3}H-norepinephrine from PC12 cells (Shin et al., 2002; Sugita et al., 2001, 2002). Notably, the C2A domains from syts I and IX failed to inhibit secretion by >20%. Syts I and IX are thought to be the primary Ca^{2+} sensors for exocytosis in PC12 cells (Fukuda et al., 2002a; Zhang et al., 2002). However, others have argued that the inhibitory activity of C2A-III and C2A-VII demonstrates that these isoforms are the major Ca^{2+} sensors for exocytosis in PC12 cells (Sugita et al., 2001, 2002). Below, we provide data that resolve this controversy.

Further experiments were performed to assess the timing of inhibition by the inhibitory C2A domains. The inhibitory effects of C2A-III and VII did not require preincubation with cracked cells; inhibition was observed whether the C2A domains were added before, concurrently with, or after the addition of Ca^{2+} (Fig. 1 C). These data indicate that the onset of inhibition occurs during a late, postdocking step in this system, consistent with previous studies of syt function (Desai et al., 2000; Earles et al., 2001).

C2A-mediated inhibition of release correlates with PIP_2 and t-SNARE–binding activity

Ca^{2+} triggers the penetration of the Ca^{2+}-binding loops of syt into the surface of lipid bilayers that contain anionic phospholipids (e.g., phosphatidylserine [PS]), and this interaction has been proposed to function as a coupling step in excitation-secretion coupling (Bai et al., 2002). Whereas PS-binding activity has been characterized for the isolated C2A domains of syts I–VIII (Li et al., 1995; Sugita et al., 2002), little is known concerning the interaction of syts with other lipids, particularly PIP_2, which has been shown to bind syt I (Schiavo et al., 1996). PIP_2 is a plasma membrane lipid that is essential for exocytosis of LDCVs (Eberhard et al., 1990; Hay and Martin, 1993; Hay et al., 1995), potentially via its interactions with syt. Therefore, we screened the C2A domains used in the inhibition studies for PS- and PIP_2-binding activity (Fig. 2, A and C). With the exception of isoforms IV, VIII, and XI, the C2A domains of the syt family bound PS-containing liposomes in response to Ca^{2+} (Fig. 2 C). The PIP_2-binding profile, however, was dramatically dif-



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Kinetics of C2A domain•membrane interactions

	25% PS			*1.5% PIP_2/25% PS*		
	k_{on} ($x10^{10}M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)	k_{on} ($x10^{10}M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
C2A I	1.82 ± 0.38	54.5 ± 6.1	3.00	1.60 ± 0.27	52.4 ± 6.3	3.28
C2A III	2.67 ± 0.26	59.8 ± 4.3	2.24	2.31 ± 0.23	16.8 ± 10.2	0.73
C2A VII	1.62 ± 0.49	57.7 ± 6.6	3.55	1.78 ± 0.14	16.5 ± 9.2	0.93

Figure 2. **Inhibition of secretion by C2A domains is correlated with PIP_2-binding activity.** GST fusions of the indicated C2A isoforms (0.13 nmole) were immobilized on glutathione-Sepharose beads and assayed for ^3H-labeled liposome binding in the presence of 2 mM EGTA (white bars) or 100 μM Ca^{2+} (black bars) as described in Materials and methods. (A) PIP_2-binding properties of C2A from syt I–XI. Liposomes were composed of 1.5% PIP_2/98.5% PC. (B) The extent of inhibition (Fig. 1 B) was plotted versus the extent of PIP_2-binding activity at 100 μM Ca^{2+} (A). Data were fitted by linear regression; r^2 values and P values are reported. (C) PS-binding properties of C2A from syt I–XI. Liposomes were composed of 25% PS/ 75% PC. (D) The extent of inhibition (Fig. 1 B) was plotted versus the extent of PS-binding activity at 100 μM Ca^{2+} (C). Note that C2A-I and -II exhibit robust PS-binding activity but fail to inhibit release. (E) Kinetic studies of C2A–PS/PC liposome interactions. Fluorescence resonance energy transfer between native tryptophan residue(s) (donor) in C2A and dansyl-PE incorporated into liposomes (acceptor; 5% with 25% PS/70% PC) was used to monitor the time course of C2A–PS/PC membrane interactions as described previously (Davis et al., 1999). Kinetic traces for each C2A (3 μM [final]) were obtained by rapid mixing with liposomes (11 nM [final]) plus Ca^{2+} (100 μM [final]). The observed rates (k_{obs}) were determined by fitting the data with a single exponential function for C2A-I and -III; data for C2A-VII were best fitted by a double exponential function. (Inset) k_{obs} is plotted as a function of the indicated liposome concentration. Note the k_{obs} of the slower component (○) of C2A-VII is not dependent on liposome concentration, indicating that it represents a conformational change or complex rearrangement, such as oligomerization (Fukuda and Mikoshiba, 2001; Wu et al., 2003), after C2A–liposome complex assembly; only the fast component (●) is considered here. Error bars represent that SD from three independent experiments. (F) PIP_2 enhances the affinity of C2A-III and C2A-VII, but not C2A-I, for membranes. Values for k_{off}, k_{on}, and K_D of the C2A–liposome interaction were calculated as described previously (Davis et al., 1999). C2A-I, -III, and -VII bind to liposomes containing 25% PS (with 70% PC and 5% dansyl-PE) with similar rate constants and affinities, whereas C2A-III and -VII exhibit a three- to fourfold lower K_D for liposomes containing 1.5% PIP_2 and 25% PS (with 68.5% PC and 5% dansyl-PE).

ferent (Fig. 2 A). PIP_2-containing liposomes bound C2A-III and C2A-VII and to a lesser extent C2A-V and C2A-X. Hence, PIP_2–C2A interactions are restricted to fewer syt isoforms than is PS-binding activity. However, as detailed below, in some syt isoforms PIP_2 binding is mediated by the C2B domain (see Fig. 6; Schiavo et al., 1996).

