Biochemical and Biophysical Research Communications 371 (2008) 781–786

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Synaptotagmin IV regulates dense core vesicle (DCV) release in LβT2 cells

Zhi-Tao Hu [a,1], Mao-Rong Chen [a,1], Zhao Ping [a], Yong-Ming Dong [a], Rong-Ying Zhang [a], Tao Xu [a,b,*], Zheng-Xing Wu [a,*]

[a] Key Laboratory of Molecular Biophysics, Ministry of Education, and Joint Laboratory of Institute of Biophysics & Huazhong University of Science and Technology, Huazhong University of Science and Technology, Luoyu Road 1037#, Wuhan 430074, PR China
[b] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

ARTICLE INFO

Article history:
Received 25 April 2008
Available online 9 May 2008

Keywords:
Synaptotagmin
Double stranded RNA-mediated interference (RNAi)
LβT2 cell
Exocytosis
Ca^{2+} sensor

ABSTRACT

Synaptotagmins (Syts) are calcium-binding proteins which are conserved from nematodes to humans. Fifteen Syts have been identified in mammalian species. Syt I is recognized as a Ca^{2+} sensor for the synchronized release of synaptic vesicles in some types of neurons, but its role in the secretion of dense core vesicles (DCVs) remains unclear. The function of Syt IV is of particular interest because it is rapidly upregulated by chronic depolarization and seizures. Using RNAi-mediated gene silencing, we have explored the role of Syt I and IV on secretion in a pituitary gonadotrope cell line. Downregulation of Syt IV clearly reduced Ca^{2+}-triggered exocytosis of dense core vesicles (DCVs) in LβT2 cells. Syt I silencing, however, had no effect on vesicular release.

Synaptotagmins (Syts) are fascinating proteins. Accumulating evidence indicates that they are Ca^{2+} sensors for synaptic vesicle and secretory granule exocytosis and play roles in vesicular priming [1,2]. Syts are a large family of proteins which are highly conserved from nematode to human [3]. The synaptotagmins are characterized by a short NH_2 terminus, one transmembrane region which interacts with the vesicular membrane and tandem cytoplasmic C2 domains [4,5]. Fifteen isoforms (Syts I-XV) have been identified in mammalian species. Among them Syt I is best characterized. It is recognized as a Ca^{2+}-sensor for synchronized neurotransmitter release [6], but its function in dense core vesicles (DCVs) in neurons and neuroendocrine cells remains under debate. Syt I has been reported to function in DCV secretion in chromaffin cells [7], have no effect on exocytosis [8,9] or to function redundantly with Syt IX in controlling DCV fusion and fusion pore kinetics in the PC12 cells [10,11].

Syt IV, contains a naturally occurring amino acid substitution at a key residue for Ca^{2+} coordination within its C2A domain (S224A in rat Syt IV), and therefore, its C2A domain does not bind calcium [12]. Syt IV exhibits Ca^{2+}-dependent binding to Syt I, and thus, it was proposed that at elevated levels, Syt IV could form part of the Ca^{2+} sensor to regulate neurotransmission [13]. Syt IV is an immediate-early gene, its expression is developmentally regulated [14], and its expression rapidly changes in response to a variety of extracellular stimuli [15,16]. Its deletion in mice leads to deficits in memory and motor performance [17,18], reduction of anxiety, and depression-like behavior [19]. The dramatic effects of Syt IV deletion on nervous system function indicate a role for this molecule in brain, likely in neurotransmitter release and/or exocytosis of DCVs. Overexpression of Syt IV reduced the peak amplitude of synaptic responses at *Drosophila* neuromuscular junctions [13] and inhibited evoked secretion in PC12 cells [20,21]. These results support a role for Syt IV, perhaps as an inhibitor of neurotransmitter and DCV release. However, Syt IV upregulation was reported to have no effect on excitatory fast synaptic transmission, fusion modes, and fusion pore kinetics in mouse hippocampal neurons [22]. Thus, the functions and the mechanisms of Syt IV in neurotransmission and vesicular exocytosis remain unclear.

The anterior pituitary gonadotrope, which has a strong exocytotic response and a highly calcium sensitive pool of vesicles (HCSP) modulated by protein kinase C [23], is a good model for endocrine secretion. However, the heterogeneity of anterior pituitary primary cultures and the scarcity of gonadotropes (~5%) in this culture limit genetic manipulation. We have previously reported that LβT2 cells, an immortal gonadotrope cell line, have a larger HCSP than that observed in primary gonadotropes, and that

* Corresponding authors. Address: Key Laboratory of Molecular Biophysics, Ministry of Education, and Joint Laboratory of Institute of Biophysics & Huazhong University of Science and Technology, Huazhong University of Science and Technology, Luoyu Road 1037#, Wuhan 430074, PR China. Fax: +86 27 87792024 (Z.-X. Wu); +86 10 64867566 (T. Xu)
E-mail addresses: xutao@ibp.ac.cn (T. Xu), ibbwuzx@mail.hust.edu.cn (Z.-X. Wu).
[1] These authors contributed equally to the work.

