RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells

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Although PC12 cells express three synaptotagmin isoforms (Syts I, IV and IX), all of which have been proposed to regulate dense-core vesicle exocytosis, it remains unknown which of the Syt isoforms acts as the major Ca^{2+} sensor for dense-core vesicle exocytosis. In the present study, it has been shown by immunoaffinity purification and immunocytochemistry that Syts I and IX, but not Syt IV, are present on the same secretory vesicles in PC12 cells. Silencing of Syt IX with specific small interfering RNA significantly reduced high KCl-dependent neuropeptide Y secretion from PC12 cells, whereas silencing of Syt I with specific small interfering RNA had no significant effect. The results indicate that Syts I and IX are not functionally equivalent and that Syt IX, and not Syt I, is indispensable for the regulation of Ca^{2+}-dependent dense-core vesicle exocytosis in PC12 cells.

Key words: Ca^{2+} sensor, endocrine cell, exocytosis, double-stranded RNA-mediated interference (RNAi), PC12 cell, synaptotagmin.

INTRODUCTION

Syts (synaptotagmins) constitute a large family of putative membrane trafficking proteins and are defined as proteins containing an N-terminal single transmembrane domain and C-terminal tandem C2 domains (reviewed in [1–5]). Syts are found in a variety of species in different phyla, and 15 different isoforms have been reported in mice and humans [3,5–9]. Although the exact functions and localization of most of the Syt isoforms remain obscure and are still matters of controversy, the best-characterized isoform Syt I, abundant on synaptic vesicles, is now widely believed to function as the major 'Ca^{2+} sensor' for neurotransmitter release as well as in the control of synaptic vesicle endocytosis [10–14]. Syt I is also found on the secretory vesicles of certain endocrine cells (e.g. chromaffin cells, pancreatic β-cell lines and PC12 cells) and has been proposed to be an endocrine Ca^{2+} sensor [15–20]. However, since Syt I-deficient PC12 cells still release catecholamines and ATP normally [21], the presence of an alternative Ca^{2+} sensor(s) for Syt I (possibly other Syt isoforms) in endocrine cells has been proposed [20,22–27]. Consistent with this notion, endogenous expression of two additional Syt isoforms, Syts IV and IX, has been reported recently at the protein level in PC12 cells, and these isoforms have been proposed to regulate dense-core vesicle exocytosis in PC12 cells [19,20,25,28,29]. However, which of the Syt isoforms (Syt I, Syt IV or Syt IX) functions as the major Ca^{2+} sensor for secretory vesicle exocytosis in PC12 cells still remains to be determined.

In the present study, the function of individual Syt isoforms has been investigated by silencing specific Syt isoforms with recently developed RNAi (double-stranded RNA-mediated interference) technology. The results showed that silencing Syt IX, but not Syt I, strongly attenuates high KCl-dependent NPY (neuropeptide Y) secretion from PC12 cells. On the basis of this finding, I propose that Syt IX is the major Ca^{2+} sensor in endocrine cells and discuss the functional relationship between Syts I and IX in dense-core vesicle exocytosis.

MATERIALS AND METHODS

Materials

Anti-Syt I, anti-Syt IV, fluorescein-labelled anti-Syt IX and anti-Rab27A rabbit polyclonal antibodies were prepared as described previously [25,30,31]. Anti-Syt I and anti-VAMP-2/synaptobrevin-2 mouse monoclonal antibodies (where VAMP stands for vesicle-associated membrane protein) were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Anti-Rab3A, anti-Rab27A, anti-Syt IX (also called anti-Syt V) and anti-Munc18-1 mouse monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, KY, U.S.A.). Anti-syntaxin I/HPC-1 mouse monoclonal antibody and anti-actin goat polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-synaptophysin and anti-SNAP-25 mouse monoclonal antibodies (where SNAP stands for soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein) were from Sigma (St. Louis, MO, U.S.A.) and Upstate Biotechnology (Lake Placid, NY, U.S.A.) respectively. Alexa Fluor 568-labelled anti-mouse IgG and Alexa Fluor 633-labelled anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR, U.S.A.). Horseradish peroxidase-conjugated anti-T7 tag mouse monoclonal antibody was from Novagen (Madison, WI, U.S.A.).

