Synaptotagmin IX Regulates Ca\(^{2+}\)-dependent Secretion in PC12 Cells*

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Synaptotagmin (Syt) I-deficient phaeochromocytoma (PC12) cell lines show normal Ca\(^{2+}\)-dependent norepinephrine (NE) release (Shoji-Kasai, Y., Yoshida, A., Sato, K., Hoshino, T., Ogura, A., Kondo, S., Fujimoto, Y., Kuwahara, R., Kato, R., and Takahashi, M. (1992) Science 256, 1821–1823). To identify an alternative Ca\(^{2+}\) sensor, we searched for other Syt isoforms in Syt I-deficient PC12 cells and identified Syt IX, an isoform closely related to Syt I, as an abundantly expressed dense-core vesicle protein. Here we show that Syt IX is required for the Ca\(^{2+}\)-dependent release of NE from PC12 cells. Antibodies directed against the C2A domain of either Syt IX or Syt I inhibited Ca\(^{2+}\)-dependent NE release in permeabilized PC12 cells indicating that both Syt proteins function in dense-core vesicle exocytosis. Our results support the idea that Syt family proteins that co-reside on secretory vesicles may function cooperatively and redundantly as potential Ca\(^{2+}\) sensors for exocytosis.

Neurotransmitter release is achieved by fusion of synaptic vesicles to presynaptic plasma membranes (i.e. exocytosis) in response to a rapid increase in Ca\(^{2+}\) ions entering through voltage-gated Ca\(^{2+}\) channels. Ca\(^{2+}\)-binding proteins (so-called “Ca\(^{2+}\) sensors”) (for reviews, see Refs. 1 and 2) must be present on the synaptic vesicles to sense such rapid increases in Ca\(^{2+}\) ions. Genetic and biochemical evidence during the past decade indicates that synaptotagmin I (Syt I),\(^1\) a Ca\(^{2+}\)-binding protein abundant in synaptic vesicles, is the most likely candidate for the major Ca\(^{2+}\) sensor for neurotransmitter release in the central nervous system (Ref. 3 and reviewed in Refs. 4–6). Syt I contains one transmembrane region at the amino terminus and two C2 domains (the C2A domain and C2B domain) in the cytoplasmic domain, and Ca\(^{2+}\) binding to the C2A domain is essential for regulating Ca\(^{2+}\)-dependent neurotransmitter release (3). Syt I is also found in the secretory granules of some endocrine cells (e.g. chromaffin cells, pancreatic β-cell lines, and PC12 cells) and has been shown to be involved in Ca\(^{2+}\)-dependent endocrine exocytosis by peptide or antibody injection experiments (7–10), suggesting a role of Syt I as an endocrine Ca\(^{2+}\) sensor (11). However, in 1992, Syt I-deficient PC12 cell lines exhibiting normal Ca\(^{2+}\)-dependent norepinephrine (NE) release were established (12). Their existence strongly contradicts the notion that Syt I is the major Ca\(^{2+}\) sensor for endocrine exocytosis (12), and the presence of an alternate Ca\(^{2+}\) sensor for Syt I in PC12 cells has been proposed (e.g. raphphilin, Doc2, calmodulin, and frequenin) (13–16). However, the actual alternative Ca\(^{2+}\) sensor to Syt I in PC12 cells has not yet been identified.

In this study we show that Syt IX is a major Syt isoform that is abundantly expressed on dense-core vesicles and regulates Ca\(^{2+}\)-dependent secretion in PC12 cells. Based on our finding, we discuss the functional relationship between Syt I and Syt IX in PC12 cells.

