Stable gene silencing of synaptotagmin I in rat PC12 cells inhibits Ca\textsuperscript{2+}-evoked release of catecholamine

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Moore, Johnnie M., Jason B. Papke, Anne L. Cahill, and Amy B. Harkins. Stable gene silencing of synaptotagmin I in rat PC12 cells inhibits Ca\textsuperscript{2+}-evoked release of catecholamine. Am J Physiol Cell Physiol 291: C270 –C281, 2006.—Synaptotagmin (syt) I is a Ca\textsuperscript{2+}-binding protein that is well accepted as a major sensor for Ca\textsuperscript{2+}-regulated release of transmitter. However, controversy remains as to whether syt I is the only protein that can function in this role and whether the remaining syt family members also function as Ca\textsuperscript{2+} sensors. In this study, we generated a PC12 cell line that continuously expresses a short hairpin RNA (shRNA) to silence expression of syt I by RNA interference. Immunoblot and immunocytochemistry experiments demonstrate that expression of syt I was specifically silenced in cells that stably integrate the shRNA-syt I compared with control cells stably transfected with the empty shRNA vector. The other predominantly expressed syt isoform, syt IX, was not affected, nor was the expression of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins when syt I levels were knocked down. Resting Ca\textsuperscript{2+} and stimulated Ca\textsuperscript{2+} influx imaged with fura-2 were not altered in syt I knockdown cells. However, evoked release of catecholamine detected by carbon fiber amperometry and HPLC was significantly reduced, although not abolished. Human syt I rescued the release events in the syt I knockdown cells. The reduction of stimulated catecholamine release in the syt I knockdown cells strongly suggests that although syt I is clearly involved in catecholamine release, it is not the only protein to regulate stimulated release in PC12 cells, and another protein likely has a role as a Ca\textsuperscript{2+} sensor for regulated release of transmitter.

RNA interference; amperometry; exocytosis

SYNAPTIC PLASTICITY of the central nervous system allows an organism to interact with environmental cues to convert external signals into a meaningful learned memory. For many organisms, these learned memories promote basic survival ability. A primary requirement presumed responsible for forming and retrieving memories is synaptic transmission. It is the dynamic control of transmitter release from vesicles that allows an electrical signal to be transmitted across a chemical synapse. Neuronal communication is highly dependent on Ca\textsuperscript{2+} to activate fusion of vesicles, and it involves coordination of many molecular events tightly regulated on a millisecond timescale. In neurons and neuroendocrine cells, release of transmitter from vesicles is mediated by three primary proteins: the vesicular protein synaptobrevin 2 (also called VAMP-2) and two plasma membrane proteins, syntaxin 1A and SNAP-25 (synaptosome-associated protein of 25 kDa) (2, 3, 44). Collectively, these three proteins form the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, an essential component of the exocytotic machinery (20). However, it is not yet understood how Ca\textsuperscript{2+} acts to control release of transmitter-filled vesicles or what proteins can act as a Ca\textsuperscript{2+} sensor.

Although a number of proteins are involved in the release of vesicles and are reported to be critical for release, one vesicle protein, synaptotagmin (syt), has been established as a Ca\textsuperscript{2+} sensor and regulator of both exo- and endocytosis (35, 40). The family of syt proteins is composed of at least 16 different genes, syt I–XVI (9). Syt I, the best studied isoform, is composed of a short intravesicular NH\textsubscript{2} terminus followed by a single transmembrane domain and two extravesicular Ca\textsuperscript{2+}-binding domains, or C2 domains, that are highly conserved among species (36, 37). Subtle differences among Syt isoforms potentially lead to a large number of possible functions within this protein family (for review, see Ref. 45). Although many of the isoforms are integral vesicle membrane proteins found in neuronal and neuroendocrine vesicles (36), the isoforms vary in their ability to bind Ca\textsuperscript{2+} and to bind phospholipids as a function of Ca\textsuperscript{2+} through their two C2 domains (1, 10, 26, 40, 42, 48, 52, 54). Syt isoforms also can form homo- and heterooligomers that lead to further complexity of the syt family (25, 56).

Evidence from a number of systems indicates that syt I is involved in vesicle docking, fusion, and recycling (for reviews, see Refs. 43, 45). For example, fast synchronous release was abolished in mice and Drosophila that lack syt I, but slow asynchronous release remained (19, 57) and even compensated for the loss of fast synchronous release (33). Early evidence that syt I plays a role in secretion came from results with antibodies, recombinant fragments, and peptides designed against specific regions of syt I protein that interfered with vesicle release (12). Furthermore, because mutations in the syt I gene disrupted the fourth-order Ca\textsuperscript{2+} dependence of synchronous release, it was proposed that syt I is a Ca\textsuperscript{2+} sensor for regulated release of transmitter (15, 28, 42, 57). However, alternative explanations may explain the observations, as syt I may act to control the expression and trafficking of other proteins, or it may physically act as a link between the vesicle release machinery and the pore of the Ca\textsuperscript{2+} channel. Syt I also is abundantly localized in endocrine vesicle membranes of adrenal chromaffin cells and pheochromocytoma (PC12) cells. Both of these cells have been well utilized to study the mechanism of Ca\textsuperscript{2+}-dependent transmitter release from vesi-
ciles. In this study, we investigated whether synt I is essential for Ca\(^{2+}\)-evoked release of catecholamines in the model neuroendocrine PC12 cells by using plasmid-based RNA interference (RNAi) to establish a cell line in which synt I levels are stably and specifically reduced. Although viability, phenotype, and resting Ca\(^{2+}\) levels are not altered compared with control cells, synt I knockdown cells exhibit significantly reduced, but not eliminated, release of catecholamines.