Immunoaffinity purification of Syt IX (or Syt I)-containing vesicles

Dynabeads M-280 (20 μl volume, wet volume) coated covalently with sheep anti-mouse IgG (Dynal, Oslo, Norway) was incubated overnight at 4 °C with the anti-Syt I antibody, anti-Syt IX antibody or control mouse IgG (5 μg) in PBS containing 0.1 % BSA. PC12 cells (two confluent 10 cm dishes) were homogenized in a homogenization buffer (5 mM Hepes/KOH, pH 7.2/5 mM EDTA/0.03 M sucrose/appropriate protease inhibitors), and after centrifugation at 800 ***g*** for 10 min, the crude membrane in the supernatant was also incubated overnight with a 50 μl volume of magnetic beads without a primary antibody for preabsorption. The

Abbreviations used: GFP, green fluorescent protein; GST, glutathione *S*-transferase; NPY, neuropeptide Y; RNAi, double-stranded RNA-mediated interference; siRNA, small interfering RNA; SNAP, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein; Syt(s), synaptotagmin(s); VAMP, vesicle-associated membrane protein.

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supernatant was then incubated with the primary antibody-coated beads for 1 h at 4 °C in PBS containing 5 % (v/v) foetal bovine serum and 2 mM EDTA. After washing the beads twice with PBS containing 2 mM EDTA for 15 min, the bound fractions were analysed by SDS/PAGE (10 % gel), transferred on to a PVDF membrane and then immunoblotted with anti-Rab27A rabbit antibody (1 μg/ml), anti-synaptophysin mouse antibody (1:5000 dilution), anti-Syt I rabbit antibody or anti-Syt IX rabbit antibody (1 μg/ml) as described previously [25,32]. Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Bucks., U.K.). The blots shown are representative of two or three independent experiments.

Immunocytochemistry

PC12 cells were cultured on glass-bottom dishes coated with collagen type IV as described previously [30,33]. The cells were fixed with 4 % (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 20 min at room temperature (25 °C), permeabilized with 0.3 % Triton X-100 in PBS for 2 min, and incubated with the blocking solution (1 % BSA and 0.1 % Triton X-100 in PBS) for 1 h at room temperature as described previously [30,33]. The cells were then incubated with the primary antibody, i.e. anti-Syt IV rabbit polyclonal antibody (5 μg/ml) and anti-Syt I mouse monoclonal antibody (1:200 dilution), and then with the secondary antibodies: Alexa Fluor 633-labelled anti-rabbit IgG (1:5000 dilution) and Alexa Fluor 568-labelled anti-mouse IgG (1:5000 dilution) for 1 h at room temperature. After washing the cells with the blocking solution, the cells were incubated with fluorescein-labelled anti-Syt IX antibody (1:200 dilution). Immunoreactive signals were analysed with a confocal fluorescence microscope (Fluoview; Olympus, Tokyo, Japan), and the images were then processed with Adobe Photoshop software (version 7.0).