PIP_2-binding activity and inhibition of catecholamine release from PC12 cells were well correlated (Fig. 2 B; $r^2 = 0.80$). This finding suggests that PIP_2-binding C2A domains disrupt the interaction of endogenous syt with PIP_2 to reduce LDCV fusion. We also found that PS-binding activity did not always result in inhibition; PS binding was only weakly correlated with inhibition (Fig. 2 D; $r^2 = 0.38$). For example, C2A-I and II failed to inhibit release by >10% despite the fact that both C2 domains bind PS to the same extent as the inhibitory C2A domains under the same conditions (e.g., C2A-III and -VII). These results suggest that inhibitory domains affect release via interactions with PIP_2 but not PS.

In contrast to the findings above, it was suggested previously that C2A domains derived from different syt isoforms inhibit secretion in PC12 cells due to their ability to bind PS in the presence of Ca^{2+} (Shin et al., 2002; Sugita et al., 2002). The lipid-binding assays illustrated in Fig. 2 were performed using single concentrations of C2A, lipid, and Ca^{2+}. It remained possible, therefore, that at 100 μM Ca^{2+} (the conditions of our release assays) C2A from syts III and VII may bind PS more tightly than C2A-I and are therefore more inhibitory. To address this, we measured the kinetics of binding of noninhibitory (C2A-I) and inhibitory (C2A-III and C2A-VII) C2 domains to liposomes composed of 25% PS and 75% phosphatidylcholine (PC). Binding was monitored via FRET and resolved in time using a stopped-flow rapid mixing approach (Davis et al., 1999). As shown in Fig. 2 E, all three C2A domains bound to PS/PC liposomes with nearly identical kinetics. From k_{on} and k_{off}, the K_d for all three domains was found to be virtually identical: 2.2–3.5 nM at 100 μM Ca^{2+} (Fig. 2 F). These data demonstrate that differences in the abilities of the C2A domains derived from syts I, III, and VII to inhibit release are not due to differences in their abilities to bind PS. We observed that C2A-VII also exhibited an additional, slow kinetic component that reflects a postmembrane-binding rearrangement, which may correspond to membrane-induced oligomerization (Fukuda and Mikoshiba, 2001; Wu et al., 2003). We also note that these kinetics data differ somewhat from those in a previous report using a mutant form of C2A-I (Davis et al., 1999); these differences are due to the use of synthetic lipids and wild-type C2A-I for the experiments shown in Fig. 2 E.

These data raise the question of whether the C2 domains of syt can sense PIP_2 in the presence of the relatively high levels of PS that are found on the inner leaflet of the plasma membrane (Breckenridge et al., 1972; Westhead, 1987). To address this, the kinetics experiments shown in Fig. 2 E were repeated using liposomes composed of 25% PS plus 1.5% PIP_2. These data are summarized in Fig. 2 F, where it is apparent that inclusion of PIP_2 increased the affinity of C2A-III and C2A-VII for membranes by three- to fourfold; C2A-I was unaffected by PIP_2 and served as a negative control. These data establish that, in an environment in which the anionic lipid is predominantly PS, the properties of at least some C2 domains are still affected by the presence of physiological levels of PIP_2 (i.e., 1–3%; Tran et al., 1993).

As noted in the Introduction, syt also forms direct contacts with syntaxin and SNAP-25, two components of the fusion complex. This interaction provides a compelling connection between the Ca^{2+} sensor for exocytosis and the fusion apparatus. We explored the idea that C2A domains might inhibit release by blocking syt–SNARE interactions by assaying the ability of C2A from syts I–XI to bind purified SNAP-25–syntaxin heterodimers (Fig. 3). Isoforms III, V, VII, and X exhibited robust Ca^{2+}-promoted binding activity, whereas binding was much weaker or devoid for the other isoforms (Fig. 3 B). The correlation between the abil-

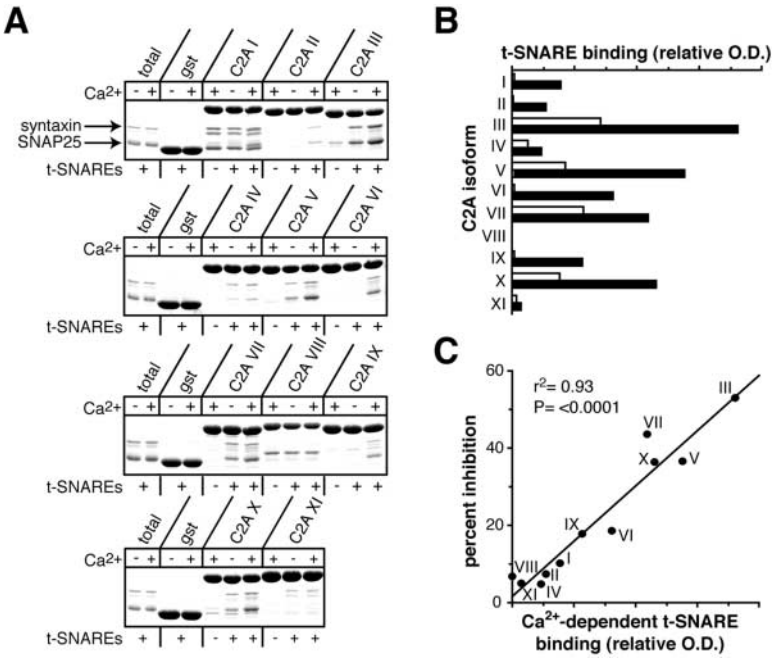

Figure 3. **Inhibitory C2A domains bind t-SNAREs.** (A) t-SNARE–binding profiles of the C2A domains derived from syts I–XI. Immobilized GST–C2A fusions (20 μg in 150 μl HBS plus 0.5% Triton X-100) and 5 μM t-SNARE hetero-dimer were incubated in 2 mM EGTA (− Ca^{2+}) or 1 mM $CaCl_2$ (+ Ca^{2+}). 20% of bound protein and 3% of total t-SNARE was subjected to SDS-PAGE and stained with Coomassie blue. (B) The C2A domains exhibit different degrees of Ca^{2+}-dependent t-SNARE binding. The t-SNARE–binding assay shown in A was quantified by densitometry, and relative optical density (O.D.) was plotted in the presence (black bars) or absence (white bars) of Ca^{2+}. (C) Inhibition of catecholamine release by C2A domains correlates with t-SNARE binding. The percentage of inhibition was plotted as a function of Ca^{2+}-dependent t-SNARE binding.