EXPERIMENTAL PROCEDURES

Antibody Purification—The anti-Syt I mouse monoclonal antibody (Ab) (SYA148) was from StressGen. The anti-Syt I-C2A rabbit Ab was prepared as described previously (17, 18). New Zealand White rabbits were immunized with the purified glutathione S-transferase (GST)-Syt IX-C2A (19), and the anti-Syt IX-C2A Ab was affinity-purified by exposure to antigen bound to Affi-Gel 10 beads (Bio-Rad) as described previously (17, 20). The cross-reactive component to Syt I was removed by incubation with glutathione-Sepharose (Amer sham Biosciences, Inc.) coupled to 1 mg of GST-Syt I-C2A proteins (20). The Abs specific for the amino-terminal domain of mouse Syt I (anti-Syt I-N) and Syt IX (anti-Syt IX-N) were raised against the following synthetic peptides: MVSSRPEALAAPVTTVATC (Syt I-N) and KTPPPDSSRIRQGAVC (Syt IX-N). The Abs were affinity-purified by exposure to antigenic peptide bound to FMP-activated Cellulolone (Seikagaku Co.) as described previously (21). Specificity of these antibodies was checked by immunoblotting using recombinant T7-tagged Syts I–XIII expressed in E. coli (22–24). Under our experimental conditions, we do not observe cross-reactivity of anti-Syt IX-C2A and anti-Syt IX-N Abs with Syt I in immunoblotting. The protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a reference. Immunoblotting was performed as described previously (20, 23).

Antibody Uptake Experiments—The purified anti-Syt I-N and anti-Syt IX-N Abs were conjugated with 5-and-6-carboxytetramethylrhodamine (Molecular Probes catalog no. C-1171) and 5-carboxyfluorescein (Molecular Probes catalog no. C-2210), respectively, according to the instructions of the manufacturer (17, 25). Nerve growth factor-differentiated PC12 cells were cultured on 35-mm glass-bottom dishes coated with collagen type IV (MaTek Corp.) in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 10% fetal bovine serum at 37 °C under 5% CO\(_2\). After washing twice with phosphate-buffered saline, the cells were immediately washed twice with phosphate-buffered saline and then fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min at room temperature as described previously (20, 22). Incorporated antibodies were analyzed with a fluorescence microscope (TE300,
Nikon) attached to a laser confocal scanner unit CSU 10 (Yokogawa Electric Corp.) and HiSCCA CCD camera (C6790, Hamamatsu Photonics). Images were pseudo-colored and superimposed with Adobe Photoshop software (Version 4.0) (20, 22).

**AIX for Ca**\(^{2+}\)**-activated Exocytosis—Assays of the Ca**\(^{2+}\)**-triggered release of [\( ^{3}H \)]NE from permeable PC12 cells were conducted as described previously (26). PC12 cells, cultured as described previously (27), were incubated with 0.5 μM [\( ^{3}H \)]NE (Amersham Biosciences, Inc.) and 0.5 mM ascorbate for 16 h at 37 °C. Cells were washed, preincubated in culture medium for two 1-h incubations, and removed from dishes by pipetting with ice-cold KGlu buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate (KGlu), 20 mM potassium acetate, 2 mM EGTA, and 0.1% bovine serum albumin). Cells were permeabilized by a single passage through a stainless steel ball homogenizer (28) and preincubated with KGlu buffer adjusted to 11 mM EGTA for 1 h on ice. Permeable cells were primed in incubations for 30 min at 30 °C in KGlu buffer containing 2 mM MgATP plus 1 mg/ml rat brain cytosol. Following two washes, the permeable cells were preincubated with antibodies where indicated for 1 h on ice and incubated at 30 °C for 3 min in triggering reactions containing KGlu buffer with free Ca**\(^{2+}\)** adjusted to 1 mM plus 0.1 mg/ml rat brain cytosol. Reactions were terminated by chilling followed by sedimentation at 2,000 × g for 10 min. [\( ^{3}H \)]NE in the supernatants and the 1% Triton X-100-solubilized cell pellets were used to calculate Ca**\(^{2+}\)**-dependent [\( ^{3}H \)]NE release as a percentage of total [\( ^{3}H \)]NE in each incubation.