Construction of pSilencer-Syt vectors for Syt silencing

To silence rat and mouse Syt I (or Syt IX), four different 19-nucleotide sequences were selected according to the manufacturer's instructions (see http://www.ambion.com/techlib/misc/siRNA_finder.html): GCTGAAGCAGAAGTTTATG (Syt I silencer site 1); AGACTTAGGGAAGACCATG (Syt I silencer site 2); GGAAGAGGAGAAACTGGGA (Syt I silencer site 3); GTCCACCGGAAAACCCTCA (Syt I silencer site 4); AATGGATGTAGGAGGACTC (Syt IX silencer site 1); CCACCATTAAGAAGAACAC (Syt IX silencer site 2); CACCCTGAACCCCTATTAC (Syt IX silencer site 3); and GAATGAGGCCATCGGGAGA (Syt IX silencer site 4). The specificity of each sequence was verified by a BLAST search of the public databases. pSilencer™ 1.0-U6 expression vectors (Ambion, Austin, TX, U.S.A.) that produce siRNAs (small interfering RNAs) targeted against Syt I or Syt IX (named pSilencer-Syt I st1–4 or pSilencer-Syt IX st1–4) were also prepared according to the manufacturer's instructions. In brief, two oligonucleotides (Syt I st1 sense, 5′-<u>GCTGAAGCAGAAGTTTATG</u>TTCAAGAGA<u>CATAAACTTCTGCTTCAGC</u>TTTTTT-3′ and Syt I st1 antisense, 5′-AATTAAAAAAGC<u>TGAAGCAGAAGTTTATG</u>TCTCTTGAA<u>CATAAACTTCTGCTTCAGC</u>GGCC-3′; the underlined sequences contribute to forming small hairpin RNAs) were chemically synthesized, and the annealed oligonucleotides encoding a short hairpin RNA were then subcloned into the *Apa*I–*Eco*RI site of the pSilencer™ 1.0-U6 vector. The efficiency and specificity of the siRNA targeted against Syt I or Syt IX were assessed by co-transfection of the pSilencer vector with pEF-T7-Syt into COS-7 cells [34] (see Figure 2A).

NPY release assay

Co-transfection of pShooter-NPY-T7-GST (where GST stands for glutathione *S*-transferase) with pSilencer-Syt into PC12 cells and the amount of NPY released were determined as described previously [35,36] with slight modification. Before the secretion assay, transfected PC12 cells were incubated with 10 μg/ml brefeldin A for 30 min to eliminate constitutive NPY secretion [19,31]. The NPY cDNA was donated by Dr W. Almers (Vollum Institute, Portland, OR, U.S.A.).

RESULTS AND DISCUSSION

Co-localization of Syts I and IX on secretory vesicles in PC12 cells

In a previous study, it was I found that Syts I, IV and IX proteins are expressed in PC12 cells, with Syts I and IX being dominant [19,25,29]. As shown in Figure 1(A), both Syts I (red) and IX (green) proteins are enriched and co-localized at the edge of the undifferentiated PC12 cells, whereas Syt IV (blue) protein is predominantly localized in the perinuclear region (i.e. the Golgi), indicating that Syt IV is not present on secretory granules [29,30,37,38]. To determine whether Syts I and IX are present on the same secretory vesicles, Syt I (or Syt IX)-containing vesicles were affinity-purified with specific anti-Syt I (or Syt IX)-coated beads (Figure 1B). As expected, Syt IX was co-purified with Syt I and vice versa (lanes 2 and 4 in the two topmost panels), whereas Syt IV was not co-purified with either Syt I or Syt IX (lanes 2 and 4 in the third panel) under my experimental conditions, consistent with the previous immunoelectron microscopic results: Syt IV is not associated with dense-core vesicles in undifferentiated PC12 cells [29]. In addition, both anti-Syt I and anti-Syt IX antibodies enriched synaptophysin (a marker for synaptic-like microvesicles) as well as Rab27A (a marker for dense-core vesicles [31,36], indicating that Syts I and IX are present on both dense-core vesicles and synaptic-like microvesicles in PC12 cells. Under the experimental conditions used in the present study, control IgG did not enrich either Syts or secretory vesicle markers (lane 3).