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.04.174

the HCSP is modulated by protein kinase A, protein kinase C and basal calcium level [24]. As a homogenous cell population these cells are also well suited for genetic manipulations.

In this study, we examined the functional roles of Syt IV and Syt I on exocytosis of DCVs using RNA interference (RNAi). We demonstrate that Syt IV is involved in Ca^{2+}-dependent exocytosis in LβT2 cells and that alteration of Syt I has no effect. The inhibition of Syt IV expression strongly reduces secretion of RRP, SRP and sustained component in response to homogenous step-like Ca^{2+}-elevations. Curiously, its overexpression has no effect on exocytosis in these cells. Analysis of the Ca^{2+}-dependence of secretion shows that down-regulation of Syt IV suppresses the RRP including the HCSP.

Materials and methods

Construction of plasmids encoding shRNA. To generate a red fluorescent protein expressing shRNA vector, mRFP was used to substitute cGFP in pRNAT-H1.1/Neo (Genescript, Scotch Plams, NJ, USA). 19-Nucleotide siRNA target sequences of mouse Syt I and Syt IV genes, GCT GAA GCA GAA GTT TAT G and GAA GCA CAG AGT GAA GAC C, respectively, were chosen to silence their expression. Annealed double stranded oligonucleotides encoding the target-sequences were subcloned into the BamHI–HindIII site of the vector pRNAT-H1.1/RFP to generate the plasmids of Syt I- and IV-shRNA. Construct integrity was verified by DNA sequencing.

The RNAi-resistant construct. The full length cDNA for Syt IV was acquired using the OneStep RT-PCR Kit (Qiagen) from total RNA of LβT2 cells generated by the RNeasy Mini Kit (Qiagen), and then inserted into the XhoI and XmaI site of pIRES2-EGFP (Clontech). In order to generate a RNAi-resistant construct, silent point mutations in the targeting site of the coding sequence have been introduced. Mutations were obtained by Quickchange site-directed mutagenesis Kit (Stratagene). The PCR forward and reverse primers carried silent third-codon point mutations, 5′-(AA)A AAA CAT AGA GTG AAG ACC-3′ (mutated nucleotides are underlined) [25].

Western blotting. Total cell protein extract was prepared by lysing cells with M-PER mammalian protein extraction reagent kit supplemented with (in μg/ml) 86 phenylmethanesulfonyl fluoride (PMSF), 50 aprotinin, and 5 leupeptin (Roche, Basel, Swissland). Western analysis was performed as described previously [26]. Briefly, primary antibody, goat anti-Syt I-IgG, diluted 1:200, and anti-Syt IV-IgG, diluted 1:100; and second antibody, HRP-conjugated rabbit anti-goat IgG, diluted 1:10,000 in blocking buffer.

Identification of subcellular localization of synaptotagmins under total internal reflection microscope (TIRFM). A total internal reflection microscope system was used to determine the subcelluar localization of synaptotagmins. The TIRFM setup was constructed based on the prismless and through-the-lens configuration as previously described [27]. Briefly, the 488-nm laser line was used for simultaneous illumination of EGFP, TDimer2 and DsRed. A dual-View Micro-Imager (Optical Insights, Dodge-Blvd, Tucson, AZ, USA) was used for imaging the green and the red fluorescence simultaneously. Images were viewed, corrected for background, processed and analyzed using TILLvisION4.01 (Till Photonics) and Adobe Photoshop 7.02.

Membrane capacitance (Cm) measurement. The cellular capacitance was measured in real time using an EPC10 amplifier (Heka Electronics, Lambrecht, Germany) in whole-cell patch clamp configuration. A sine + DC protocol was applied using the Lockin extension of the Pulse program (Heka Electronics). The cells were voltage clamped at a holding potential of −70 mV and a sine wave voltage command with amplitude of 40 mV and frequency of 1042 Hz was applied. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz.

Ca^{2+}-uncaging and $[Ca^{2+}]_i$ measurement. A homogenous global $[Ca^{2+}]_i$ elevation was generated by photolysis of the Ca^{2+}-caging compound, nitrophenyl-ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (NP-EGTA, Molecular Probes, Carlsbad, CA, USA), with the UV light source as previously described [28]. The NP-EGTA containing pipette solution contained (in mM): 110 CsCl, 5 NP-EGTA, 2 NaCl, 4 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.2 furaptra or 0.2 fura-6F, and 35 Hepes, adjusted to pH 7.2 using CsOH or HCl (osmolarity, 300 mOsm). Unless otherwise indicated, the free Ca^{2+} concentration of the pipette solution was determined to be 300–400 nM. $[Ca^{2+}]_i$ was measured using either the single wavelength or dual wavelength excitation method for ramp and flash experiments respectively as suggested by Grynkiewicz et al. [29].