**EXPERIMENTAL PROCEDURES**

**Antibodies.** The anti-syt I and anti-β-actin mouse monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). The anti-syt II and anti-syt VII goat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-syt III rabbit antibody was a generous gift from Dr. Bryan A. Wolf (University of Pennsylvania, Philadelphia, PA). The anti-syt IX mouse antibody was obtained from BD Biosciences-Pharmingen (termly syt V; San Diego, CA) and recognizes the isoform whose amino acid sequence begins with MPPEP. Anti-SNAP-25 and anti-VAMP-2 rabbit antibodies were obtained from Alomone Labs (Jerusalem, Israel); anti-syntaxin I mouse antibody was obtained from Medical and Biological Laboratories (Nagoya, Japan); and anti-synaptophysin I mouse monoclonal antibody was obtained from Chemicon (Temecula, CA). For immunoblots, horse-radish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology and also recognize the Cruz molecular weight standards. For immunocytochemistry, secondary goat-antimouse FITC-conjugated antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Plasmids.** We constructed a plasmid designed to express short hairpin RNAs (shRNAs) from a U6 RNA polymerase III promoter and to allow selection of stably transfected cells with G418 (6). The vector (pG418-shRNA) was analyzed using extensive restriction enzyme digestion and partial sequencing. A human syt I plasmid was purchased from American Type Culture Collection (GenBank accession no. BC058917).

**Design of shRNA insert.** Target sites for silencing synt I were selected using Target Finder (Ambion, Austin, TX). Two complementary DNA oligonucleotides incorporating the chosen target site AGACCTAGGAAGACCATG (bp 846–864 of GenBank accession no. X52772), a loop sequence, and the reverse complement of the target site were designed. A nine-nucleotide loop sequence was used, TTCAAGAGA (4). The pair of 56-bp DNA oligonucleotides was purchased from Integrated DNA Technologies (Corvaline, IA), 5’ phosphorylated, and PAGE purified. The pair of oligonucleotides was annealed and ligated into pG418-shRNA digested with Xbal and BpiI. The resulting plasmid, termed shRNA-Syt I, was sequenced to ensure that the insert was present and correct.

**Cell culture.** An early passage of rat PC12 cells was maintained in culture according to standard methods (21, 22). Cells were grown in medium that consisted of RPMI 1640, 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37°C, 5% CO\(_2\) incubator. The cells were passaged after 1–2 days until use.

**Transfection.** Cells were transfected with 4 µg of transfection grade shRNA-syt I plasmid DNA or shRNA plasmid that did not contain an insert (referred to as control transfection, or CT) per 35-mm tissue culture plate of cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (23). Cells were replated 24 h later. Selection media that contained 100 µg/ml G418 (Sigma, St. Louis, MO) was added to the cells 24 h after replating. This concentration of G418 was found to be optimal to kill untransfected cells. Cells grew in selection medium up to ~28 days, and single colonies were picked from the plates and expanded for freezing and testing for stable incorporation of the shRNAs.

**PCR.** Drug-resistant cell lines were screened for insertion of shRNA plasmids into the genome by PCR with genomic DNA, plasmid-specific primers, and MasterTaq PCR reagents (Eppendorf, Westbury, NY). PCR reactions were performed with 35 cycles of 95°C for denaturing (30 s), 54°C for annealing (1 min), and 72°C for elongating (1 min). A final elongation step was run for 10 min at 72°C. The plasmid-specific primers were designed to match sequences on either side of the multiple cloning site to determine whether the plasmid had incorporated into the genome. The forward primer matched the plasmid sequence 100-bp 5’ from the KpnI site in the multiple cloning site (GGCGATTAAGTTGGTAAAGC) and was used with the reverse primer that was situated about 100 bp into the PGK polyA tail and 3’ to the multiple cloning site (GGCTGAA-GACGAGATCAGC). This set of primers resulted in a PCR product of ~570 bp for the empty plasmid (shRNA) and ~600 bp for the plasmid that contained the insert (shRNA-syt I). Because PC12 cells can develop spontaneous resistance to G418, we determined that cell lines that tested positive with the primers described above also tested positive for the neomycin cassette. A second set of primers was designed to match sequences within the neomycin cassette; the forward primer was GACAATCGCGCTGTCCTGATG, and the reverse primer was ACCATGATATTCGGCAAGCA. Incorporation of the neomycin resistance cassette resulted in a PCR product of 512 bp with this primers.

**Immunoblot analysis.** Cells were grown in culture to confluence, removed from the plates, washed, and lysed by resuspension in buffer containing 20 mM Tris, pH 7.5, 1% Triton X-100, 10% glycerol, 2 mM EDTA, and 0.1% protease inhibitor cocktail (Sigma). After 30 min on ice, the cells were sonicated for three short bursts and centrifuged for 2 min at 16,000 g at 4°C. Portions of the supernatant were used to determine protein concentration (see below) or mixed with lithium dodecyl sulfate (LDS) loading buffer (Invitrogen). Crude membranes were prepared from rat cerebellum by homogenization for 10 s on ice in 20 mM Tris, pH 7.5, 2 mM EDTA, and 0.25 M sucrose using a Polytron. After incubation for 1 min on ice, the homogenization was repeated three times. The tissue was further disrupted in a Dounce homogenizer with 15 strokes and centrifuged for 10 min at 1,000 g at 4°C. The supernatant was collected and centrifuged for 30 min at 16,000 g at 4°C. The supernatant was removed, and the pellet was resuspended in homogenization buffer without sucrose. Protein concentration was determined with Coomassie Plus protein assay reagent (Pierce, Rockford, IL) with BSA as a standard. The protein was mixed with LDS loading buffer and heated to 70°C for 10 min.