Silencing of Syt IX, but not Syt I, with specific siRNA attenuates dense-core vesicle exocytosis in PC12 cells

To determine which of the Syt isoforms (Syt I or Syt IX) is the predominant Ca^{2+} sensor for dense-core vesicle exocytosis in PC12 cells, Syt I (or Syt IX) was specifically knocked-down by RNAi technology [39,40]. Four pSilencer vectors were constructed, which produce specific siRNA targeted against Syt I (or Syt IX) (named Syt silencer st1–4) as described in the Materials and methods section. As shown in Figure 2(A), the Syt I silencer st1 strongly suppressed expression of recombinant T7-Syt I (lane 2 in the upper left panel) when the pSilencer vector and pEF-T7-Syt I were co-transfected into COS-7 cells. It is important to note that the Syt I silencer st1 had no effect on the expression of T7-Syt IX (lane 6 in the upper right panel), confirming the specificity of the siRNA. Similarly, the Syt IX silencer st1 strongly suppressed expression of T7-Syt IX (lane 2 in the upper right panel), but not of T7-Syt I (lane 6 in the upper left panel). In addition, the Syt IX silencer st1 had no effect on the expression of other Syt isoforms (e.g. Syt IV or Syt VII) that have been proposed to function in PC12 cells [23,41,42] (results not shown).

When the pSilencer-Syt I (or Syt IX) st1 was co-transfected with pEGFP-C1 into PC12 cells, endogenous Syt I (or Syt IX) protein levels decreased significantly in the GFP (green fluorescent protein)-expressing cells (arrows) compared with the non-transfected cells (Figure 2B). Selective knock-down of Syt I (or Syt IX) with specific siRNA was further confirmed by

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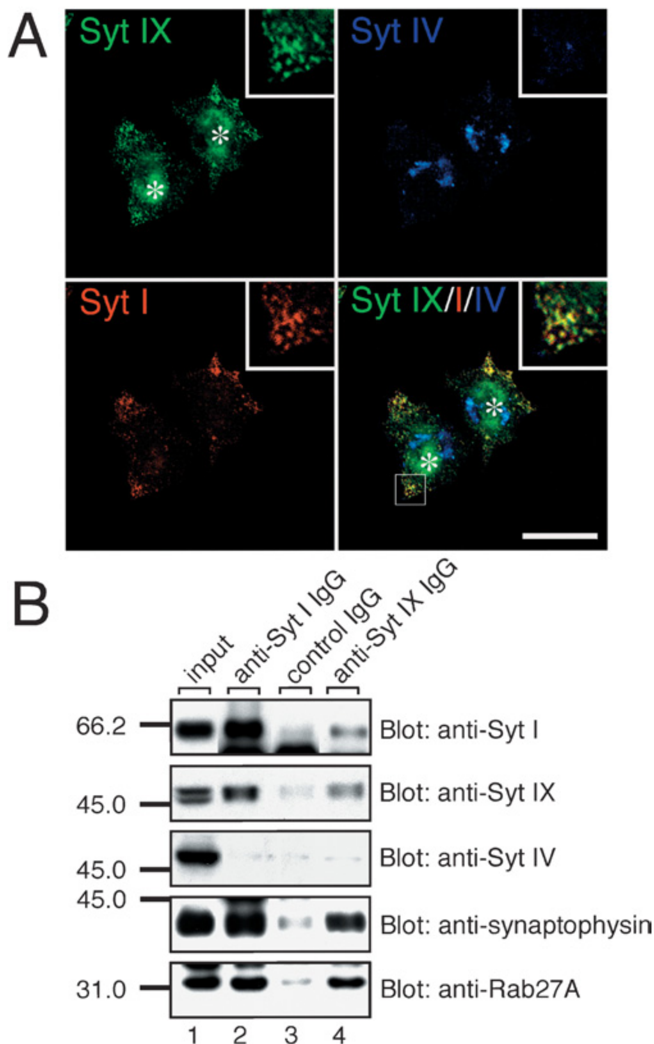

Figure 1 Expression of Syts I and IX on the same secretory vesicles in PC12 cells