**Miscellaneous Procedures—SDS-polyacrylamide gel electrophoresis and immunoblotting analyses were performed as described previously** (23). Immunostaining of Syt I and IX in PC12 cells was also performed as described previously (20, 22). Preparation of GST fusion proteins and the phosphatidylserine/phosphatidylcholine (PS/PC; 1:1, w/w) (or PC) lipid binding assay were also carried out as described elsewhere (19, 29). Syt I-deficient PC12 cells were isolated as a G418-resistant cell line following transfection with a pcDNA3.1 plasmid containing a rat Syt I cDNA sequence (22–109) in reverse orientation. Immunoblotting indicated the complete absence of Syt I protein but normal levels of Syt IX, syntaxin 1A, VAMP2, and SNAP-25.

**RESULTS AND DISCUSSION**

Synaptotagmin has been found to represent a large protein family in both vertebrates and invertebrates, and 13 isoforms have been identified in the rat and mouse (4, 23, 24) (Fig. 1C). Since several other presynaptic proteins show functional redundancy in the brain (e.g. synapsins, complexins, and SV2a) (30–32), we hypothesized that other Syt isoforms may compensate for the function of Syt I in the Syt I-deficient PC12 cells. To test this hypothesis, we generated a specific antibody against each Syt isoform (Syt I–XIII) and examined its expression in normal and Syt I-deficient PC12 cell lines (PC12-a and -b). Quantitative analysis by using the recombinant Syts I–XIII with a T7 tag indicated that only the Syt IX isofrom (formally called Syt V) (33, 34) is expressed as abundantly as the Syt I isofrom in both PC12 cell lines but that it is less abundant in brain (Fig. 1, A and B). The most important finding was that the Syt IX expression level is dramatically reduced in PC12-b cells (less than 5% of that of normal PC12-a cells) and that the Syt IX expression level is significantly higher than that of normal PC12 cells (Fig. 1B), suggesting that Syt IX may substitute for Syt I function in PC12-b cells. Consistent with this hypothesis, the phylogenetic distance between invertebrate Syt I and mouse Syt IX is indistinguishable from that between invertebrate Syt I and mouse Syt I (Fig. 1C, boxed), and the Ca**\(^{2+}\)**-dependent phospholipid binding properties of Syts I, II, and IX are the same in terms of affinity for Ca**\(^{2+}\)** and specificity for phospholipids (26, 29). The C2A domain of Syt IX fused to GST has no PS/PC liposome binding activity in a Ca**\(^{2+}\)**-dependent manner but did not bind PC liposomes alone, irrespective of the presence of Ca**\(^{2+}\)** (Fig. 1D).

If Syt IX compensates for the function of Syt I in Syt I-deficient PC12 cells, the Syt IX would be expected to be localized to dense-core vesicles. Immunocytochemical studies indicated that Syt IX was present at the tips of neurites where dense-core vesicles are enriched, and it closely co-localized with Syt I (Fig. 2, Syt IX in green (A), Syt I in red (B), and overlay in yellow (C)). The specificity of the anti-Syt IX antibody was confirmed by incubation with antigenic peptide (Fig. 2, D and E). To examine the dynamics of Syt IX molecules during Ca**\(^{2+}\)**-dependent exocytosis, Abs directed against the luminal domain of Syt IX conjugated to fluorescein and the luminal domain of Syt I conjugated to rhodamine were added to the culture medium (24, 35), and the cells were stimulated with a low or high concentration of KCl. Uptake of both the fluorescein-Syt IX Ab and rhodamine-Syt I Ab into neurites and cell body occurred only at depolarization KCl concentrations (Fig. 3, A–D and G–J). Co-localization of the fluorescein-Syt IX Ab and the rhodamine-Syt I Ab (Fig. 3, C and I, arrowheads) indicated that Syt I and IX proteins are present in the same vesicles that undergo Ca**\(^{2+}\)**-dependent exocytosis.