Total lysates were electrophoresed on a 10% NuPAGE Bis-Tris gel (Invitrogen), transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA), and blocked overnight at 4°C in 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20. The membrane was incubated with primary antibody (all 1:1,000 dilutions except syntrophin, used at 1:5,000 dilution), followed by a horse-radish peroxidase-conjugated secondary antibody used at dilutions that ranged from 1:30,000 to 1:50,000 (Santa Cruz Biotechnology). Immunoreactive bands were detected and visualized using ECL Advance reagent (GE Healthcare, Piscataway, NJ), exposed to X-ray film, and developed. A positive control tissue or cellular lysate that is known to express the particular syt isoform protein of interest was included on each immunoblot. Rat cerebellar tissue was used for synt I, II, and IX, and the RINm5F pancreatic β-cell line was used for synt III and VII (generously provided by Dr. John Corbett, St. Louis University, St. Louis, MO). All immunoblots are representative of three or more independent experiments. Quantitative analysis of the immunoblots was performed with ImageJ (http://rsb.info.nih.gov/ij/) and normalized to arbitrary densitometry units of β-actin.
Immunocytochemistry. Cells were replated to collagen-treated Permanox chamber slides and fixed 1 (PC12 cells) or 2 days later (NGF-PC12 cells) with 2% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100, blocked with 2 mg/ml BSA, and incubated with anti-syt I mouse monoclonal antibody (1:5 dilution) or anti-syt IX mouse antibody (1:200 dilution), blocked, and incubated with a secondary goat-anti-mouse FITC-conjugated antibody (1:200; Jackson Immunoresearch Laboratories). The cells were washed, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and sealed. Images were acquired using a scanning confocal microscope (Bio-Rad MRC 1024; Hercules, CA) with a 4× zoom on a 60× oil-immersion lens (NA 1.4). For the human syt I rescue experiments, cells were permeabilized with 0.1% Triton X-100, blocked with 10% normal goat serum in PBS, and incubated with anti-syt I mouse monoclonal antibody (1:5 dilution), blocked, and incubated with a secondary rhodamine red-X-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (1:2,000; Jackson Immunoresearch Laboratories), sealed, and mounted as described above. Images were acquired using a 3× zoom on a 60× oil-immersion lens (NA 1.4). All immunocytochemistry results are representative of three or more independent experiments.

Ca$^{2+}$ imaging. Intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]) were measured using ratiometric fluorescence imaging with fura-2. Cells were replated 4–8 h before experimentation, loaded with fura-2, and prepared for recording as described previously (23). For each experiment, 20–40 cells were selected and individually imaged using a commercial imaging system (Incytim2; Intracellular Imaging, Cincinnati, OH). Image pairs were obtained every 10 s at 340- and 380-nm wavelengths. Backgrounds were subtracted from each wavelength, and the 340-nm image was divided by the 380-nm image to provide a ratiometric image. Ratios were converted to free [Ca$^{2+}$] by comparing data with fura-2 calibration curves made in vitro by adding fura-2 (50 µM free acid) to solutions that contained known Ca$^{2+}$ concentrations (0 to 602 nM; Molecular Probes-Invitrogen). Cells were exposed to a high-K$^+$ solution that contained (in mM) 50 KCl, 87 NaCl, 1 MgCl$_2$, 5 CaCl$_2$, 10 d-glucose, and 12 HEPES (pH 7.3). Resting Ca$^{2+}$ level was determined from the average of measurements from all cells in four independent experiments during the 60 s before stimulation. The change in Ca$^{2+}$ was calculated from the five points during the peak change in Ca$^{2+}$ levels that occurred in response to stimulation.

Amperometry. Carbon fibers (6-µm diameter; Goodfellow, Huntingdon, UK) were threaded into electrodes, pulled with a vertical puller, sealed with epoxy, painted with Sylgard (Dow-Corning, Midland, MI), and stored at 95°C until use. A newly cut surface of a carbon fiber electrode (5) was gently pressed against the side of a cell, because the collection efficiency of detectable events is thought to provide the highest yield in this recording configuration (5, 39). The electrode was backfilled with 3 M KCl and clamped at +700 mV versus an Ag-AgCl ground with an NPI amplifier (ALA Scientific, Westbury, NY) to detect the oxidized catecholamine transmitter. The signal was low-pass filtered at 2 kHz and digitized at 70 kHz (8-pole Bessel, WPI Instruments, Hamden, CT). A 16-bit analog-to-digital converter (National Instruments, Austin, TX) was interfaced with custom-written software (graciously provided by Dr. Aaron P. Fox, University of Chicago, Chicago, IL), acquired at 10 kHz, and stored on a personal computer. Root mean square (RMS) noise was typically <1 pA. Amperometric spike features such as amplitude, quantal charge, and kinetic parameters were analyzed using custom-written analysis software (graciously provided by Dr. Eugene Mosharov, Columbia University, New York, NY). The detection threshold for an event was set for five times the baseline RMS noise level, and no trace was analyzed with RMS noise >2.5 pA. Overlapping events, those whose spike had not returned to baseline before the next event, were not considered in the analysis even though they were uncommon events. Rise time was measured over 10–90% of the spike’s maximal amplitude. The area under individual amperometric spikes is equal to the charge (Q, measured in pC) per release event.

Recording. Cells were treated with NGF for 5–10 days, replated to glass coverslips, and cultured overnight. Before recordings, cells were preloaded with 200 nM norepinephrine (Sigma) for 2–5 h at 37°C. Recordings were conducted on adherent cells exposed to constant and uniform perfusion (~1 ml/min) in a recording chamber with an approximate volume of 200 µl. Cells were perfused for 5–10 min with Hanks’ buffered salt solution (HBSS), and recording was begun during the HBSS perfusion for ~20 s during recording for basal release before changing to a high (50 mM) K$^+$ solution for stimulation of transmitter release for the remainder of the 4-min recording period. All experiments were performed at ambient temperature, 22–24°C.

Catecholamine measurements. Catecholamines were identified and quantified using high-performance liquid chromatography (HPLC)–electrochemical detection, as previously described (7, 11), from cells treated with NGF for 10–12 days. Release was stimulated with a 50 mM K$^+$ solution. The system consists of a Varian Pro-Star solvent delivery system and a model 9000 autosampler (Varian) coupled to a C18 column and an ESA Coulochem II detector. Separations were performed isocratically with the use of a filtered and degassed mobile phase consisting of 12% methanol, 0.1 M sodium phosphate, 0.2 mM sodium octyl sulfate, and 0.1 mM EDTA, adjusted to pH 2.7 with phosphoric acid. A computer was used to collect and store the chromatograms that were analyzed using Varian Star software.

