Non-polarized distribution of synaptotagmin IV in neurons: evidence that synaptotagmin IV is not a synaptic vesicle protein

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Abstract

Synaptotagmin IV (Syt IV) expression is regulated by neuronal development and by depolarization in the brain and in neuronal cell cultures. In cultures, immunocytochemical analysis has shown that Syt IV is localized at the Golgi and at the tips of growing neurites, but little was known about associations between Syt IV and vesicles or organelles [J. Neurochem. 74 (2000) 518]. In this study we performed an electron microscopic (EM) analysis of developing mouse neocortex to determine the exact localization of Syt IV in native mouse tissues. In neurons of layer II/III, Syt IV was found to be localized in the dendrites and axons, and at the Golgi in the cell body. Some Syt IV signals were clearly associated with vesicles and/or organelles, but EM and cell fractionation studies showed no Syt IV signals at synaptic vesicles. Detection of fluorescence protein-tagged Syt IV (Syt IV-EGFP) in hippocampal neurons also showed the presence of Syt IV-EGFP vesicles or organelles in the axons and dendrites. These results suggest that Syt IV regulates non-polarized membrane trafficking in neurons, which may be involved in synaptic plasticity or neuronal development.

Keywords: Synaptotagmin; Immediate early genes; Depolarization; Golgi; Neuronal development; Synaptic plasticity

1. Introduction

Recent genetic, biochemical, and structural studies indicate that proteins involved in membrane trafficking (e.g. soluble NSF attachment protein receptor (SNARE) proteins and the Rab family) are evolutionarily conserved and occur in many isoforms. Since each isoform is found in a distinct subcellular location, such as the endoplasmic reticulum (ER), Golgi, endosomes, or lysosomes, the isoforms are thought to regulate specific membrane trafficking events (for reviews, see Söllner and Rothman, 1996; Novick and Zerial, 1997; Jahn and Südhof, 1999; Lin and Scheller, 2000). Synaptotagmin is one such protein family and is thought to be involved in regulated membrane fusion (for reviews, see Fukuda and Mikoshiba, 1997; Schiavo et al., 1998; Marquèze et al., 2000; Südhof, 2002). Although SNARE proteins, composed of syntaxin, vesicle-associated membrane protein (VAMP), and synaptosome-associated protein 25 (SNAP-25), are basic machinery for membrane fusion, synaptotagmin as well as SNARE proteins is necessary for regulated membrane fusion. For example,
synaptotagmin I (Syt I), a well-characterized isoform abundant in synaptic vesicles, plays an essential role in the calcium-dependent neurotransmitter release, probably functioning as a calcium sensor at synapses (Mikoshiba et al., 1995; Mochida et al., 1997; Fernandez-Chacon et al., 2001). At least 13 synaptotagmins have been identified in mice (Fukuda et al., 1999; Fukuda and Mikoshiba, 2001; Craxton, 2001, and references therein), and all members have a single transmembrane region and two C2 domains (known as the C2A and C2B domains). It is speculated that each synaptotagmin has a distinct subcellular localization, the same as SNAREs or Rabs, and plays a role in a distinct regulated fusion event at that site, and consistent with this speculation, differential distribution of Syt I, III, IV, VI and VII has recently been reported (Butz et al., 1999; Fukuda and Mikoshiba, 1999; Ibata et al., 2000; Berton et al., 2000; Martinez et al., 2000; Sugita et al., 2001; Fukuda et al., 2002).