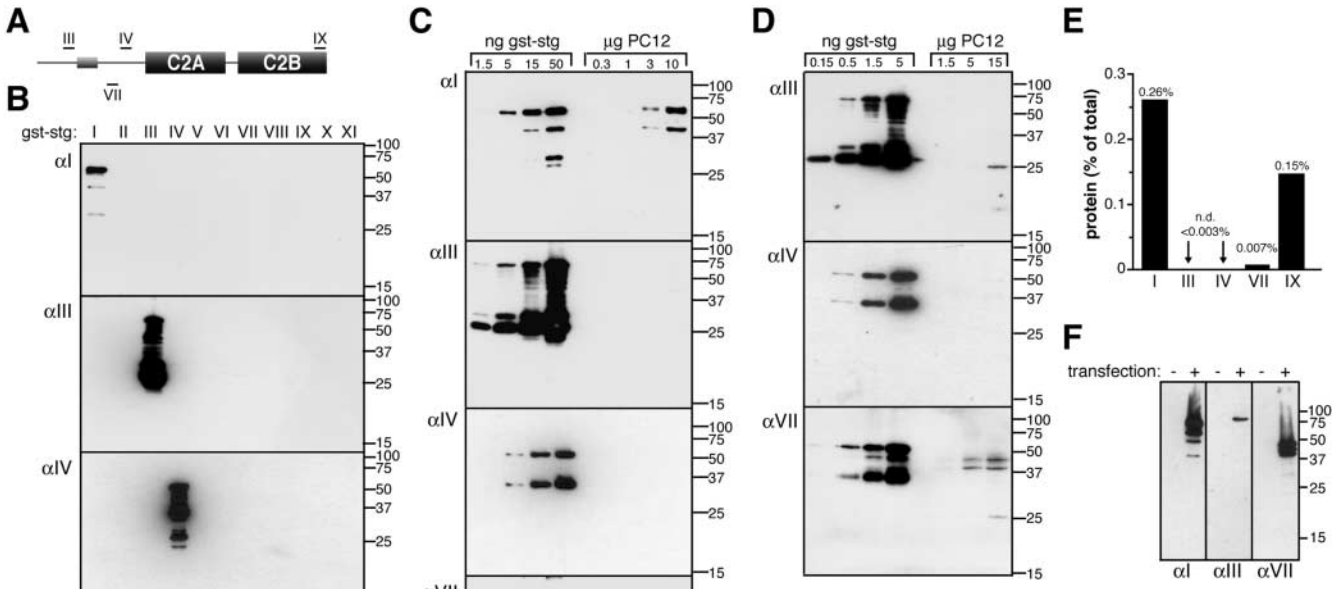

Figure 4. **Syts I and IX are the major syt isoforms in PC12 cells.** (A) Peptides corresponding to the indicated regions of syts III, IV, VII, and IX were used to produce polyclonal antibodies as described in Materials and methods. (B) Specificity of the anti-syt (α-syt) antibodies. 100 ng of the indicated GST–syt fragments lacking the C2B domain (αI, III, IV, and VII) or composed of only the cytoplasmic domain (αIX) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibody. Truncated syt fragments were used due to their relative ease of expression and isolation compared with full-length syts. Recombinant protein preparations often contained multiple proteolytic fragments as a result of bacterial expression; quantification of recombinant standards was based on the abundance of the band corresponding to the molecular weight of the intact protein. Each of the antibodies is specific for the syt it is raised against. For αI, mAb 41.1 was used (Chapman and Jahn, 1994). (C) Syt expression levels in PC12 cells. The indicated amounts of recombinant standards and postnuclear PC12 cell membranes (total protein) were subjected to SDS-PAGE and immunoblot analysis using the indicated antibodies. (D) Syt VII is a low abundance Ca^{2+} sensor in PC12 cells. Immunoblots for syts III, IV, and VII were performed as described in C with reduced amounts of standards and increased amounts of cell membranes as indicated. (E) Blots shown in C and D were quantified by densitometry and plotted as a percentage of total protein from membranes. Since multiple bands were detected for each isoform expressed, quantitation was based on total immunoreactivity from cell extracts by summing the density of all bands. (F) Anti–syt-III and -VII antibodies detect syt III and VII expressed in fibroblasts. HEK cells were transfected with expression constructs (pCI-neo; Promega) for syt isoforms I, III, or VII (+), or empty vector (−), and probed with their respective antibody.

ity of C2A domains to bind neuronal t-SNAREs in the presence of Ca^{2+} and to inhibit release was striking (Fig. 3 C; $r^2 = 0.93$); the extent of inhibition by a C2A domain is directly proportional to its ability to engage t-SNAREs. These results suggest that inhibitory C2A domains inhibit catecholamine release from PC12 cells by disruption of endogenous syt–SNARE interactions and by perturbing syt–PIP_2 interactions.

Expression levels of syt isoforms in PC12 cells

The experiments described above provide strong evidence that C2A domains inhibit regulated secretion in PC12 cells by binding PIP_2 and/or t-SNAREs, presumably by blocking endogenous syt–PIP_2–t-SNARE interactions to disrupt a late step in the fusion reaction (Fig. 1 A). Next, we asked which isoforms of syt are being interfered with by the exogenous C2 domains characterized above. There has been considerable controversy regarding which syt isoforms are expressed in PC12 cells. Zhang et al. (2002) performed immunoblot studies using antibodies directed against syt isoforms I–XIII. Syt isoforms I, IV, and IX were the only isoforms that were detected in PC12 cells. All three isoforms have been localized to LDCVs (Wang et al., 2001; Fukuda et al., 2002a, 2003). Syt I and IX were expressed at much higher levels than syt IV and appear to function as redundant Ca^{2+} sensors for LDCV fusion (Shoji-Kasai et al., 1992; Fukuda et al., 2002a). In contrast, others have proposed that syts III and VII serve as the major Ca^{2+} sensors in PC12 cells (Sugita et al., 2001, 2002; Shin et al., 2002). To resolve this controversy, we generated antibodies against syts III, VII, and IX and quantified the abundance of each isoform in PC12 cells.