Results

Syt I and Syt IV have a vesicular distribution in LβT2 cells and were down-regulated by the transient expression of Syt I- and IV-shRNA

Different isoforms of Syts, which may mediate different pathways of membrane trafficking and secretion, have various subcellular localizations [30]. To identify the distribution of Syt IV and Syt I in LβT2 cells, we co-expressed a Syt IV-EGFP chimera and DsRed tagged NPY, a marker for DCVs [31]. The co-transfection revealed that in LβT2 cells Syt IV was localized on the DCVs (Fig. 1B). Phogrin (phosphatase on the granules of insulinoma cells), another marker for labeling DCVs [32], was co-localized with NPY-DsRed as shown in Fig. 1C, thus phogrin-EGFP and Syt I-TDimer2 were co-expressed for identification of vesicular distribution of Syt I. As shown in Fig. 1A, fluorescent puncta of Syt I-TDimer2 co-localize to a large degree with those of phogrin-EGFP, indicating that Syt I is located on DCVs in LβT2 cells.

The expression of the mRNA and the proteins of Syt I and Syt IV in LβT2 cells were measured by performing RT-PCR (data not shown) and Western blotting (Fig. 2). To determine whether Syt I and Syt IV are involved in the exocytosis of dense core vesicles in LβT2 cells, they were specifically knocked-down by RNAi technology. As shown in Fig. 2A, the expression of Syt I- and Syt IV-shRNA strongly suppressed expression of their target genes in the cells. The silencing efficiencies estimated by Western blot were ∼82% and ∼80% for Syt I- and IV-shRNA plasmids, respectively.

Gene silencing of Syt I with small interfering RNA had no significant effect on the exocytosis of DCVs

The effects of Syt I on exocytosis in LβT2 cells were measured using high time resolution capacitance measurements to record the secretory response to step-like and ramp-like calcium elevations induced by photolysis. Exocytotic responses in mock-transfected cells (plasmid with a vector only expressing mRFP) were similar to responses in untransfected cells (data not shown) and these two data sets were pooled. Silencing of Syt I had no effect on the amplitude or the kinetics of exocytotic responses to step-like calcium elevations (Fig. 3A and B). We next used a ramp increase of $[Ca^{2+}]_i$ photolysed by a weak steady UV illumination to analyze the Ca^{2+}-dependence of secretion in Syt I-silenced and control cells as described in our previous report [24]. No significant differences in secretion were found between Syt I-silenced cells and controls (Fig. 3C and D). The magnitude of the highly calcium sensitive pool (HCSP) and remaining RRP were the same in The Syt I-silenced cells and the controls. The calcium stimuli were the same in both populations (Fig. 3C, upper panel). The results indicate that Syt I is not involved in the vesicular secretion in LβT2 cells.

Syt IV knockdown attenuated dense core vesicle exocytosis in LβT2 cells

Since Syt IV can substitute for Syt I in Ca^{2+}-dependent neurotransmitter release [33] we then asked whether Syt IV plays a role