Syt IV was first identified as the fourth member of the synaptotagmin family (Hilbush and Morgan, 1994) and characterized as an immediate early gene induced by depolarization or forskolin in PC12 cells and rat brain (Vician et al., 1995). Syt IV expression in neurons is also regulated during development (Berton et al., 1997; Ibata et al., 2000), and analysis of knock-out mice has suggested that Syt IV is involved in some forms of memory related to the hippocampus (Ferguson et al., 2000). The exact subcellular localization of Syt IV (synaptic vesicle vs. other unknown structures), however, remains a matter of controversy, although this information is quite important to understanding the molecular mechanism of the regulation of learning and memory by Syt IV via vesicular trafficking. Our previous immunocytochemical studies showed that Syt IV is localized at the Golgi and the tips of neurites in PC12 cells and cultured hippocampal neurons (Ibata et al., 2000; Berton et al., 2000). Although these findings suggest that Syt IV functions at the Golgi and the tips of neurites, the localization studies were performed by using in vitro dissociated cultures and analyzed only at the immunocytochemical level. Therefore, the possibility that Syt IV is also present at synaptic vesicles, as proposed by other researchers (Thomas et al., 1999; Littleton et al., 1999; Ferguson et al., 2000), cannot be excluded.

In this study, we examined the exact localization of Syt IV by electron microscopy (EM), subcellular fractionation, and time-lapse imaging of Syt IV-EGFP expressed in hippocampal neurons. We found that Syt IV is localized at vesicle- and organelle-structures, but not synaptic vesicles, both in axons and dendrites. On the basis of these results, we discuss the possible role of Syt IV in the neurites of developing neurons and in synaptic plasticity.

2. Methods

2.1. Immunohistochemistry

Six- and 13-day-old mice were deeply anesthetized with Nembutal (60 mg kg⁻¹) and transcardially perfused with a physiological saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4 (PB). After removing the brains and postfixing them in the same fixative for 4–6 h at 4 °C, they were cryoprotected in 30% sucrose at 4 °C for 24–48 h, and 35-µm frozen sections were cut on a freezing microtome. All procedures involving the experimental animals were approved by the RIKEN Animal Care Committee. The sections were rinsed three times in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), and then placed in 6% normal goat serum, 0.3% Triton X-100 in PBS (PBS-T-NGS buffer) for 1 h at room temperature. After incubating the sections for 12 h at 4 °C with rabbit anti-Syt IV antibody (1.8 µg ml⁻¹; Ibata et al., 2000) in a PBS-T-NGS buffer and washing them with PBS, the procedure described in the instructions supplied with the avidin biotinylated-peroxidase complex (ABC) kit from Vector Laboratories was followed. Peroxidase activity was visualized with 0.01% H₂O₂ and 0.05% diaminobenzidine tetrahydrochloride.

2.2. Electron microscopy

Six- and 13-day-old mice were deeply anesthetized with Nembutal and transcardially perfused with 4% paraformaldehyde and 3.8% acrolein in 0.1 M PB. The brains were removed, placed in 4% paraformaldehyde for 30 min, and then in a solution of 0.1 M PB for 12–24 h at 4 °C. The specimens were cut into 1 mm-thick sections, placed in 2% BSA, 10% normal goat serum, and 0.01% Triton X-100 in 0.1 M PB for 4 h at room temperature and then incubated with anti-Syt IV antibody (3.6 µg ml⁻¹) for 60 h at 4 °C. Anti-rabbit IgG conjugated with 1 or 5 nm gold particles was used as the secondary antibody, and silver intensification was performed with the IntensM kit (Amersham Biosciences, Inc.). The sections were then osmicated, and dehydrated in graded ethanol, and embedded in Araldite. Ultrathin sections containing Syt IV signals were examined with an electron microscope (1200EX, JEOL).

2.3. Subcellular fractionation

The brains of 5–8-day-old mice were homogenized in 9 volumes of ice-cold 5 mM HEPES, pH 7.2, in a glass–Teflon homogenizer (1300 rpm, 10 strokes), and the homogenate was centrifuged for 10 min at 900 × g. The supernatant was then recentrifuged for 10 min at 900 × g to remove cellular debris and nuclei, and the supernatant was recovered as the S1 fraction. The S1 fraction
was then loaded on a 0.15–0.9 M linear sucrose gradient and spun at 22 000 rpm (83 000 g max) for 4 h at 4 °C in a rotor (SW41Ti; Beckman Instruments). The first fraction, equivalent to the loaded volume, and successive 0.5 ml fractions were collected from the top, and 20 μl of each fraction was processed for immunoblot analysis as described previously (Fukuda et al., 1999). Anti-Syt I and anti-synaptophysin mouse monoclonal antibodies were obtained from StressGen Biotechnologies Corp. and Sigma Chemical Co., respectively.