Syt I rescue. We confirmed by sequencing that the target sequence of the shRNA for syt I differed by four base pairs from the human syt I plasmid. Because of this four-nucleotide difference, human syt I should not be silenced by the shRNA. Syt I knockdown cells were treated with NGF and transiently transfected for 2 days with the human syt I plasmid before experimentation.

Statistical analysis. All data are displayed as means ± SE, and Student’s t-test was used to compare significance.

RESULTS

Stable cell lines generated to express shRNAs against syt I. We utilized the plasmid-based approach to RNAi because it allows the generation of stable cell lines. Such stably transfected cell lines continuously transcribe shRNAs from an RNA polymerase III type promoter (e.g., U6 or H1 promoters). These shRNAs are effective in silencing genes (4, 31, 58). This approach has the advantage that it does not require expensive oligonucleotides or RNase-free conditions, and it allows for generation of stably transfected cell lines in which all cells express homogeneous levels of a particular protein. This was an important aspect of our experiments, because we knew from preliminary experiments (not shown) that only 15% or less of our particular line of PC12 cells routinely are transiently transfected, an efficiency that is fairly low for RNAi purposes.

To detect a significant reduction in protein expression and to confirm gene silencing by immunoblot analysis, a relatively high transfection efficiency rate is required, ~85% or greater. Therefore, we stably transfected cells with a plasmid designed to make hairpin RNAs and generated a homogeneous population of cells that express shRNAs.

PC12 cells were stably transfected with two different plasmids, the shRNA-syt I plasmid and a plasmid that lacked an insert that was used as a positive control for the transfection (referred to as control transfection, or CT). Colonies were expanded, and genomic DNA was prepared from each of the stable cell lines. PCR was used to test for incorporation of the shRNA plasmid into genomic DNA with specific primers that
recognized a sequence of the plasmid flanking the insert (and that does not recognize any known genomic sequence), or within the neomycin resistance cassette of the plasmid.

**RNAi stably knocks down syt I expression.** We tested whether syt I expression was specifically “silenced” in cells stably transfected with shRNA-syt I using immunoblot analysis. Lysates were made from cells expressing the shRNA designed to recognize and reduce the expression of syt I (shRNA-syt I) and from cells stably transfected with the empty vector (CT). Expression of proteins from these cells was compared with expression of syt I in untransfected PC12 cells and rat cerebellum as controls. Blots were probed with an anti-syt I antibody to detect a band of 65 kDa, the expected apparent molecular weight. Six of the seven shRNA empty plasmid cell lines (CT) tested positive for the plasmid with PCR and did not exhibit a reduction in expressed syt I as expected. In contrast, the four shRNA-syt I cell lines that tested positive for the shRNA insert by PCR exhibited varying degrees of reduction in expressed syt I protein. We continued experimentation with one of each of the stable cell lines, and all experiments described were performed with lines termed CT and shRNA-syt I. In addition, both of these lines tested positive for incorporation of neomycin resistance cassette by PCR. Figure 1A, top, shows a blot probed with the anti-syt I antibody. Both the untransfected PC12 cells (control) and the empty vector stable cells (CT) have a detectable band of protein at 65 kDa compared with cells expressing the shRNA designed specifically to knockdown syt I (shRNA-Syt I). Syt I levels in the knockdown cells were 0.02% of those in control cells (Fig. 1A, bottom), which was not different from zero.

**shRNA-syt I is specific for syt I.** Because the ultimate goal was to specifically knockdown syt I and not affect expression of any other endogenously expressed syt isoforms, we established which Ca^{2+}-binding syt isoforms are expressed endogenously in our particular line of PC12 cells by performing immunoblot analysis using antibodies directed against each isoform. We tested for the syt isoforms I, II, III, VII, and IX, because previous reverse-transcriptase PCR experiments (unpublished results) revealed that the cells make transcript for these Ca^{2+}-binding isoforms. Syt IX is the most abundant isoform expressed, and Syt I is the second most abundant isoform (for example, see Fig. 1A). (Note: There has been confusion in the literature between the two isoforms syt V and IX. We call the syt isoform that begins with the amino acid sequence MFPEP “syt IX.” The specific antibody we purchased to recognize this isoform is termed anti-syt V.) Syt III and VII are expressed in much lower abundance (for example, see Fig. 1C, where protein lysate was loaded at 2 and 3 μg/lane, respectively, for detection with syt III and VII antibody). Syt II could not be detected in any PC12 cells even when 20 μg/lane protein amounts were loaded (data not shown). These results are similar to published reports that syt I and IX are the primary isoforms expressed in PC12 cells and that either syt IV (16) or VII occurs in substantially lower abundance (51, 59) depending on the individual PC12 cell line.