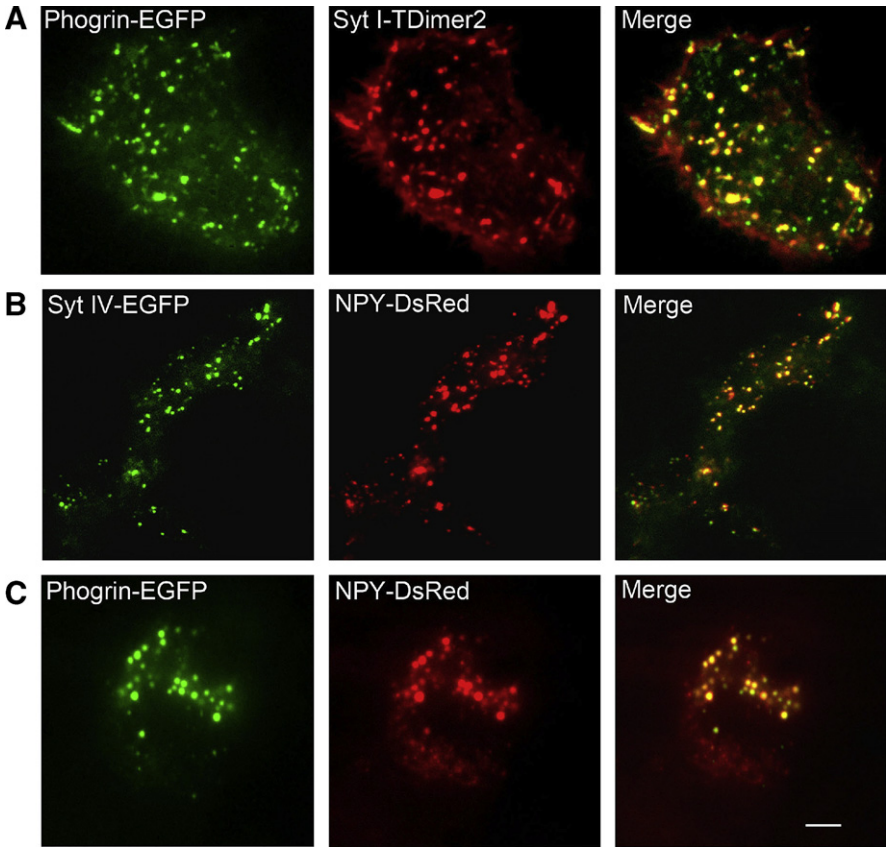

Fig. 1. Vesicular localization of Syt I and Syt IV in LβT2 cells. (A) Phogrin-EGFP (left) and the Syt I-TDimer2 (middle) are co-localized as shown in the overlay (right). (B) Colocalization of Syt IV-EGFP (left) with NPY-DsRed (middle) showed by their good overlay (right). (C) Colocalization of Phogrin-EGFP and NPY-DsRed. The scale bar represents 5 μm.

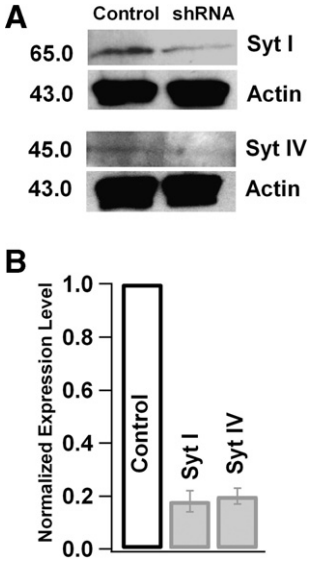

Fig. 2. Syt I and IV expression are knocked-down by their gene-target shRNAs. (A) Western blot analysis shows Syt I and IV were reduced to low levels in the LβT2 cells transfected with the shRNA-Syt I and shRNA-Syt IV plasmids when compared to the mock-transfected cells. (B) Summary of the expression levels of Syt I and IV in the controls and the silenced cells.

in the exocytosis of DCVs in LβT2 cells. As shown in Fig. 4A, inhibition of Syt IV expression resulted in a significant reduction in exocytosis as compared to controls. Detailed kinetic analysis of exocytosis revealed that the amplitude of the rapidly releasable pool (RRP, 120.5 ± 10.9 fF in the silenced cells versus 234.9 ± 35.3 fF in the controls; $^{***}P < 0.001$) and the slowly releasable pool (SRP, 457.4 ± 38.9 fF v.s. 691.6 ± 82.8 fF in the silenced and controls; $^{**}P < 0.01$) of vesicles as well as the slope of sustained component (41.3 ± 6.8 fF/s in the silenced v.s. 67.1 ± 8.8 fF/s in the controls; $^{**}P < 0.01$) was reduced significantly (Fig. 4D). There were no changes in the time constants of RRP and SRP release (data not shown). To determine the specificity of exocytotic effects of gene silencing, we used the Syt IV-shRNA plasmid and the Syt IV gene carrying three point mutations (see materials and methods) which resists the inhibition of the silencing sequence [34]. As shown in Fig. 4B, the expression of the mutant plasmid did not change the flash response in these cells, excluding an off-target effect of the gene silencing which is a general concern in knockdown experiments.

It is interesting that the down-regulation of Syt IV also decreased the size of the highly Ca^{2+}-sensitive pool (HCSP) and the remaining RRP without changing the Ca^{2+}- dependence of either pool (Fig. 4E–H). The sizes of HCSP and the remaining RRP were decreased from 61.6 ± 6.2 fF and 820.9 ± 72.6 fF in the controls to 35.2 ± 4.8 fF and 438.7 ± 56.8 fF in the silenced cells, respectively, with statistical significance $P < 0.001$ and $P < 0.01$ for each pool.

Previous studies suggest that upregulation of Syt IV is a protective mechanism to reduce neural activity [12,13]. Overexpression of Syt IV at the *Drosophila* neuromuscular junction reduced the peak amplitude of synaptic responses [13]. Similar evidence was reported in PC12 cells, in which Syt IV overexpression inhibits evoked secretion [20,21]. To test whether upregulation of Syt IV suppressed endocrine secretion in LβT2 cells we transfected the wild-type Syt IV plasmid into the cells. Surprisingly, our data