(**A**) Co-localization of Syt IX (green) with Syt I (red), but not with Syt IV (blue), in undifferentiated PC12 cells. Asterisks indicate non-specific nuclear staining by the fluorescein-labelled anti-Syt IX antibody. Note that Syts I and IX co-localized well at the cell periphery (yellow in insets), whereas Syt IV was mainly localized in the Golgi [29,30,33]. Scale bar, 20 μm. (**B**) Co-purification of Syts I and IX with secretory vesicle markers in PC12 cells. Immunoaffinity purification of Syt I (or Syt IX)-containing vesicles was performed as described in the Materials and methods section. Note that Syt IX-containing vesicles contain Syt I, synaptophysin and Rab27A, but not Syt IV (lane 4). The positions of the molecular-mass standards ($\times 10^{-3}$) are shown on the left.

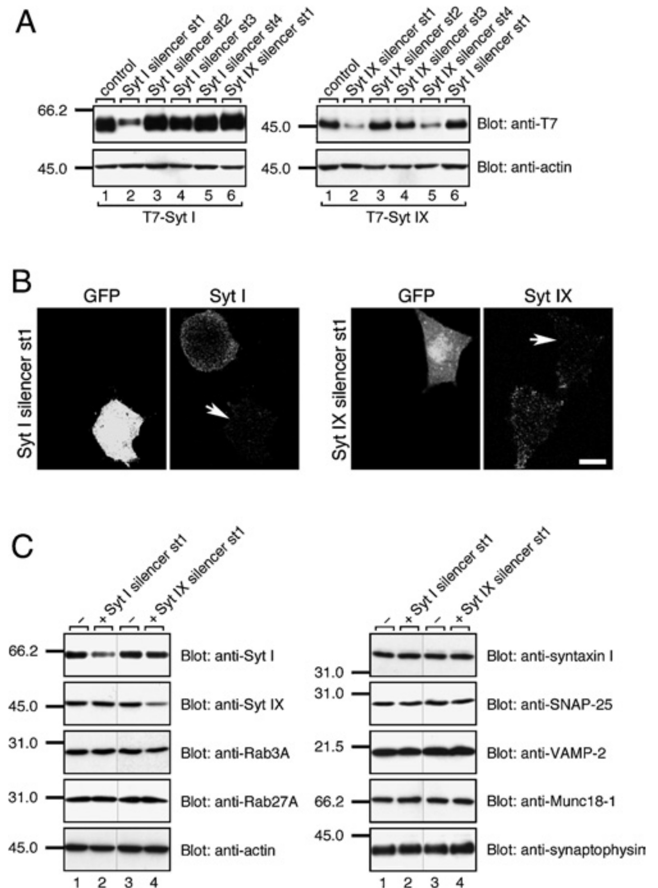

Figure 2 Silencing Syt I or Syt IX in PC12 cells with RNAi

(**A**) Efficiency and specificity of siRNA targeted against Syt I (left panel) or Syt IX (right panel). pSilencer-Syt and pEF-T7-Syt were co-transfected into COS-7 cells. Cells were harvested 3 days after transfection and homogenized in 1 % SDS and then analysed by immunoblotting with horseradish peroxidase-conjugated anti-T7 tag antibody (upper panels) and anti-actin antibody (lower panels). Note that Syt I silencer st1 efficiently silenced the expression of T7-Syt I (lane 2 in the left upper panel) but had no effect on the expression of T7-Syt IX (lane 6 in the right upper panel). The lack of effect of the Syt I silencer st2–4 is probably attributable to the inefficient formation of double-stranded RNA (dsRNA) because of the steric hindrance caused by the secondary or ternary structure of mRNA. (**B**) Silencing of endogenous Syt I or Syt IX in PC12 cells with RNAi. pSilencer-Syt I/IX st1 vector was co-transfected with pEGFP-C1 into PC12 cells. Cells were fixed, permeabilized and stained with anti-Syt I or anti-Syt IX mouse monoclonal antibody 3 days after transfection (right panels). Note that the Syt I (or Syt IX) immunostaining was significantly reduced in the GFP-expressing (i.e. siRNA-expressing) cells (arrows in the right panels), compared with the control non-transfected cells. Scale bar, 10 μm. (**C**) Specific silencing of endogenous Syt I or Syt IX with RNAi. Expression levels of other proteins involved in regulated exocytosis [SNAREs (SNAP receptors), Rabs, Muc18-1 and synaptophysin] in PC12 cells were unaltered by Syt silencing. The positions of the molecular-mass standards ($\times 10^{-3}$) are shown on the left.