**Fig. 1.** RNA interference (RNAi) is specific for targeting and knocking down expression of synaptotagmin I (syt I). A, top: syt I expression is knocked down in stably transfected cell lines. Immunoblot analysis shows that syt I is reduced to undetectable levels in the cells stably transfected with the short hairpin RNA (shRNA)-syt I plasmid (lane 2) compared with either control untransfected PC12 cells (lane 1) or cells transfected with a plasmid that does not contain an shRNA insert (CT; lane 3). All lanes were loaded at 2 μg/lane. Expression of syt II, the most abundant isoform, is unaffected in the cells expressing the shRNA-syt I plasmid (0.1 μg/lane). Expression of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins involved in vesicle release or expression of a structural protein, β-actin, also was unaffected (1 μg/lane). A, bottom: syt I expression was significantly reduced compared with control PC12 cells and normalized to arbitrary densitometry units of β-actin (n = 5, P < 0.001). This is the only significant difference between any expressed proteins on the blots. B: increasing amounts of loaded lysate (shown above each lane) for the syt I knockdown cells show that there was no detectable syt I expression for loaded lysate to 20 μg/lane. C: knocking down expression of syt I did not cause an increase of expression of syt II or VII. Lane 1 shows lysates made from control tissues: cerebellum for syt II and a pancreatic β cell line, RINm5F cells, for syt III and VII. Lane 2 shows lysates from control untransfected PC12 cells; lane 3, from syt I knockdown cells; and lane 4, from CT cells (syt II and III, 2 μg/lane, and syt VII, 3 μg/lane).
To determine whether suppression of protein expression is specific for syt I, we performed immunoblot analysis to test whether the shRNA-syt I and CT cell lines exhibited a reduction in protein expression for syt IX, the most abundantly expressed syt isoform and one that is similar in sequence to Syt I. We also tested whether additional proteins involved in regulated secretory vesicle release were altered in their expression, such as SNAP-25, VAMP-2 (or synaptobrevin), syntaxin I, and a structural protein, β-actin. Figure 1A shows that cells stably transfected with shRNA-syt I specifically suppressed the expression of syt I. The shRNA-syt I cells were tested for low abundance expression by using increasing concentrations of loaded protein amounts. Even with 20 μg of lysate protein, the shRNA-syt I cells did not express detectable syt I protein (Fig. 1B). Syt IX was not affected by the shRNA-syt I, because the shRNA-syt I cells express syt IX at levels equivalent to that of the CT cells. Furthermore, none of the proteins tested exhibited any significant changes in expressed protein levels in the syt I knockdown cells compared with the CT cells. Thus RNAi targeted against syt I effectively and specifically knocked down expression of syt I in stably transfected cells.

In addition to specificity of knockdown, a primary concern with RNAi is whether suppression of one gene product results in compensatory upregulation of closely related gene products. We used immunoblot analysis to determine whether syt II, III, or VII was upregulated. Figure 1C shows that neither syt III nor syt VII exhibit an upregulation of protein expression in the shRNA-syt I cells compared with the CT cells. Each of the isoforms is shown compared with control tissue, cerebellum (syt II) or RINm5F pancreatic β cells (syt III and VII). Syt II was not detectable as expected.

Neither syt I protein expression nor knockdown of syt I decreases with time in culture. Because PC12 cells are maintained in culture for multiple passages, it was important to verify that endogenous syt I protein levels do not decrease with time in culture and therefore do not provide a false interpretation of RNAi in syt I knockdown stable cells. Immunoblot analysis was used to evaluate the expression levels of syt I with time in passage for PC12 cells and stably transfected shRNA-syt I cells. The expression levels of syt I in control cells did not decrease with time in culture for at least seven passages in control cells (data not shown). Furthermore, syt I in the stably transfected cell line remained stably knocked down for at least 9 wk when the cells were cultured in the presence of selection media.

To determine whether the cellular expression patterns of syt I and IX were affected in the syt I knockdown cells, we performed immunocytochemistry on both undifferentiated and NGF-treated cells to detect syt I and syt IX in untransfected control cells, shRNA-syt I cells, and CT cells. Figure 2 shows confocal images of single cells expressing syt I (A) and syt IX (B). The syt I knockdown cells did not express detectable syt I, whereas control cells and cells stably transfected with the shRNA plasmid did express syt I. Syt IX expression was not affected in the syt I knockdown cells. From the images, regardless of NGF treatment, both syt I and IX appear to be expressed in the cytoplasm of the cell and along the surface of the cellular membrane, but are largely excluded from the nucleus. These data are similar to those reported for syt I and IX proteins localized to vesicle membrane (16, 17, 29). This experiment confirms at the cellular level that RNAi targeted against syt I completely and specifically knocked down expression of syt I in individual PC12 cells without affecting the other abundantly expressed syt IX protein.

Stable syt I knockdown cells are viable and appear to be differentiated with NGF. One concern with selection of a stable cell line incorporating copies of a plasmid in the genome is viability of the cell line. Each of the cell lines used for experimentation had been passaged at least seven times, with early passages brought up from a freeze and continued in culture. In this way, it was determined that RNAi does not alter the viability of PC12 cells that are stably transfected with the shRNAs. Using increasing concentrations of G418 in selection media, we determined that a high concentration of G418 was required to kill shRNA-syt I cells. The shRNA-syt I cells were viable until concentrations of G418 exceeded 3,000 μg/ml, a 30-fold greater concentration than that required to kill untransfected cells. In addition, the morphology of PC12 cells was evaluated using photography and visualization after no treatment with NGF or after treatment with NGF to differentiate the cells toward a more sympathetic neuronal phenotype (50). The cellular morphology of the shRNA-syt I cells was indistinguishable from that of either control cells or CT cells.

![Fig. 2. Expression of syt I is abolished in single cells. PC12 (−NGF) or NGF-treated PC12 cells (+NGF) were prepared for immunocytochemistry with a primary anti-syt I mouse antibody (A) or a primary anti-syt IX mouse antibody (B). Representative photos are shown for control untransfected cells (control) and stable cells transfected with either shRNA-Syt I or empty vector (CT). Scale bar, 10 μm.](http://ajpcell.physiology.org/)

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treated with NGF, all of the cells exhibited larger and more flattened cell bodies with processes that extended from the cell bodies, and the shRNA-syt I cells were indistinguishable from either control or CT cells.

**Stable syt I knockdown cells have normal resting Ca$$^{2+}$$ levels and respond to high-K$$^{+}$$ stimulation.** To determine whether cells stably transfected with shRNA-syt I have altered resting Ca$$^{2+}$$ levels or altered Ca$$^{2+}$$ influx in response to stimulation, we performed Ca$$^{2+}$$ imaging experiments on individual cells with the Ca$$^{2+}$$ indicator fura-2. Figure 3 shows averaged fura-2 imaging experiments. Each panel shows a representative group of cells imaged from one dish of cells. Average Ca$$^{2+}$$ concentration is plotted as a function of time for control PC12 cells (Fig. 3A), shRNA-syt I cells (Fig. 3B), and CT cells (Fig. 3C). For each group of cells, data were averaged at each time point for all of the cells imaged. The cells were stimulated with 50 mM K$$^{+}$$ solution (solid bar above each graph). Average values for resting Ca$$^{2+}$$ and the change in [Ca$$^{2+}$$]$_i$ are shown in the summary graph (Fig. 3D). Syt I knockdown cells did not exhibit any differences in resting Ca$$^{2+}$$ compared with control untransfected or CT cells. When the cells were stimulated, syt I knockdown cells responded with a somewhat greater change in Ca$$^{2+}$$ influx ($P < 0.01$) that is most likely due to the cells lacking Ca$$^{2+}$$ binding sites, because each syt I molecule can coordinate 5 Ca$$^{2+}$$ ions (14, 38).