2.4. Hippocampal cultures and transfections

Hippocampal primary neurons were prepared from fetal rats at embryonic day 18 (E18). The hippocampus was dissected and dissociated with papain, and 2 × 10⁵ neurons per poly L-lysine-coated 35 mm dish were grown with Neurobasal medium and B27 supplement (Ibata et al., 2000). Calcium-phosphate mediated transfection into 7 DIV hippocampal neurons was achieved according to the procedure described by Köhrmann et al. (1999). Seven days after transfection time-lapse imaging of Syt IV-EGFP (enhanced green fluorescence protein; Clontech) in 14 DIV hippocampal neurons (see below) was performed to avoid the mis-localization of exogenous protein by the short duration after transfection.

2.5. Construction of the Syt IV-EGFP fusion protein

The coding region of Syt IV and EGFP was separately amplified by the polymerase chain reaction (PCR) using specific primers with appropriate restriction enzyme sites. Two amplified PCR fragments were connected between the Syt IV-3′ MunI (CAATTG) site and the EGFP-5′ EcoRI (GAATTC) site, and thus the linker between Syt IV and EGFP was 5′-CAATTC-3′, which encodes Gln and Phe. The Syt IV-EGFP fragment was then subcloned into the modified pEF-BOS mammalian cell expression vector (Mizushima and Nagata, 1990; Fukuda et al., 1994, 1999).

2.6. Time-lapse microscopy

Hippocampal neurons on a glass-bottom dish were examined with an Olympus IX70 inverted microscope equipped with a 100 × magnification oil immersion objective lens. The imaging solution was 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES, pH 7.4, and it was maintained at 37 °C. Sequential images were acquired with a micromax CCD camera and Metaflow software. The exposure time was 100 ms, and the time lapse was 1 s.

3. Results

3.1. Immunohistochemical analysis of Syt IV localization in neocortical layers

Our previous study by immunoblot analysis revealed that Syt IV expression is developmentally regulated and that its expression peaks in mouse hippocampus at around postnatal day (PD) 5 (Ibata et al., 2000). Consistent with this finding, Syt IV mRNA is also developmentally regulated (Berton et al., 1997). Similar expression patterns were observed by immunohistochemical analysis with the anti-Syt IV specific antibody: the immunoreaction was stronger in sections from PD6 (Fig. 1A) than from PD13 (data not shown). On PD6, the strongest Syt IV signals were detected in layers II/III and V of the neocortex (Fig. 1A). In layers II/III, prominent signals were seen in the somatodendritic compartment of the pyramidal neurons. These signals probably correspond to the signals of the Golgi seen in

![Fig. 1. Immunohistochemical analysis of the Syt IV protein in the PD6 mouse cortex. (A) Layers II/III and V of the neocortex stained strongly with anti-Syt IV specific antibody. The arrows in A point to axon bundles from layers II/III and V. Note that the somatodendritic compartments can be seen to be strongly stained at the higher magnification (B and C). (C) Higher magnification of the hatched box in B. The scale bars in A–C represent 200, 50, and 10 μm, respectively.](image)
the previous immunocytochemical studies. The signals appeared to continue in the direction of the dendrite (Fig. 1B and arrows in Fig. 1C), but the strong Syt IV signals were also observed in axon bundles from layer II/III and V (white arrows in Fig. 1A). These Syt IV signals should be specific, because in the absence of primary antibody we could not observe any Syt IV signals (data not shown). These results indicated that Syt IV was localized at both the axons and dendrites in native mouse tissues, consistent with the results of our previous immunocytochemical analysis (Ibata et al., 2000; Berton et al., 2000).