immunoblotting (Figure 2C). Expression of siRNA against Syt I (or Syt IX) did not alter the protein expression levels of other membrane-trafficking proteins (syntaxin I, SNAP-25, VAMP-2, Munc18-1, synaptophysin, Rab3A and Rab27A) or actin. Dose-dependent down-regulation of endogenous Syt I (or Syt IX) protein in PC12 cells was also investigated (Figures 3A and 3B). When 2 μg of pSilencer-Syt st1 vector was transfected into PC12 cells, the endogenous Syt I and IX expression levels were reduced to approx. 50 and 60–70 % respectively, of their levels in non-transfected cells. Since the maximum transfection efficiency into PC12 cells by LIPOFECTAMINE™ 2000 reagent was estimated to be 50 % (as determined by GFP fluorescence), Syt I was almost completely knocked-out in siRNA-expressing cells, and at least 80 % of Syt IX was knocked down.

Finally, the effect of specific silencing of Syt I (or Syt IX) on high KCl-dependent NPY secretion from PC12 cells was investigated. As shown in Figure 3(C), NPY secretion was reduced in a Syt IX-silencer-dose-dependent manner (shaded bars). However, it was surprising to note that silencing of Syt I had no significant effect on NPY secretion under the experimental conditions used (black bars). Both Syt I and IX silencers (2 μg each) were also simultaneously introduced into PC12 cells, but no additive effect was observed (results not shown), indicating that Syt I is dispensable for dense-core vesicle exocytosis in PC12 cells. It should be noted that there was a close correlation between Syt IX expression levels and NPY secretion in PC12 cells (open circles in Figure 3D), whereas Syt I expression levels

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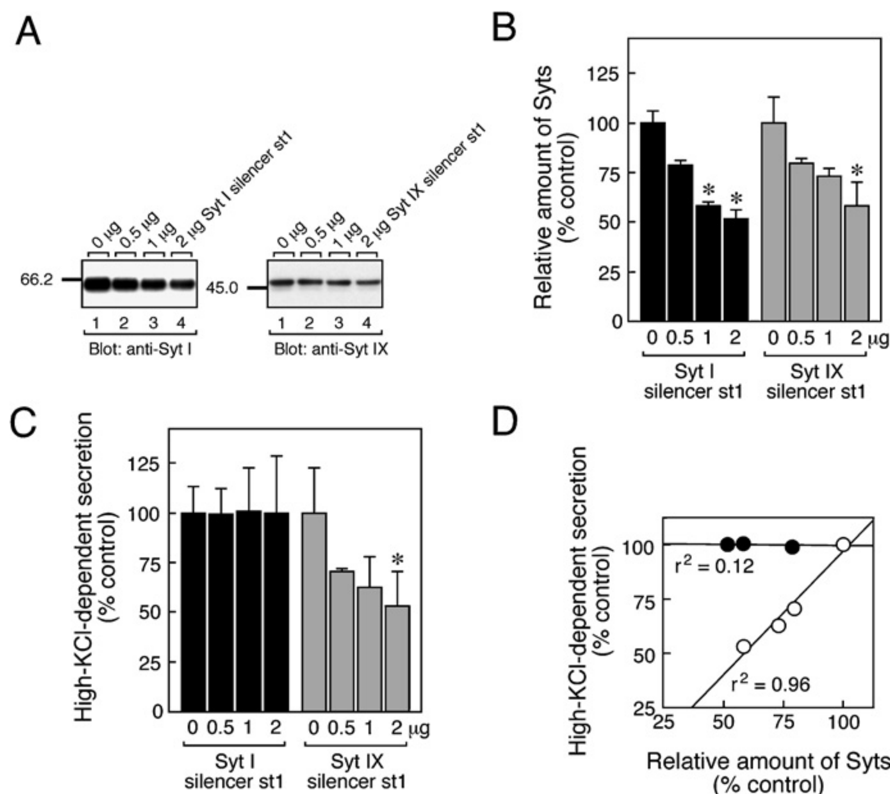