Peptides corresponding to nonconserved segments of syt III, VII, and IX were used to raise isoform-specific antibodies (Fig. 4 A). In the case of syt VII, the peptide corresponded to a region present in all predicted splice variants (Sugita et al., 2001). Antibodies to syt IV, which was previously reported to be expressed at low levels in PC12 cells (Zhang et al., 2002), were also generated. After affinity purification, antibodies were screened for specificity. mAb

41.1 was used to detect syt I (Chapman and Jahn, 1994). As shown in Fig. 4 B, all five antibodies recognized only the appropriate syt isoform. The antibodies were then used to carry out quantitative immunoblot analysis of PC12 cell membranes using recombinant protein standards (Fig. 4 C). Syts I and IX were readily detected and accounted for ~0.26 and 0.15%, respectively, of the total membrane protein (Fig. 4, C and E). The ratio of syt I to syt IX (~1.6) is similar to the value (~1.3) reported previously (Zhang et al., 2002). Syts III, IV, and VII were not detected at the level of sensitivity adequate for isoforms I and IX (Fig. 4 C). In an effort to detect these isoforms, we decreased the levels of the standards loaded onto the blots, and we increased the level of PC12 cell membranes (Fig. 4 D). Under these conditions, the syt VII antibody yielded bands at ~45, 40, and 26 kD. Together, these bands represent ~0.007% of the total protein (Fig. 4 E) and is 37 times less abundant that syt I. The multiple syt VII bands are likely to reflect splice variants (Sugita et al., 2001) or degradation products. A similar banding pattern was seen in HEK cells transfected with full-length syt VII, although the intensity of the 26-kD band was significantly reduced relative to the higher molecular weight bands (Fig. 4 F). In brain extracts, higher molecular weight bands (~76 kD) and a virtually identical banding pattern to that reported by Sugita et al. (2001) were observed (unpublished data), indicating that these antibodies recognize the same antigens. A faint band at ~26 kD became apparent in the high sensitivity syt III blots, but bands expected for the full-length protein were not observed (Fig. 4 D). It is not clear whether the 26-kD band is the result of nonspecific binding, cross reactivity, or a degradation product of the full-length protein. Transfection of syt III in HEK cells gave rise to a single band at ~80 kD (Fig. 4 F), consistent with a previous report (Fukuda et al., 1999). Hence, this antibody is able to recognize syt III expressed in mammalian cells. On some blots, a faint syt IV band was present, but the level of expression was too low to quantify.

Given these data, syts I and IX are likely to be the primary syts in PC12 cells. It cannot be ruled out that syt VII may play a role in Ca^{2+}-dependent LDCV exocytosis. At present, it has not been possible to localize native syt VII using currently available antibodies. In transiently transfected PC12 cells, syt VII–GFP fusion proteins were initially reported to localize to the cell periphery, and it was suggested that they were targeted to the plasma membrane (Sugita et al., 2001). In a later study, using stable PC12 cell lines, syt VII-GFP was shown to colocalize with syt I and trans Golgi markers (Fukuda et al., 2002b). Thus, syt VII appears to be on an organelle that participates in Ca^{2+}-triggered exocytosis. However, our immunoblot data indicate that this isoform is at least 30 times less abundant that syt I. Furthermore, >50% of the syt VII detected in our blots is truncated and may lack an intact cytoplasmic domain (Fig. 4, D and E).

In syt I and IX, critical effector interactions require tandem C2 domains or are localized to the C2B domain

If syts I and IX are the primary Ca^{2+} sensors in PC12 cells, why do the C2A domains derived from these isoforms fail to inhibit catecholamine release? One possible explanation is that syt–effector interactions important for exocytosis are localized to either the C2B domain or to the tandem C2 domains of syt I and IX. Indeed, in syt I the C2B domain but not the C2A domain binds PIP_2 (Schiavo et al., 1996) and inhibits secretion from PC12 cells (Desai et al., 2000). Furthermore, in syt I both C2A and C2B are needed for high affinity t-SNARE–binding activity (Chapman et al., 1996; Davis et al., 1999; Gerona et al., 2000). In the following paragraphs, we further explore this possibility.

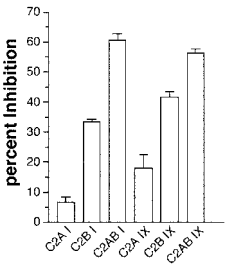

Figure 5. **The C2B and C2A-C2B domains from syt I and syt IX inhibit exocytosis.** C2A, C2B, and C2A-C2B domains (10 μM) of syt isoforms I and IX were assayed for their ability to inhibit exocytosis as described in Fig. 1 B.

Recombinant C2B-I copurifies with a bacterial contaminant that appears to be RNA (Ubach et al., 2001; Wu et al., 2003; unpublished data). Others have reported that removal of this contaminant disrupts inhibition mediated by C2B-I (Sugita et al., 2001; Shin et al., 2003). For our studies, recombinant proteins including t-SNAREs and C2 domains were treated with nucleases and high salt buffers, which completely remove bacterial contaminants. We found that contaminant-free C2B-I retained inhibitory activity (Fig. 5; Desai et al., 2000). Thus, inhibition of secretion is not an artifact due to the presence of the contaminant.

If syts I and IX are the major Ca^{2+} sensors for LDCV exocytosis in PC12 cells, one would expect that their cytoplasmic domains (C2A-C2B) would interfere with endogenous syt I/IX–effector interactions and block release. This was indeed the case: the cytoplasmic domains of syts I and IX (10 μM) inhibited exocytosis by 61 and 56%, respectively (Fig. 5). As in the case of syt I, most of the inhibitory activity of syt IX mapped to the C2B domain; again, C2A-I and -IX were poor inhibitors. We next investigated whether the inhibition of release mediated by fragments of syts I and IX was correlated with PS, PIP_2, and/or t-SNARE–binding activity.