We had previously shown that inhibition of Ca**\(^{2+}\)**/phospholipid binding to the C2A domain of Syt I by anti-Syt I-C2A Ab blocks neurotransmitter release in the squid giant synapse and superior cervical ganglion neurons (17, 18). Because the role of Syts in Ca**\(^{2+}\)**-dependent exocytosis in PC12 cells is unclear (12),

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\(^{2}\)X. Zhang, M. J. Kim-Miller, M. Fukuda, J. A. Kowalchyck, and T. F. J. Martin, submitted for publication.
we introduced Syt C2A antibodies into permeabilized PC12 cells (26–28) to assess their effect on Ca^{2+}-dependent NE release. The Ab against the C2A domain of Syt IX (anti-Syt IX-C2A) inhibited Ca^{2+}-dependent NE release in a dose-dependent manner with maximal inhibition at about 50% (Fig. 3K, left, circles), whereas a preimmune Ab did not have any significant effect (Fig. 3K, left, squares). Similar results were obtained when the anti-Syt I-C2A Ab was introduced into permeable PC12 cells with maximal inhibition at about 50% (Fig. 3K, left, triangles). To analyze the functional relationship between Syts I and IX in NE release, the anti-Syt I-C2A and anti-Syt IX-C2A Abs were simultaneously introduced into PC12 cells. No significant additive effect of these two antibodies was observed at maximally effective concentrations of both antibodies (Fig. 3K, middle).

To assess whether antibody inhibition could result from steric effects or cross-linking of bivalent IgGs, we tested Fab fragments of the Abs. Fab fragments from either the Syt IX-C2A or the Syt I-C2A Ab strongly inhibited Ca^{2+}-dependent NE release from wild type PC12 cells with maximal inhibition exceeding 80% (Fig. 3K, right, open symbols). As anticipated, the Syt I-C2A Fab fragments failed to inhibit Ca^{2+}-dependent NE release from Syt I-deficient PC12 cells, whereas the Syt IX-C2A Fab fragments were fully inhibitory (Fig. 3K, right, closed symbols). The greater inhibitory effect of the Fab fragments compared with IgGs suggests that full access of larger IgGs to the C2A domain may be limited, which could be due to C2A domain-protein interactions that may be important for transmitter secretion (for a review, see Ref. 4). Alternatively, cross-linking of Syt I or IX isoforms by bivalent IgGs may allow residual Syt function in NE release. The results for Fab fragments indicate that inhibition of either Syt I or Syt IX in wild type PC12 cells is sufficient to block Ca^{2+}-dependent exocytosis. This suggests that Syts I and IX function interdependently, which is consistent with our finding that both reside on the same vesicles and with our previous studies showing that Syts I and IX can form Ca^{2+}-dependent hetero-oligomers (36).

In summary, several lines of evidence indicate that Syt IX is a major Syt isoform that is required for Ca^{2+}-dependent secretion in PC12 cells. First, in wild type PC12 cells, Syt IX is
expressed as abundantly as Syt I, and in Syt I-deficient PC12 cells, the expression of Syt IX is up-regulated (Fig. 1). No other Syt isoforms including Syt VII, a recently proposed plasma membrane Ca^{2+} sensor (37), are expressed abundantly (although Syt IV, a third isoform expressed in PC12 cells, is present at <5% of the level of Syt I). Second, both Syt I and Syt IX are present on the same vesicles that undergo Ca^{2+}-dependent exocytosis (Figs. 2 and 3). Third, the Ca^{2+}-dependent binding of the Syt IX C2A domain to phospholipids (Fig. 1) is similar to that of the Syt I C2A domain (19, 29), and similar Ca^{2+}-dependent interactions of Syt I (38) and Syt IX with SNAP-25 have been characterized. Last, Fab fragments from either Syt I-C2A or Syt IX-C2A Abs almost completely inhibited Ca^{2+}-dependent NE release in PC12 cells (Fig. 3). Based on these results, we propose that PC12 cells utilize two Ca^{2+} sensors, Syts I and IX, on the same vesicles that function cooperatively to mediate the Ca^{2+} triggering of exocytosis. This hypothesis can account for the finding that Syt I-deficient PC12 cells exhibit normal Ca^{2+}-dependent NE release; Syt IX can function as an alternate Ca^{2+} sensor.

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