3.2. Electron microscopic analysis of Syt IV in neocortex layers II/III and V

To examine the localization of Syt IV more clearly and in greater detail, we performed EM analysis of Syt IV in mouse neocortex layers II/III and V, because layers II/III and V stained well, as described above. The numbers of gold particles in anti-Syt IV-antibody-treated or control (no primary antibody) specimens were counted, and the ratios of the particles to the areas were calculated (Table 1). As expected, specific Syt IV signals (i.e. gold particles) were mainly associated with the Golgi and vesicles/organelles around the Golgi (Fig. 2A), but interestingly, Syt IV signals were also observed from the proximal dendrite to the postsynapse (Fig. 2B–E), although the signals decreased in the distal portions. In some cases, gold particles in the dendrite were associated with vesicles/organelles or plasma membranes (Fig. 2C and D, arrow). The weaker association of Syt IV signals with vesicles/organelles was due to: (i) poorer preservation of the membrane of vesicles/organelles in this EM study or (ii) the distance between exact localization of Syt IV and gold particle being approximately 20 nm apart. These Syt IV signals were definitely caused by recognition of Syt IV proteins, we hardly detected any differences in the density of gold particle between control and anti-Syt IV staining sections from PD13 (data not shown), in which Syt IV protein expression was very low. We also observed Syt IV-positive signals in axons shown by arrows in Fig. 1A. However, no Syt IV signals were detected on synaptic vesicles at the presynapse (Fig. 2E), consistent with our previous immunocytochemical analysis showing less colocalization of Syt IV and Syt I in the same vesicles/organelles in PC12 cells and cultured hippocampal neurons (Ibata et al., 2000).

3.3. Fractionation of PD6-9 mouse brain on a sucrose gradient

To further confirm that Syt IV proteins were not localized at synaptic vesicles, we performed sucrose-gradient fractionation of a hypotonic-buffer-treated infant mouse brain homogenate. When fractionation was performed with a 0.15–0.9 M sucrose gradient, one sharp peak and a residual broad peak were observed for synaptic vesicle markers Syt I and synaptophysin (Fig. 3).
3, top and bottom panels). The former peak represented the synaptic vesicle-enriched fraction appearing at around 0.4 M (Fig. 3, bar), while the latter peak caused by membrane compartments larger or denser than synaptic vesicles. By contrast, Syt IV exhibited a mobilization that was similar to the latter peak of Syt I and synaptophysin, but it was hardly detected at all in the synaptic vesicle fraction (Fig. 3, middle panel). These results also support our conclusion that Syt IV proteins are not localized at synaptic vesicles, but are present at other membrane compartments.

3.4. Time-lapse imaging of the EGFP-tagged Syt IV protein expressed in cultured hippocampal neurons

Finally, we performed time-lapse imaging of fluorescence protein (EGFP)-tagged Syt IV expressed in the hippocampal neurons to determine whether Syt IV-containing vesicles/organelles migrate along neurites. This fusion protein showed similar localization to endogenous Syt IV (Berton et al., 2000; Fukuda et al., 2001), indicating that the C-terminal fusion of EGFP did not affect the correct membrane targeting of Syt IV (Fukuda et al., 2001). When we took frames at a ratio of 100 ms s⁻¹ for 100 s, about 30% (33.0 ± 5.6%, from 71 vesicles/organelles of three cells) of Syt IV-EGFP containing vesicles/organelles in the axon and about 75% (74.3 ± 6.6%, from 65 vesicles/organelles of three cells) of Syt IV-EGFP containing vesicles/organelles in the dendrite migrate from their initial location. These data also indicated Syt IV-containing vesicles/organelles are distributed uniformly and migrate along both axons and dendrites.