**Syt I knockdown cells exhibit reduced secretion.** Amperometry is a useful tool for detecting release of catecholamine from individual cells. Analysis of single amperometric events provides a kinetic component of stimulated release. Comparing the individual events between control and shRNA-syt I knockdown cells and performing kinetic analysis of many events provides a means to consider how the release mechanism has been altered by reducing the expression of the vesicle protein syt I. To accomplish this, we preloaded norepinephrine into control (untransfected) PC12 cells, shRNA-syt I knockdown cells, and CT cells. Catecholamine secretion from individual vesicles was stimulated by high (50 mM)-K$$^{+}$$ depolarization and detected using amperometry. Figure 4A shows a representative amperometric trace measured from control and shRNA-syt I cells. The amperometric trace was recorded for ~20 s in a basal level K$$^{+}$$ solution before switching to the high-K$$^{+}$$ stimulating solution. Stimulated release events are shown as upward events that occurred after high-K$$^{+}$$ solution was applied. The control cell (Fig. 4A, left) had 157 events after the stimulating solution was applied, whereas the shRNA-syt I cell (Fig. 4A, right) had 64 events. These cells are representative of the average number of events for the control cells (160 ± 52 events), the syt I knockdown cells (60 ± 22 events), and CT cells (155 ± 37 events) (Fig. 4C). The total number of events analyzed was 1,119 events for control cells ($n = 7$), 417 events for syt I knockdown cells ($n = 7$), and 1,399 events for CT cells ($n = 9$). Although the averaged number of events per cell was not significantly different (Student’s t-test), there was an approximately two-thirds reduction in the total number of events in the shRNA-syt I knockdown cells compared with the control cells.

![Fig. 3](http://ajpcell.physiology.org/) Resting and stimulated Ca$$^{2+}$$ levels are similar in syt I knockdown cells compared with control cells. Ca$$^{2+}$$ imaging was performed with fura-2 to measure resting [Ca$$^{2+}$$]$_i$, level and changes in Ca$$^{2+}$$ in response to stimulation with 50 mM K$$^{+}$$ (solid bars). A representative experiment for each group of cells is plotted as the average Ca$$^{2+}$$ level (±SE) vs. time for untransfected control cells (A), cells stably expressing shRNA-syt I plasmid (B), or CT cells (C). The summary graph shows the average resting Ca$$^{2+}$$ and change in Ca$$^{2+}$$ during stimulation (D). The data represent an average of 183 control cells, 199 shRNA-Syt I cells, and 152 shRNA cells. *$P < 0.01$. 

**Fig. 3.** Resting and stimulated Ca$$^{2+}$$ levels are similar in syt I knockdown cells compared with control cells. Ca$$^{2+}$$ imaging was performed with fura-2 to measure resting [Ca$$^{2+}$$]$_i$, level and changes in Ca$$^{2+}$$ in response to stimulation with 50 mM K$$^{+}$$ (solid bars). A representative experiment for each group of cells is plotted as the average Ca$$^{2+}$$ level (±SE) vs. time for untransfected control cells (A), cells stably expressing shRNA-syt I plasmid (B), or CT cells (C). The summary graph shows the average resting Ca$$^{2+}$$ and change in Ca$$^{2+}$$ during stimulation (D). The data represent an average of 183 control cells, 199 shRNA-Syt I cells, and 152 shRNA cells. *$P < 0.01$. 

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The amperometric trace was expanded to analyze individual events (Fig. 4B). ShRNA-syt I cells exhibited a drastically reduced spike amplitude compared with the control cells. The majority of events rose from baseline rapidly as single spikes and decreased back to baseline at a slower rate. In some instances, the single spike was preceded by a slower and smaller elevation from baseline that is referred to as the "foot," believed to be caused by slow release of transmitter from a partially open fusion pore (8).

Expression of human syt I can rescue catecholamine secretion. To determine whether the reduced secretion in shRNA-syt I knockdown cells is actually due to lack of syt I, we attempted to rescue secretion by transiently transfecting the shRNA-Syt I knockdown cells with a plasmid designed to express human syt I. Human syt I differs from rat syt I in 4 of the 19 nucleotides in the shRNA target region. Therefore, human syt I mRNA should not be subject to RNAi elicited by the shRNA used in this study, which targets rat syt I. Using immunocytochemistry, we established that human syt I is indeed expressed in syt I knockdown cells compared with control cells. Release of stimulated catecholamine is expressed as a percentage of total content of norepinephrine or dopamine content of the cells (n = 6 for each set of cells).
Expression of green fluorescent protein was used to identify transfected cells. The average number of release events per cell measured in the syt I knockdown cells expressing human syt I (114 events ± 10, n = 7 cells, 798 total events) was not significantly different from the number of release events measured in either the control untransfected or CT cells (Fig. 4C). Because expression of human syt I rescued the secretion deficit observed in syt I knockdown cells, the decrease in secretion most likely is due to lack of syt I and is unlikely to be due simply to clonal variability between cell lines.

**Catecholamine release is reduced determined by HPLC.** To confirm that the reduction in release events measured in single cells with amperometry is reflective of a reduction of release from the cell lines, we used HPLC to quantify stimulated release of catecholamine from fields of cells. NGF-treated cells were stimulated with high-K+ solution for 5 min to evoke Ca2+-dependent vesicle release of the catecholamines norepinephrine and dopamine. Figure 4E shows the stimulated fractional release of norepinephrine and dopamine expressed as a percentage of total norepinephrine or dopamine content of the cells, respectively. Average stimulated fractional release of norepinephrine was reduced by 58% and that of dopamine by 51% in syt I knockdown cells compared with control cells (P < 0.001, n = 6 for each set of cells). Average basal fractional release was <0.85% (data not shown) for the same sets of experiments.