The isolated C2B domains of both syt I and IX exhibit little PS-binding activity (Fig. 6 A; note that C2B-I binds PS tightly when tethered to C2A [Bai et al., 2002]). Thus, there is no correlation between PS-binding activity and inhibition of catecholamine release by the C2 domains from syts I and IX (Fig. 6 B; $r^2 = 0.02$). In contrast, there is a clear correlation between the ability of fragments of syts I and IX to bind PIP_2 and to inhibit catecholamine release (Fig. 6, C and D; $r^2 = 0.93$).

We next determined whether the ability of syts I and IX fragments to block release was also correlated with their ability to bind t-SNAREs. In a GST pull-down assay, the C2B and the C2A-C2B domains of syts I and IX exhibited strong binding to syntaxin–SNAP-25 t-SNARE heterodimers (Fig. 7, A and B). Consistent with previous reports, GST-C2A exhibited little t-SNARE–binding activity (Chapman et al., 1996; Davis et al., 1999). There was a strong correlation be-

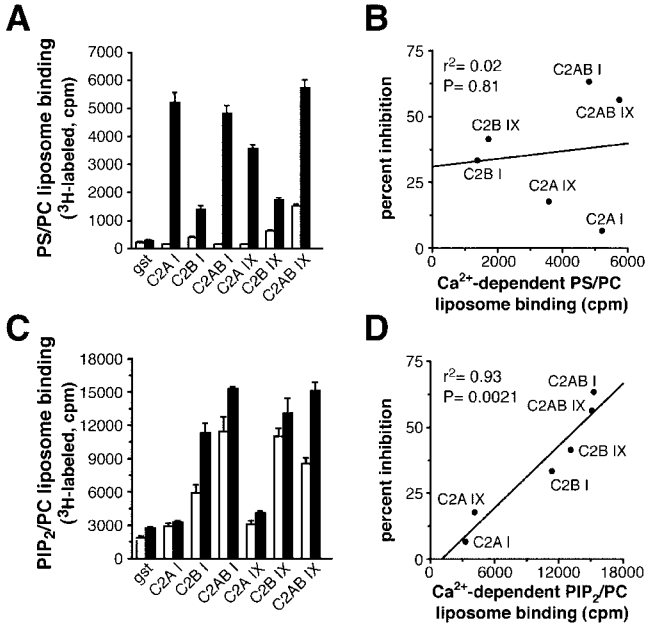

Figure 6. **PIP$_2$-binding activity and inhibition of exocytosis are correlated and localized to the C2B domains of syts I and IX.** (A) PS-binding activity of the indicated syt I/IX constructs was assayed as described in the legend to Fig. 2 C. (B) PS-binding activity does not correlate with inhibition of secretion. The percentage of inhibition of catecholamine release (Fig. 5) was plotted as a function of Ca^{2+}-dependent PS/PC liposome-binding activity and fitted by linear regression. For syts I and IX, PS binding is not necessary for inhibition since the C2B domains bind PS weakly yet inhibit release. (C) PIP$_2$-binding activity of the indicated syt I/IX constructs was assayed as described in the legend to Fig. 2 A. (D) PIP$_2$ binding is correlated with inhibition of secretion. The percentage of inhibition (Fig. 5) was plotted as a function of Ca^{2+}-dependent PIP$_2$ binding (C) and fitted by linear regression.

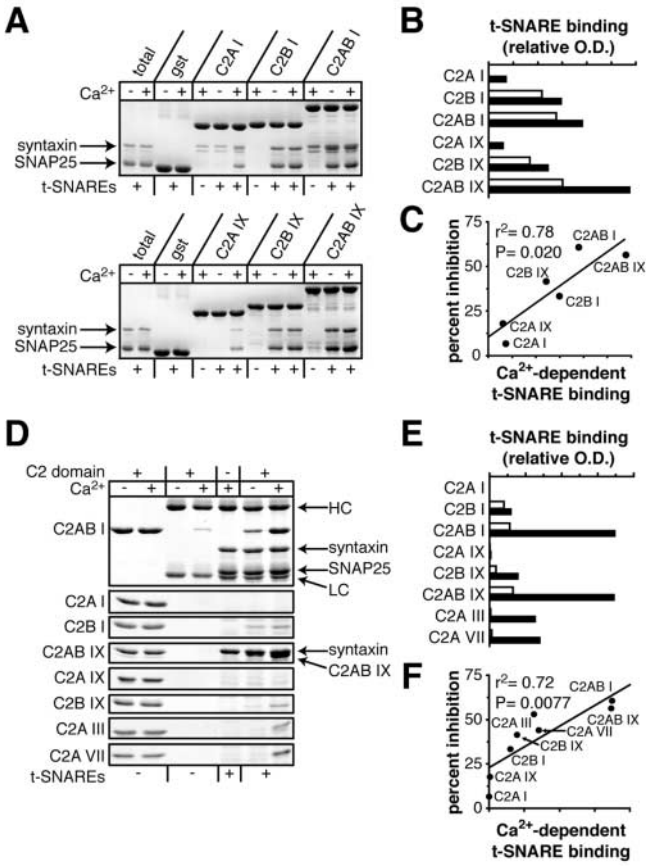

Figure 7. **Inhibitory fragments of syt I and IX bind t-SNAREs.** (A) GST-C2A, -C2B, or -C2A-C2B (3 μM) from syts I and IX was immobilized on beads and assayed for t-SNARE heterodimer- (5 μM) binding activity, as described in Materials and methods, in the presence of EGTA (2 mM) or Ca^{2+} (1 mM). Proteins were visualized by staining with Coomassie blue. (B) Quantitation of t-SNARE–binding activity was determined by densitometry of bands shown in A. EGTA, white bars; Ca^{2+}, black bars. (C) Inhibition of secretion correlates with t-SNARE–binding activity. The percentage of inhibition (Fig. 5) is plotted as a function of Ca^{2+}-dependent t-SNARE–binding activity (B). (D) Coimmunoprecipitation of syt I and IX with t-SNAREs. Soluble syt fragments (2 μM) were incubated with soluble t-SNARE heterodimer (2 μM) in 2 mM EGTA or 1 mM Ca^{2+} for 1 h. Syntaxin was then immunoprecipitated by adding mAb HPC-1 and protein G–Sepharose. Immunoprecipitates were subjected to SDS-PAGE; gels were stained with Coomassie blue. (E) Quantitation of t-SNARE–binding assayed by coimmunoprecipitation. Quantitation was performed by densitometry as described in B. (F) The percentage of inhibition (Fig. 5) was plotted as a function of Ca^{2+}-dependent t-SNARE binding as determined by coimmunoprecipitation in E.