Figure 3 Syt IX silencing, but not Syt I silencing, caused reduction of dense-core vesicle exocytosis in PC12 cells

(**A**) Dose–response effect of pSilencer-Syt on endogenous Syt I or Syt IX expression in PC12 cells. The Syt I (or Syt IX) expression level was reduced in a dose-dependent manner, with maximal inhibition approx. 50 % (or 60–70 %). The positions of the molecular-mass standards ($\times 10^{-3}$) are shown on the left. (**B**) Graph of fold decreases in values from (**A**). The intensity of the bands in (**A**) was quantified with Lane Analyzer (version 3.0; ATTO Corp., Tokyo, Japan). Results are expressed as means ± S.E.M. for three independent experiments normalized to the protein content of control cells. (**C**) Dose–response effect of pSilencer-Syt on high KCl-dependent NPY secretion. The NPY-T7-GST secretion assay was performed as described previously [35,36]. Results are expressed as percentages of NPY-T7-GST secretion in control samples. Bars represent the means ± S.E.M. for three determinations. Results shown are representative of at least three independent experiments. Note that Syt IX silencing resulted in a significant reduction in NPY secretion (shaded bars), whereas Syt I silencing had no effect (black bars). *$P < 0.05$, Student's unpaired *t* test. (**D**) The extent of inhibition of NPY secretion (in **C**) was plotted against the relative amount of Syts (in **B**; ○, Syt IX and ●, Syt I). Data were fitted by linear regression and r^2 values are reported.

were independent of NPY secretion (closed circles). This finding is surprising and unexpected, since Syts I and IX were previously supposed to play redundant roles in secretory vesicle exocytosis in PC12 cells [20,25] and two C2 domains of Syts I and IX exhibit the same biochemical properties tested so far [20,25,43,44].

Conclusions

In the present study, it was found that specific silencing of Syt IX, but not of Syt I, with siRNA significantly reduced dense-core vesicle exocytosis in PC12 cells. Although NPY secretion was not completely inhibited by siRNA (approx. 40–50 % of the control cells), the residual activity is probably mediated by the other Syt IX molecules that were not knocked-down by the siRNA treatment, rather than by Syt I molecules (i.e. compensatory effect). Actually, it was found by co-immunostainings of Syt IX and NPY that approx. 40 % of the NPY-positive cells contain a normal level of Syt IX (i.e. co-transfection efficiency is approx. 60 %; results not shown), indicating that NPY secretion occurs normally in these cells. Since Syt IX is also expressed in other endocrine cells (e.g. chromaffin cells, AtT20 cells and pancreatic β-cell lines) (M. Fukuda, unpublished work, [45]), it is proposed that Syt IX is the major Ca^{2+} sensor for dense-core vesicle exocytosis in various endocrine cells and that Syt I may be involved in this process through Ca^{2+}-dependent interaction with Syt IX via C2 domains [46,47]. Alternatively, Syt I may be involved in fast exocytosis (or kinetics of exocytosis) but not required for the total amount of Ca^{2+}-triggered exocytosis [18].

Note added in proof (received 26 March 2004)

While this manuscript was being prepared for publication, the role of Syt IX in insulin secretion in pancreatic β-cells has been reported (see [45] for details).

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