**Quantal charge and kinetic characteristics are reduced in syt I knockdown cells.** Quantification of the individual events shows that peak amplitude was reduced in shRNA-syt I cells. Peak amplitude of the averaged events was 15.6 ± 0.9 pA for control cells (n = 1,119) and 7.2 ± 0.6 pA for syt I knockdown cells (n = 417; P < 0.01) (Fig. 5A). Similarly, the kinetic rate of rise was significantly reduced from 27.3 pA/ms (±2.3 pA/ms) in control cells to 13.4 pA/ms (±1.9 pA/ms; P < 0.01) in syt I knockdown cells (Fig. 5B). The rising phase was reduced from 1.35 ms (±0.05 ms) in control cells to 1.11 ms (±0.08 ms) in syt I knockdown cells (P < 0.01). The bar histograms shown in Fig. 5, A and B, for peak amplitude and rate of rise kinetic parameters reveal that the distribution of events is different in the syt I knockdown cells compared with control cells.

To compare the kinetics of the events, we chose two cells from each of the control and shRNA-syt I knockdown cells that had a similar number of events (~170). To show the kinetics of the fusion pore opening, these averaged spikes from two individual cells are depicted in Fig. 6A: one control and one shRNA-syt I knockdown cell. The spike amplitude, rising phase, falling phase, time to half-width, and quantal charge were measured as indicated by arrows for the averaged spike event from each cell. The quantal charge was calculated by integrating the area under the curve. From this comparison, the difference between the amplitudes and rate of rise is apparent. These data can be interpreted to mean that the fusion pore opening does not occur with the same kinetics for the syt I knockdown cells as for the control cells.

Average quantal charge, half-width, and falling phase of all events were analyzed for control (n = 1,119) and shRNA-syt I knockdown (n = 417) cells. Each of the three parameters was...
Fig. 6. Quantitative analysis reveals that the amplitude, quantal charge, and kinetics are reduced in the syt I knockdown cells. A: two cells were chosen from each of the control and shRNA-syt I knockdown cells that had a similar number of events (~170). Spike amplitude (Amp), rising phase (RP), falling phase (FP), time to half-width (T1/2), and quantal charge (Q) were measured as shown by arrows for the averaged spike event from each cell. Quantal charge was calculated by integrating the area under the curve. The kinetic parameters of quantal charge (B), half-width (C), and falling phase (D) were significantly reduced in the syt I knockdown cells compared with control cells. *P < 0.01.
significantly ($P < 0.01$) reduced in the shRNA-syt I knockdown cells compared with control cells (Fig. 6, B–D). The number of oxidized molecules ($N$) was calculated using the Faraday equation $N = Q/ne$, where $Q$ is total quantal charge measured, $n$ is two electrons per oxidized molecule of detectable transmitter molecule of norepinephrine, and $e$ is the elemental charge ($1.603 \times 10^{-19}$ C). From the calculation with total quantal charge per group of cells, the total number of transmitter molecules released, $N$, is $12.56 \times 10^7$ molecules released from the control cells and $2.34 \times 10^7$ molecules released from the synt I knockdown cells, an approximately sixfold reduction in transmitter molecules released.

### DISCUSSION

We have generated a PC12 cell line stably transfected with a plasmid expressing an shRNA insert that targets a specific region of synt I mRNA. Expression of this shRNA specifically and completely knocked down the expression of synt I protein without affecting cell survival, the ability of cells to respond to treatment with NGF, or stimulated Ca$^{2+}$ influx. The lack of synt I reduced, but did not abolish, secretion of catecholamines from the cells, suggesting that additional protein(s) may act as a Ca$^{2+}$ sensor for regulated release. A primary advantage of generating stable knockdown cell lines is that electrophysiological measurements can be performed on single cells within a homogenous population that have equivalently reduced levels of one specific protein. Other advantages to using RNAi include lack of developmental problems and lethality often observed in knockout mice, as well as simultaneous or sequential knockdown of multiple proteins. Furthermore, expression of most proteins can be knocked down within a relatively short period of time, with relative ease and at a lower cost to the researcher compared with generating knockout mice. By establishing stable knockdown cell lines, the uncertainty that the targeted protein is knocked down to the same extent in each cell is reduced.

Synt I knockdown was specific in that expression of synt I was abolished in the synt I knockdown cells, whereas other vesicle and structural protein expression levels were unaffected. Synt I knockdown cells did not exhibit either a reduction or compensatory upregulation of other endogenously expressed synt isoforms that bind phospholipids in a Ca$^{2+}$-dependent manner, including the highly abundant synt IX isoform or the much less abundant synt III or VII isoforms. Synt II, an isoform that is closely related to synt I in sequence and that has been postulated to be functionally complementary to synt I, is an isoform that is not expressed in the cells as protein even though the transcript is made in these cells. Synt II expression was not upregulated when synt I was abolished in the synt I knockdown cells.

We have begun to characterize the functional effects of removing synt I on catecholamine secretion from the neuroendocrine cells. Synt I knockdown cells exhibited reduced secretion events as measured using amperometry and reduced fractional catecholamine release as measured using HPLC. As a test of specificity of the shRNA, expression of human synt I not targeted by the shRNA reversed the effects of the synt I knockdown by functionally rescuing the secretion events. Quantitative kinetic analysis of the amperometric events shows that amplitude, quantal charge, rate of rise, half-width, and falling phase all were significantly reduced in the synt I knockdown cells compared with control cells. The amplitude of the spike events was reduced to 50% of control, and quantal charge was reduced, which translated to a sixfold decrease in catecholamine molecules released. This result may be due to 1) reduced time or size of pore opening, 2) reduced vesicle size and thus volume of stored transmitter, or 3) reduced numbers of transmitter molecules packaged in vesicles. Although our current results do not allow us to directly distinguish among these three possibilities, the measurements of reduced rate of rise time, reduced half-width, and reduced falling phase of the amperometric spike events in synt I knockdown cells are consistent with the idea that the fusion pore may remain open for a shorter time than in control cells.