tween t-SNARE–binding activity and inhibition of secretion by the syts I and IX fragments (Fig. 7 C; $r^2 = 0.78$). These data further support a model in which syt–SNARE and syt–PIP$_2$ interactions are essential steps in Ca^{2+}-triggered exocytosis. Furthermore, these studies establish the idea that the biochemical activity of a given C2 domain cannot be predicted by its position in a parent polypeptide. In some cases, an activity that is localized to the first C2 domain (C2A) in one isoform might only be manifest in the second C2 domain (C2B) of another isoform.

Several studies concluded that tandem C2 domains are needed for syt I to efficiently bind t-SNAREs (Chapman et al., 1995, 1996; Davis et al., 1999; Gerona et al., 2000; Earles et al., 2001). However, in the GST pull-down experiment presented in Fig. 7 A, the isolated C2B domain of syt I bound the t-SNARE complex nearly as effectively as C2A-C2B (Fig. 7, A and B). Thus, it appears that either the GST moiety, or the immobilization of proteins onto beads, can affect interactions with SNAREs. Therefore, we tested the ability of the C2 domains derived from syts I and IX to bind t-SNAREs using a solution-based binding assay. Soluble syt fragments were incubated with soluble t-SNAREs, and t-SNARE complexes were immunoprecipitated using an antibody directed against syntaxin (Fig. 7, D and E). C2A-C2B from syt I and IX engaged t-SNAREs much more effectively than did the isolated C2 domains. The C2B domains of syts I and IX exhibited a low level of SNARE-binding activity; binding of the C2A domains could not be detected in this assay system (Fig. 7). For comparison, we tested the ability of the C2A domains from syts III and VII, which again inhibit catecholamine release, to engage SNAREs using this assay system. In contrast to C2A-I and C2A-IX, both C2A-III and C2A-VII exhibited significant levels of t-SNARE–binding activity (Fig. 7, D and E). These data further support the idea that inhibitory C2 domains interfere with secretion by interacting with t-SNAREs (Fig. 7 F; $r^2 = 0.72$).

We reiterate that the two assays used to monitor syt–SNARE interactions yield differences in the extent and Ca^{2+} dependence of binding, particularly in the case of C2B and C2A-C2B from syt I and IX (Figs. 3 and 7). These differences might explain some of the apparent discrepancies that arose from previous studies in which binding of syt I to SNAREs was either Ca^{2+} dependent (Chapman et al., 1995; Davis et al., 1999) or largely Ca^{2+} independent (Schiavo et al., 1997). In the former study, a coimmunoprecipitation approach was used, and in the latter study syt I fragments were immobilized on beads as GST fusion proteins. It is important to note that native syt I–SNARE interactions are likely to be Ca^{2+} dependent in vivo, since cross-linking experiments of endogenous syt to SNAP-25 in semiintact PC12 cells is strongly dependent on increases in Ca^{2+} (Zhang et al., 2002). Furthermore, syt and t-SNAREs coimmunoprecipitate from brain extracts in a Ca^{2+}-promoted manner (Chapman et al., 1995).

Discussion

The data reported here are consistent with the hypothesis that members of the syt family confer Ca^{2+} sensitivity to membrane fusion through Ca^{2+}-dependent interactions with PIP_2 and t-SNAREs. These effector molecules are localized to the target membrane where they play a critical role in exocytosis (Jahn and Südhof, 1999; Holz et al., 2000). PIP_2 is thought to function via recruitment of proteins to their sites of action (Cremona and De Camilli, 2001). Our results are consistent with the idea that PIP_2 recruits the cytoplasmic domain of syt toward the target membrane during exocytosis. This trans interaction might contribute to the ensemble of vesicle/target membrane interactions that pull the bilayers together to ultimately mediate fusion. Syt–PIP_2 interactions could also enhance binding of syt to t-SNAREs. We note that in our studies, all of the C2 domains that bound to t-SNAREs also bound to PIP_2 and vice versa (r^2 values range from 0.651–0.786, depending on the assay system used). It is possible that C2 domains evolved to recognize both effectors in a mutually dependent way (detailed below).

Syt I and IX are the major isoforms in PC12 cells, and both isoforms require relatively high levels of Ca^{2+} ($[Ca^{2+}]_{1/2}$ ~100 μM) to bind t-SNAREs (Chapman et al., 1995; unpublished data). This value is well above the $[Ca^{2+}]_{1/2}$ for catecholamine release from these cells (~1–20 μM; Klenchin et al., 1998; Earles et al., 2001), raising the concern that syt–SNARE interactions may not occur at physiologically relevant Ca^{2+} concentrations in situ. However, the affinity of syt for Ca^{2+} is markedly enhanced by anionic lipids (Brose et al., 1992), indicating that syt–SNARE interactions may occur at lower Ca^{2+} concentrations in the presence of membranes. Consistent with this idea, syt appears to be able to simultaneously interact with both SNAREs and membranes (Davis et al., 1999). Furthermore, native syt I cross-links to SNAP-25 in PC12 cell membrane fragments in a Ca^{2+}-dependent manner. Cross-linking was largely complete at 10 μM Ca^{2+}, perhaps owing to the presence of lipid bilayers (Zhang et al., 2002). We have attempted to directly measure anionic lipid induced shifts in the Ca^{2+} requirements for syt–t-SNARE–binding activity but have been hampered by the formation of large aggregates (unpublished data). Thus, in our biochemical assays we used high $[Ca^{2+}]$ in order to drive efficient syt–SNARE interactions. In a recent report, it was proposed that Sr^{2+} triggers exocytosis without activating syt I–t-SNARE interactions (Shin et al., 2003). However, other studies indicate that Sr^{2+} is able to promote syt–t-SNARE interactions (Chapman et al., 1995; unpublished data).