4. Discussion

This study showed the ultrastructural localization of Syt IV in developing mouse neocortex. Syt IV is localized at the Golgi, surrounding regions in the cell body, and along both the axons and dendrites. Syt IV signals were also found to sometimes be associated with vesicle/organelle-structures that were clearly distinct from synaptic vesicles (Fig. 2E and Fig. 3), quite unlike Syt I, which regulates synaptic vesicle trafficking in presynapses. As far as we know, Syt IV is the first synaptotagmin isofrom that has been found to exhibit a non-polarized distribution in neurons. By contrast, Syt I is a presynapse-specific isofrom that regulates synaptic vesicle trafficking. By analogy with Syt I’s role in Ca²⁺-regulated exocytosis of synaptic vesicles, we speculate that Syt IV drives a specific membrane fusion event at the Golgi in cell bodies and at the vesicles/organelles located in the neurites.

Since Syt IV expression is developmentally regulated and induced by neuronal activity, the function of Syt IV may be related to neuronal development and plasticity. Indeed, Syt IV knock-out mice exhibited abnormalities in some forms of memory related to the hippocampus and in motor performance (Ferguson et al., 2000). Thus, it is tempting to speculate that Syt IV-containing vesicles/organelles in the Golgi, axons, and dendrites may contain molecules that are crucial to such phenomena and that Syt IV may regulate their trafficking to the postsynapse during neuronal development or plastic changes. Candidate cargoes are glutamate (AMPA) receptors or adhesion molecules, because these glycoproteins must be properly modified at the Golgi and are known to be inserted into postsynaptic plasma membranes during synaptogenesis or synaptic plasticity (Kiss et al., 1994; Nayak et al., 1998; Shi et al., 1999). Further work is needed to determine the specific cargo of Syt IV-containing vesicles/organelles. An attempt to identify cargo molecules is now under way in our laboratory.

Another possible function of Syt IV is regulation of the biogenesis of secretory granules by the trans-Golgi network or the Golgi. Consistent with this, exogenously expressed Syt IV has been shown to inhibit the release of neuropeptide ACTH (adrenocorticotropic hormone) by interfering with the formation of mature secretory granules (Eaton et al., 2000).

In summary, we have presented several lines of evidence that Syt IV is not a synaptic vesicle protein and that it is present at uncharacterized vesicle/organellar-stuctures both in the axons and dendrites. Based on these results, we suggest that Syt IV may be involved in exocytic events other than synaptic vesicle exocytosis.

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References

Craxton, M. 2001. Genomic analysis of synaptotagmin genes. Geno-
Eaton, B.A., Haugwitz, M., Lau, D., Moore, H.P. 2000. Biogenesis of
Fukuda, M., Mikoshiba, K. 1997. The function of inositol high-
Ko¨hrmann, M., Haubensak, W., Hemraj, I., Kaether, C., Lessmann,
Lin, R.C., Scheller, R.H. 2000. Mechanisms of synaptic vesicle
Littleton, J.T., Serano, T.L., Rubin, G.M., Ganetzky, B., Chapman,
Marquize, B., Berton, F., Seagar, M. 2000. Synaptotagmins in
Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K.,
Mizushima, S., Nagata, S. 1990. pEF-BOS, a powerful mammalian
Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H., Mikoshiba, K.
Mikoshiba, K., Ibata et al. / Neuroscience Research 43 (2002) 401
No
dent exocytosis of lysosomes in fibroblasts. J. Cell Biol. 148, 1141–
Novick, P., Zerial, M. 1997. The diversity of Rab proteins in vesicle
Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J.,
Sugita, S., Han, W., Butz, S., Liu, X., Fernandez-Chacon, R., Lao, Y.,
Südhof, T.C. 2001. Synaptotagmin VII as a plasma membrane Ca2+
Vician, L., Lim, I.K., Ferguson, G., Tocco, G., Baudry, M., Hersch-