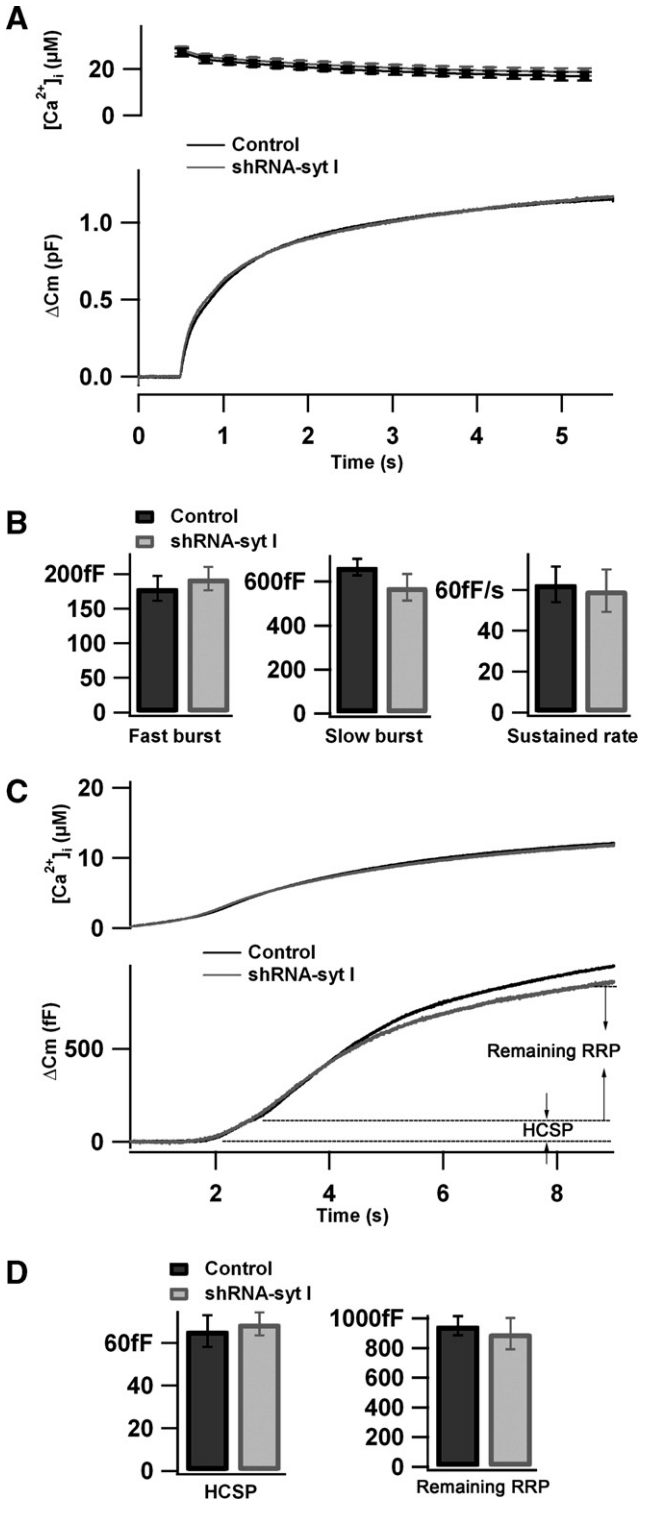

Fig. 3. Gene silencing of Syt I had no effect on Ca^{2+}-dependent exocytosis in LβT2 cells. (A) Mean exocytotic responses (capacitance increment, ΔCm, bottom panel) elicited by a step-like elevation of $[Ca^{2+}]_i$ (upper panel) generated by photolysis of caged Ca^{2+} in control ($n = 29$; black line and symbols) and in Syt I gene silenced ($n = 24$; gray line and symbols) LβT2 cells. (B) Summary of the different kinetic components in the control and Syt I knockdown cells. (C) Capacitance responses (lower panel) to ramp-like increases of $[Ca^{2+}]_i$ (upper panel) in control ($n = 17$; black line and symbols) and the Syt I gene silenced ($n = 18$; gray line and symbols) LβT2 cells. (D) Summary of the effects of Syt I silence on the amplitude of the HCSP and the remaining RRP in LβT2 cells.