These data might provide an explanation for the correlation between the ability of the C2 domains characterized here to bind to both t-SNAREs and PIP_2. In this model, PIP_2 is the relevant lipid that increases the affinity of syt for Ca^{2+} such that syt can engage t-SNAREs at physiological levels of Ca^{2+}. Previous studies indicate that Ca^{2+}-enhanced binding of syt to t-SNAREs regulates SNARE complex assembly or function (Littleton et al., 2001). Indeed, the SNARE complex does not appear to assemble until arrival of the Ca^{2+} signal in permeabilized PC12 cells (Chen et al., 1999). Furthermore, mutations in *Drosophila* syt I are associated with a loss of SDS-resistant SNARE complexes (Littleton et al., 2001), further supporting the idea that syt I is required for the assembly of stable SNARE complexes.

A recent study using mutant versions of C2A-I and C2A-VII indicated that inhibition of catecholamine release from cracked PC12 cells was correlated with the Ca^{2+} requirements for binding to PS (Shin et al., 2002); C2A domains with high apparent Ca^{2+} affinities inhibited release, whereas those with lower affinities did not. This led the authors to suggest that syt functions during exocytosis by interacting with PS rather than with SNARE proteins. However, in these experiments C2 domains with relatively low apparent affinities for Ca^{2+} did not become inhibitory at Ca^{2+} concentrations that were sufficient to drive efficient binding to PS (Shin et al., 2002), arguing that C2A–PS interactions cannot mediate inhibition. Also, in our experiments the Ca^{2+}-triggered PS-binding activity of all of the C2 domains was saturated with Ca^{2+}, yet some PS-binding C2 domains failed to inhibit release (Fig. 1 B). Moreover, we found that C2A-I, a noninhibitory domain, and C2A-III and -VII, which are both potent inhibitors of release, bind PS-containing liposomes to the same extent, and with the same dissociation constants, at Ca^{2+} concentrations identical to those used in our secretion assays (Fig. 2, E and F). These data clearly demonstrate that PS-binding activity is not sufficient to block release under the conditions of our assay system. Finally, isolated C2B domains from syts I and IX lack strong PS-binding activity (Fig. 6 A) yet inhibit catecholamine release from PC12 cells (Fig. 5). In summary, PS-binding activity is neither necessary nor sufficient to block release.

It is possible that higher concentrations of PS-binding C2 domains could block exocytosis in our assay system. However, we note that a 100-nm liposome that contains 25% PS can bind ~225 syt molecules (Bai and Chapman, 2003). PS is a ubiquitous and abundant constituent of synaptosomal membranes (7–18 mol%; Breckenridge et al., 1972; Westhead, 1987). Therefore, PC12 cells (~10 μm in diameter) harbor a large number of C2 domain–binding sites, making them difficult to saturate without having nonspecific effects on other interactions within cracked cells. In contrast, PIP_2 is present at ~10-fold lower concentrations in cellular mem-

branes (Tran et al., 1993), and we estimate that in our release assays t-SNAREs are present at sub μM concentrations (~0.4 μM SNAP-25 and ~0.06 μM syntaxin; data unpublished). Thus, the levels of free PIP_2 and t-SNAREs are likely to be strongly affected by addition of μM concentrations of recombinant C2 domains.

What then is the in vivo function of the noninhibitory PS-binding C2A domains? Our quantitative immunoblots demonstrate that syts I and IX are the major Ca^{2+} sensors in PC12 cells (Fig. 4), yet their isolated C2A domains fail to inhibit release. The first clue as to the function of C2A-I was provided by genetic studies in *Drosophila*. A mutant allele, *AD1*, harbors a single point mutation that truncates the protein after the C2A domain (DiAntonio and Schwarz, 1994). The synaptic physiology of *AD1* flies is distinct from wild-type or syt-null flies (DiAntonio and Schwarz, 1994; Littleton et al., 1994). *AD1* exhibits some degree of synchronous release that is absent from null mutants (Yoshihara and Littleton, 2002). These data suggest that the role of C2A is to accelerate fusion. Other groups have mutated the C2A domain, but a consensus as to the effects of these mutations has not been reached (Fernández-Chácon et al., 2001, 2002; Robinson et al., 2002).

In our acute disruption experiments, inhibition of secretion could involve blockade of additional syt–effector interactions. For example, the ability of C2B-I to inhibit secretion from cracked cells is likely to also involve perturbation of native syt oligomerization (Desai et al., 2000; Littleton et al., 2001). This interpretation was recently called into question by data showing that recombinant C2B-I contains tightly bound bacterial contaminants. It was suggested that removal of the contaminant abolishes the ability of this domain to oligomerize in response to Ca^{2+} (Ubach et al., 2001); we have confirmed this finding. However, using EM to visualize syt bound to lipid bilayers that contain anionic phospholipids, we observed that membranes restore Ca^{2+}-triggered oligomerization activity (Wu et al., 2003). A future challenge will be do develop quantitative assays to determine whether there is a correlation between oligomerization activity and inhibition of secretion by C2 domains.