The partial reduction of catecholamine secretion seen in our synt I knockdown cells is similar to the $\sim 60\%$ reduction in catecholamine release measured using real-time amperometry in cracked PC12 cells after the introduction of recombinant C2A-C2B domain from synt I (51). The C2A-C2B domain presumably competes with endogenous Syt I for binding to Ca$^{2+}$ and the SNARE complex, thus inhibiting the usual function of synt I. However, our data vary from results in chromaffin cells obtained from synt I knockout mice (53). In their studies of secretion from Ca$^{2+}$-dialyzed chromaffin cells in adrenal slices, the authors found that the Ca$^{2+}$ dependence of release was altered, exocytosis of large dense-core vesicles was delayed, and Ca$^{2+}$-dependent fusion rates were slowed as measured using membrane capacitance techniques (53). They did not detect differences in amperometric release events between wild-type and synt I knockout cells. One possibility for the differences between our study and that of Voets et al. (53) may be the stimulation protocols for vesicle release. Because Voets et al. used Ca$^{2+}$ dialysis to stimulate, the release machinery may have become saturated with Ca$^{2+}$ and caused the subsequent shift in Ca$^{2+}$ dependence of vesicle release, whereas our stimulation protocol may not have saturated the release machinery. This possible explanation would fit well with results measured in wild-type chromaffin cells that exhibit a strong Ca$^{2+}$ dependence for release of vesicle content; that is, for small Ca$^{2+}$ influx, only a fraction of the contents of vesicles is released, but with strong stimulation and hence a large Ca$^{2+}$ influx, all of the vesicle content is released (13).

In a spontaneously occurring mutant line of PC12 cells that lacked the synt I isoform, Ca$^{2+}$-dependent secretion of dopamine as measured by HPLC was normal compared with that in wild-type PC12 cells (41). This observation led to the suggestion that neuroendocrine cells do not require synt I and that an alternative protein may function as the Ca$^{2+}$ sensor for regulated release. Yet another experimental difference between our results and the data for mutant PC12 cells (41). Our data differ from the mutant PC12 cell data, because the synt I knockdown cells exhibited differences in release of catecholamine compared with the parent cell line. It is not known at this time whether the differences between our results may arise from methodological measurement of secretion, variations between the PC12 cell lines, or the proteins that act to regulate Ca$^{2+}$-dependent release. Yet another experimental difference between our results and the data for mutant PC12 cells that lack synt I is that the mutant cells exhibited an upregulation of synt IX expression compared with control cells, whereas our synt I knockdown cells did not exhibit any upregulation in synt IX. Even so, our results agree with the interpretation that additional proteins may function as regulators of evoked vesicle release.
Previous work has led to the suggestion that a protein other than Syt I has a role as a Ca\(^{2+}\) sensor in rapid, regulated release. Syt I deletion studies performed in various organisms such as *Drosophila* (29), *Caenorhabditis elegans* (34), and mice (19) revealed that syt I was required for fast synaptic release of neurotransmitter. In syt I knockout mice, fast synchronous release was abolished, but slow asynchronous release was still evident (19) and even increased to match the synchronous release of wild-type neurons, which led to the hypothesis that syt I inhibits slow asynchronous release (32, 33). Furthermore, upon either altering or neutralizing the Ca\(^{2+}\)-binding pockets of the C2 domains by point mutagenesis, the Ca\(^{2+}\) dependency of release was shifted, which suggested that the C2A domain cannot represent the Ca\(^{2+}\) sensor in its entirety but, rather, is a necessary but not sufficient component of the sensor required for fast neurotransmission in the central nervous system (42). Additional experiments in a variety of systems have shown that interfering with either the C2A or C2B domain, or with Ca\(^{2+}\) ions, SNARE complex proteins, and/or phospholipid binding of syt I, results in altered secretion of vesicles (18, 27, 30, 49, 55).

To account for the remaining Ca\(^{2+}\)-dependent fusion events, cells would require an additional protein to compensate as a Ca\(^{2+}\) sensor. Likely candidates include one of the additional Syt isoforms endogenously expressed by the cells, such as syt IX, or the less abundant syt isoforms, syt III or VII. Our results showing that syt I and IX are the two most abundant isoforms of syt expressed agree well with other reports of syt isoform expression of syt VII have been reported in PC12 cells (51). There is a discrepancy as to whether syt III is expressed; for example, Tucker et al. (51) did not detect syt III, but Sugita et al. (47) did detect this isoform. Both syt III and VII have been proposed to act as Ca\(^{2+}\) sensors (24, 46, 47), because recombinant fragments of the C2 domains of these isoforms inhibited secretion from cracked PC12 cells (51), and syt VII can function as a high-affinity Ca\(^{2+}\) sensor for secretion of large dense-core vesicles in PC12 cells (24).

In conclusion, this study has established a neuroendocrine cell line that utilizes the plasmid-based RNAi method to stably, specifically, and completely knock down expression of syt I in a homogenous population of cells. Release studies show that syt I knockdown cells have reduced release events and reduced amounts of transmitter released that may be due to a reduced open time of the fusion pore. These results support a role for syt I as a functional Ca\(^{2+}\) sensor, but not an exclusive Ca\(^{2+}\) sensor. RNAi-induced effects are unlikely to be due to a lack of Ca\(^{2+}\) ions, given that Ca\(^{2+}\) influx was similar between knockdown and control cells. Furthermore, it is unlikely that one of the other endogenously expressed syt isoforms or the closely related isoform, syt II, has compensated functionally for the lack of syt I.

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**REFERENCES**

SECRETION IS INHIBITED IN SYNAPTOTAGMIN I KNOCKDOWN CELLS