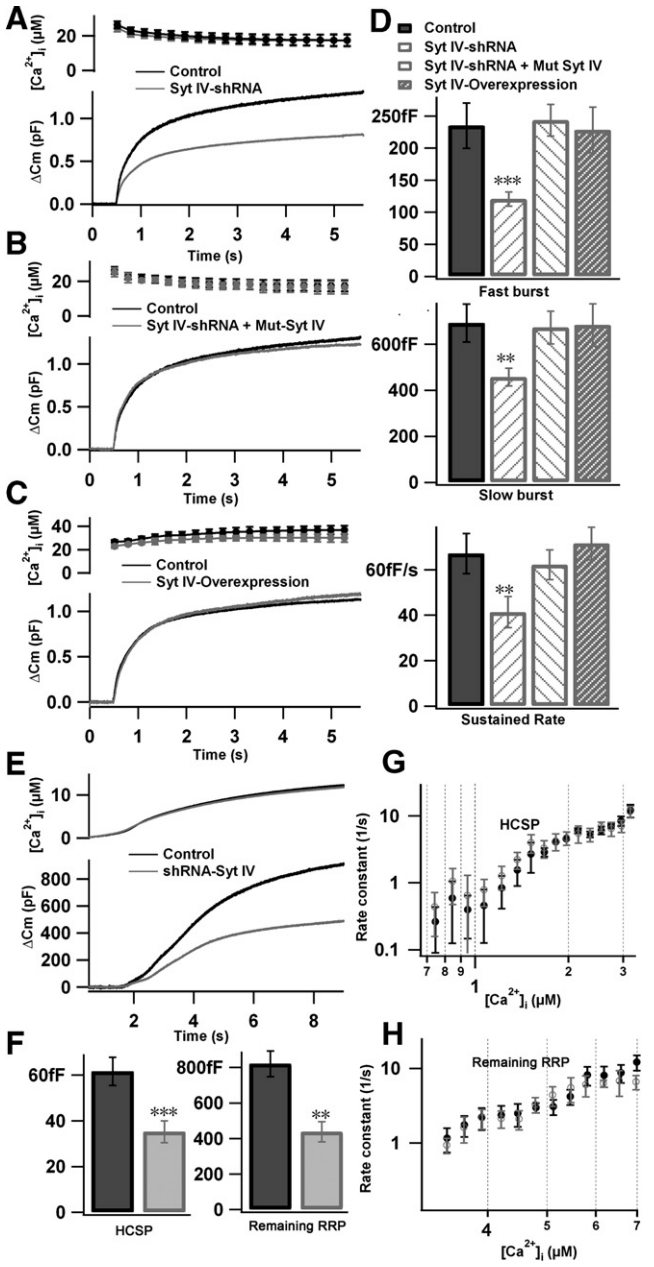

Fig. 4. siRNA knockdown of Syt IV leads to reduction of exocytosis. (A) Mean exocytotic responses (capacitance increment ΔCm, bottom panel) elicited by a step-like elevation of $[Ca^{2+}]_i$ (upper panel) in control ($n = 25$; black line and symbols) and Syt IV gene silenced ($n = 22$; gray line and symbols) LβT2 cells. (B) Co-transfection of Mut-Syt IV and Syt IV-shRNA plasmids rescued secretion to the control levels. (C) Ca^{2+}-dependent exocytosis was not altered by upregulation of Syt IV. (D) Summary of different kinetic components in control and Syt IV knockdown, Syt IV rescue and Syt IV overexpressed cells. (E) The membrane capacitance increment (Cm, bottom panel) and the associated Ca^{2+} concentration (upper panel) recorded in Syt IV gene silenced cells ($n = 21$) and the control cells ($n = 46$) in calcium ramp experiments. (F) Summary of the effects of Syt IV silencing on the size of HCSP and remaining RRP. (G,H) Double logarithmic plots of the exocytotic rate constants versus calcium concentrations for HCSP and remaining RRP.

clearly showed that overexpression of Syt IV had no effect on the secretion in this cell line, as shown in Fig. 4C. This result agrees with the observation that overexpression of Syt IV has no effect on neurotransmission in mouse hippocampal neurons [22].

Discussion

Syt I is likely a Ca^{2+} sensor for synaptic vesicle (SV) release and may play a similar role in dense core vesicles (DCVs) release [6,7].

Syt IV is structurally homologous to Syt I, so it is tempting to speculate that Syt IV may also function as a Ca^{2+} sensor for exocytosis. However, Syt IV is known to have different Ca^{2+}-binding properties than those of Syt I. Syt IV carries a mutation of a single amino acid known to be critical for coordinating Ca^{2+} binding and this substitution results in the Syt IV C2A domain being insensitive to Ca^{2+}. In spite of this, the C2B domain of Syt IV mediates Ca^{2+}-dependent homo-oligomerization [26], suggesting that although the C2A domain is Ca^{2+} insensitive, the whole protein does sense Ca^{2+} through the C2B domain. The observation that Syt IV can replace Syt I in mediating Ca^{2+}-dependent synaptic transmission [33,35,36] is consistent with this proposal.

The expression level of Syt I is much higher than that of Syt IV (unpublished results). Surprisingly, an 82% reduction in Syt I had no effect on exocytosis. The amplitude, kinetics and Ca^{2+}-dependence of secretion was unchanged. This may indicate that there is redundancy of Syts as observed in PC12 cell [11].

The size of the exocytotic burst (RRP and SRP) are an indicator of vesicle priming in resting conditions and indicate vesicle fusion in response to stimuli. The slope of sustained component indicates the rate of the recruitment of releasable vesicles which then fuse at the high Ca^{2+} level after a flash [37]. Our results in control, Syt IV-silenced and rescued cells show that down-regulation of Syt IV reduces the amplitude secretion of the RRP and SRP, and also the slope of sustained component in LβT2 cells. These results clearly demonstrate that knockdown of Syt IV inhibits not only the fusion and also the recruitment of releasable vesicles. Puzzlingly, the overexpression of Syt IV does not up-regulate its function. The capacitance changes of secretion triggered by ramp $[Ca^{2+}]_i$ elevation consist of two components, indicating two tiers of vesicles with different Ca^{2+}-sensitivities. The HCSP consists of vesicles with a high Ca^{2+}-sensitivity, probably endowed by a different fusion machinery [38]. The secretion of the remaining RRP is believed to occur by the fusion of vesicles via a release machinery with a lower calcium sensitivity. In this study we found that the silencing of Syt IV reduces the amplitudes of both HCSP and remaining RRP without changing their Ca^{2+}-cooperativities (Fig. 4F–H). This result suggests Syt IV acts as a Ca^{2+}-sensor for the release of both HCSP and less calcium sensitive RRP. The inhibitory effects of Syt IV knockdown on every exocytotic kinetic component indicate that Syt IV not only plays roles in vesicular fusion with plasma membrane, but also is involved in the vesicle trafficking and maturation process in cells. The molecular mechanism of Syt IV's roles in exocytosis needs further study.