Unexpected differences between syt isoforms are beginning to emerge. For example, some effectors that bind to the C2B of isoform I (e.g., PIP_2) can bind to the C2A domain of a different isoform (e.g., syt VII). Thus, syts are biochemically more complex than initially appreciated. It is not clear why effector interactions are shuffled between tandem C2 domains, but these findings should be taken into account when single C2 domains are used for perturbation studies. We note that in several reports, the ability of a syt C2 domain to inhibit fusion has been interpreted as evidence for its function in the pathway under study (Sugita et al., 2001, 2002). For example, it has been argued that syt III is a major Ca^{2+} sensor in PC12 cells because the C2A domain derived from this isoform is potent inhibitor of release (Sugita et al., 2002). However, we could not detect any syt III in PC12 cells (Fig. 4). This is not unexpected: whereas the complete cytoplasmic domains of syts that regulate a given fusion step should inhibit secretion, fragments of irrelevant syt isoforms or even other molecules can also block secretion if they are able to bind the targets of the endogenous syt isoforms.

In summary, our data support a model in which syt triggers LDCV fusion in PC12 cells by interacting with PIP_2 and t-SNAREs, two effector molecules that are localized to the target membrane. We propose that these interactions facilitate fusion by pulling the vesicle and target membranes together to facilitate SNARE complex assembly.

Materials and methods

Recombinant proteins

cDNA encoding rat syt I (Perin et al., 1990), III (Mizuta et al., 1994), and IV (Vician et al., 1995) was provided by T.C. Südhof (University of Texas Southwestern Medical Institute, Dallas, TX), S. Seino (Chiba University, Chiba, Japan), and H. Herschman (University of California, Los Angeles, Los Angeles, CA), respectively. cDNA encoding mouse syts II and V–XI (Fukuda et al., 1999) was provided by M. Fukuda (Institute of Physical and Chemical Research, Saitama, Japan). The C2A, C2B, and C2A-C2B domains of syt I (the G374 form) and the C2A domain of syt III were prepared as described previously (Littleton et al., 1999; Desai et al., 2000; Earles et al., 2001). Syt fragments were subcloned into pGEX 2T-1 or 4T-1 (Amersham Biosciences) as described previously (Chapman et al., 1996). The aa residues encoding the C2A-C2B and C2A domains of each isoform are as follows: I, 96–421, 96–265; II, 139–423, 139–265; III, 290–569, 290–421; IV, 152–425, 152–278; V, 218–491, 218–349; VI, 143–426, 143–273; VII, 134–403, 134–262; VIII, 97–395, 97–238; IX, 104–386, 104–235; X, 223–501, 223–360; and XI, 150–430, 150–286. Syts lacking the C2B domain were prepared as follows: I, 1–265; II, 1–267; III, 1–423; IV, 1–280; V, 1–349; VI, 1–273; VII, 1–260; VIII, 1–234; IX, 1–232; X, 1–357; and XI, 1–283. Amino acid residues 215–386 encode the C2B domain of syt IX. All recombinant constructs were confirmed by DNA sequencing and expressed as GST fusion proteins. Proteins were purified by affinity chromatography with glutathione Sepharose 4B (Amersham Biosciences) and washed with nucleases and high salt buffer to remove bacterial contaminants as described in Wu et al. (2003). Soluble syt fragments were prepared by thrombin cleavage (Chapman et al., 1996).

The pTW34 construct for expression of the t-SNARE heterodimer composed of mouse his_6–SNAP-25 and rat syntaxin 1a was provided by T. Weber (Mount Sinai School of Medicine, New York, NY) and purified by NiNTA chromatography (Weber et al., 1998; Parlati et al., 1999).

Binding assays

All protein–protein interactions were performed in 150 μl of HBS (50 mM Hepes-NaOH, 100 mM NaCl, pH 7.4) with 0.5% Triton X-100 plus 2 mM EGTA or 1 mM $CaCl_2$. For GST pull-down assays, samples were incubated for 1 h at 4°C followed by three washes with binding buffer. For immunoprecipitation studies, soluble syt (C2A-C2B) was incubated with t-SNARE heterodimer plus 1 mg/ml BSA for 1 h at 4°C. Complexes were immunoprecipitated using an antisyntaxin mAb (7.5 μl HPC-1; provided by R. Jahn [Max Planck Institute for Biophysical Chemistry, Goettingen, Germany]) and 25 μl of a 50% slurry of protein G–Sepharose fast flow (Amersham Biosciences) as described (Chapman et al., 1995). Immunoprecipitates were washed three times in binding buffer, solubilized in SDS sample buffer, and subjected to SDS-PAGE; proteins were visualized by staining with Coomassie blue.

Radiolabeled liposome-binding assays were performed as described in Davis et al. (1999). L-3-phophatidyl(*N*-methyl-^{3}H)choline-1,2-dipalmitoyl was purchased from Amersham Biosciences. Error bars represent the SD from three separate determinations.

Real-time secretion assays

Catecholamine release from cracked PC12 cells was monitored using rotating disc electrode voltammetry was as described (Earles et al., 2001). Cumulative release was determined by measuring the amplitude of the release profile 80 s after the addition of 100 μM Ca^{2+}. For each experiment, SEM is shown for three to five independent determinations.

Antibody generation and quantitative immunoblot analysis

Peptides corresponding to nonconserved regions of syt III (residues 41–52; sequence: C-IRGYPRGPDADI), IV (residues 102–112; sequence: C-NGNFPKTNPKA-amide), VII (residues 86–96; sequence: C-PGQTPHDESDR-amide), and IX (residues 375–386; sequence: C-RPPDRARPIPAP) were synthesized, conjugated to keyhole limpet hemocyanin using a Pierce Chemical Co. KLH Conjugation kit, and used to immunize rabbits. Each

peptide was generated with an NH_2-terminal cys residue, which was used to couple it to a matrix for affinity purification using a Pierce Chemical Co. Sulfolink Kit; immobilization and purification were performed according to the manufacturers instructions.

Quantitative immunoblot analysis of syt expression was performed on postnuclear membranes isolated from cultured PC12 cells. Membranes were prepared by incubating cells in hypotonic buffer (10 mM Tris-HCl, pH 7.0, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 20 min at 4°C followed by homogenization (30 strokes, Dounce glass/glass). A postnuclear supernatant was prepared by centrifugation at 400 *g* for 2 min. Membranes were collected by centrifugation at 21,000 *g* for 15 min and solubilized in 1% SDS. Protein concentrations were determined by BCA (Pierce Chemical Co.).

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