Acknowledgments

The authors thank Dr. Pamela L. Mellon for supplying the LβT2 cell line. We are grateful to Prof. Dr. Jens Rettig and Dr. David R. Stevens for modifying the manuscript. This work was supported by grants from the National Science Foundation of China (30670502, 30500117), the Science Foundation of Hubei province (2007ABA020) and the Major State Basic Research Program of P.R. China (2006CB503900, 2006CB910700).

References

[1] X. Shao, C. Li, I. Fernandez, X. Zhang, T.C. Sudhof, J. Rizo, Synaptotagmin–syntaxin interaction: the C2 domain as a Ca^{2+}-dependent electrostatic switch, Neuron 18 (1997) 133–142.
[2] S. Sugita, O.H. Shin, W. Han, Y. Lao, T.C. Sudhof, Synaptotagmins form a hierarchy of exocytotic Ca^{2+} sensors with distinct Ca^{2+} affinities, EMBO J. 21 (2002) 270–280.
[3] M. Craxton, Genomic analysis of synaptotagmin genes, Genomics 77 (2001) 43–49.
[4] J. Bai, E.R. Chapman, The C2 domains of synaptotagmin-partners in exocytosis, Trends Biochem. Sci. 29 (2004) 143–151.
[5] M.S. Perin, N. Brose, R. Jahn, T.C. Sudhof, Domain structure of synaptotagmin (p65), J. Biol. Chem. 266 (1991) 623–629.
[6] M. Yoshihara, J.T. Littleton, Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release, Neuron 36 (2002) 897–908.
[7] T. Voets, T. Moser, P.E. Lund, R.H. Chow, M. Geppert, T.C. Sudhof, E. Neher, Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I, Proc. Natl. Acad. Sci. USA 98 (2001) 11680–11685.
[8] M. Fukuda, RNA interference-mediated silencing of synaptotagmin IX, but not synaptotagmin I, inhibits dense-core vesicle exocytosis in PC12 cells, Biochem. J. 380 (2004) 875–879.
[9] Y. Shoji-Kasai, A. Yoshida, K. Sato, T. Hoshino, A. Ogura, S. Kondo, Y. Fujimoto, R. Kuwahara, R. Kato, M. Takahashi, Neurotransmitter release from synaptotagmin-deficient clonal variants of PC 12 cells, Science 256 (1992) 1820–1823.
[10] K.L. Lynch, T.F. Martin, Synaptotagmins I and IX function redundantly in regulated exocytosis but not endocytosis in PC12 cells, J. Cell Sci. 120 (2007) 617–627.
[11] D. Zhu, W. Zhou, T. Liang, F. Yang, R.Y. Zhang, Z.X. Wu, T. Xu, Synaptotagmin I and IX function redundantly in controlling fusion pore of large dense core vesicles, Biochem. Biophys. Res. Commun. 361 (2007) 922–927.
[12] C. von Poser, K. Ichtchenko, X. Shao, J. Rizo, T.C. Sudhof, The evolutionary pressure to inactivate. A subclass of synaptotagmins with an amino acid substitution that abolishes Ca^{2+} binding, J. Biol. Chem. 272 (1997) 14314–14319.
[13] J.T. Littleton, T.L. Serano, G.M. Rubin, B. Ganetzky, E.R. Chapman, Synaptic function modulated by changes in the ratio of synaptotagmin I and IV, Nature 400 (1999) 757–760.
[14] D. Xi, H. Chin, H. Gainer, Analysis of synaptotagmin I–IV messenger RNA expression and developmental regulation in the rat hypothalamus and pituitary, Neuroscience 88 (1999) 425–435.
[15] E.M. Denovan-Wright, R.A. Newton, J.N. Armstrong, J.M. Babity, H.A. Robertson, Acute administration of cocaine, but not amphetamine, increases the level of synaptotagmin IV mRNA in the dorsal striatum of rat, Brain Res. Mol. Brain Res. 55 (1998) 350–354.
[16] G. Tocco, X. Bi, L. Vician, I.K. Lim, H. Herschman, M. Baudry, Two synaptotagmin genes, Syt1 and Syt4, are differentially regulated in adult brain and during postnatal development following kainic acid-induced seizures, Brain Res. Mol. Brain Res. 40 (1996) 229–239.
[17] G.D. Ferguson, S.G. Anagnostaras, A.J. Silva, H.R. Herschman, Deficits in memory and motor performance in synaptotagmin IV mutant mice, Proc. Natl. Acad. Sci. USA 97 (2000) 5598–5603.
[18] G.D. Ferguson, L. Vician, H.R. Herschman, Synaptotagmin IV: biochemistry, genetics, behavior, and possible links to human psychiatric disease, Mol. Neurobiol. 23 (2001) 173–185.
[19] G.D. Ferguson, H.R. Herschman, D.R. Storm, Reduced anxiety and depression-like behavior in synaptotagmin IV (−/−) mice, Neuropharmacology 47 (2004) 604–611.
[20] H.B. Machado, W. Liu, L.J. Vician, H.R. Herschman, Synaptotagmin IV overexpression inhibits depolarization-induced exocytosis in PC12 cells, J. Neurosci. Res. 76 (2004) 334–341.
[21] C.T. Wang, R. Grishanin, C.A. Earles, P.Y. Chang, T.F. Martin, E.R. Chapman, M.B. Jackson, Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles, Science 294 (2001) 1111–1115.
[22] J.T. Ting, B.G. Kelley, J.M. Sullivan, Synaptotagmin IV does not alter excitatory fast synaptic transmission or fusion pore kinetics in mammalian CNS neurons, J. Neurosci. 26 (2006) 372–380.
[23] H. Zhu, B. Hille, T. Xu, Sensitization of regulated exocytosis by protein kinase C, Proc. Natl. Acad. Sci. USA 99 (2002) 17055–17059.
[24] H.S. Liu, Z.T. Hu, K.M. Zhou, Y.M. Jiu, H. Yang, Z.X. Wu, T. Xu, Heterogeneity of the Ca^{2+} sensitivity of secretion in a pituitary gonadotrope cell line and its modulation by protein kinase C and Ca^{2+}, J. Cell Physiol. 207 (2006) 668–674.
[25] A. Almeida, J.P. Bolanos, S. Moreno, Cdh1/Hct1-APC is essential for the survival of postmitotic neurons, J. Neurosci. 25 (2005) 8115–8121.
[26] D.M. Thomas, G.D. Ferguson, H.R. Herschman, L.A. Elferink, Functional and biochemical analysis of the C2 domains of synaptotagmin IV, Mol. Biol. Cell 10 (1999) 2285–2295.
[27] Z.X. Wu, S. Xia, L. Xu, L. Bai, T. Xu, Dynamic imaging of single secretory granule in cultured PC12 cells, Acta Biochim. Biophys. Sin. 35 (2003) 381–386.
[28] T. Xu, M. Naraghi, H. Kang, E. Neher, Kinetic studies of Ca^{2+} binding and Ca^{2+} clearance in the cytosol of adrenal chromaffin cells, Biophys. J. 73 (1997) 532–545.
[29] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.
[30] B. Marqueze, F. Berton, M. Seagar, Synaptotagmins in membrane traffic: which vesicles do the tagmins tag?, Biochimie 82 (2000) 409–420
[31] L. Bai, D. Zhu, K. Zhou, W. Zhou, D. Li, Y. Wang, R. Zhang, T. Xu, Differential properties of GTP- and Ca^{2+}-stimulated exocytosis from large dense core vesicles, Traffic 7 (2006) 416–428.
[32] C. Wasmeier, J.C. Hutton, Molecular cloning of phogrin, a protein–tyrosine phosphatase homologue localized to insulin secretory granule membranes, J. Biol. Chem. 271 (1996) 18161–18170.
[33] I.M. Robinson, R. Ranjan, T.L. Schwarz, Synaptotagmins I and IV promote transmitter release independently of Ca^{2+} binding in the C(2)A domain, Nature 418 (2002) 336–340.
[34] P. Lassus, J. Rodriguez, Y. Lazebnik, Confirming specificity of RNAi in mammalian cells, Sci. STKE (2002) L13.

[35] J.M. Mackler, J.A. Drummond, C.A. Loewen, I.M. Robinson, N.E. Reist, The C(2)B Ca^{2+}-binding motif of synaptotagmin is required for synaptic transmission in vivo, Nature 418 (2002) 340–344.
[36] C.T. Wang, J.C. Lu, J. Bai, P.Y. Chang, T.F. Martin, E.R. Chapman, M.B. Jackson, Different domains of synaptotagmin control the choice between kiss-and-run and full fusion, Nature 424 (2003) 943–947.
[37] J. Rettig, E. Neher, Emerging roles of presynaptic proteins in Ca^{2+}-triggered exocytosis, Science 298 (2002) 781–785.
[38] Y. Yang, T.J. Craig, X. Chen, L.F. Ciufo, M. Takahashi, A. Morgan, K.D. Gillis, Phosphomimetic mutation of Ser-187 of SNAP-25 increases both syntaxin binding and highly Ca^{2+}-sensitive exocytosis, J. Gen. Physiol. 129 (2007) 233–244.