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The C-terminal transmembrane region of synaptobrevin binds synaptophysin from adult synaptic vesicles

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Abstract

Synaptophysin and synaptobrevin are abundant membrane proteins of neuronal small synaptic vesicles. In mature, differentiated neurons they form the synaptophysin/synaptobrevin (Syp/Syb) complex. Synaptobrevin also interacts with the plasma membrane-associated proteins syntaxin and SNAP25, thereby forming the SNARE complex necessary for exocytotic membrane fusion. The two complexes are mutually exclusive. Synaptobrevin is a C-terminally membrane-anchored protein with one transmembrane domain. While its interaction with its SNARE partners is mediated exclusively by its N-terminal cytosolic region it has been unclear so far how binding to synaptophysin is accomplished. Here, we show that synaptobrevin can be cleaved in its synaptophysin-bound form by tetanus toxin and botulinum neurotoxin B, or by botulinum neurotoxin D, leaving shorter or longer C-terminal peptide chains bound to synaptophysin, respectively. A recombinant, C-terminally His-tagged synaptobrevin fragment bound to nickel beads specifically bound synaptophysin, syntaxin and SNAP25 from vesicular detergent extracts. After cleavage by tetanus toxin or botulinum toxin D light chain, the remaining C-terminal fragment no longer interacted with syntaxin or SNAP 25. In contrast, synaptophysin was still able to bind to the residual C-terminal synaptobrevin cleavage product. In addition, the His-tagged C-terminal synaptobrevin peptide 68–116 was also able to bind synaptophysin in detergent extracts from adult brain membranes. These data suggest that synaptophysin interacts with the C-terminal transmembrane part of synaptobrevin, thereby allowing the N-terminal cytosolic chain to interact freely with the plasma membrane-associated SNARE proteins. Thus, by binding synaptobrevin, synaptophysin may positively modulate neurotransmission.

Keywords: Synaptophysin; Synaptobrevin; Clostridial neurotoxins; Syp/Syb complex; SNARE complex

Introduction

Synaptophysin and synaptobrevin are highly abundant membrane proteins of small synaptic vesicles. Synaptobrevin forms two mutually exclusive protein complexes,

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one with the plasma membrane proteins syntaxin and SNAP25 to form the SNARE complex, and a second with synaptophysin. In contrast to the well-known key function of synaptobrevin in exocytotic membrane fusion, the physiological role of synaptophysin is still little understood, especially given the fact that synaptophysin deletion mutants do not develop phenotypic changes (Eshkind and Leube, 1995; McMahon et al., 1996). So far, synaptophysin has been reported to be a cholesterol-binding protein and thus probably helps to maintain the membrane integrity of synaptic vesicles during exocytotic/endocytotic cycles (Thiele et al., 2000). In addition, synaptophysin-expressing neurons are better donors for synaptic terminals when analyzed in competition with synaptophysin-deficient neurons (Tarsa and Goda, 2002). A further function of synaptophysin is the formation of the synaptophysin/synaptobrevin (Syp/Syb) complex. The Syp/Syb complex is developmentally upregulated in neurons (Becher et al., 1999a) and does not occur in neuroendocrine cells (Becher et al., 1999b). Blockade of NMDA receptors during neuronal development in culture prevents the upregulation of the Syp/Syb complex, probably by locking synaptic vesicles in an immature state (Bacci et al., 2001). Developmental upregulation of the Syp/Syb complex appears to involve a cross-talk between the post- and the presynaptic sites. In this respect, the Syp/Syb complex is a molecular marker for mature synapses. In adult brains, the Syp/Syb complex increases when synaptic activity is enhanced persistently by repetitive stimulation over weeks (Hinz et al., 2001). It appears that the Syp/Syb complex provides synaptobrevin for exocytotic membrane fusion since it is dissociated upon short-term stimulations (min) in a calcium-dependent manner (Pennuto et al., 2002; Reisinger et al., 2004). The interaction between the two proteins has been shown to be sensitive to reducing and high-salt conditions (Edelmann et al., 1995; Becher et al. 1999a), and critically depends on the cholesterol content of the membrane (Mitter et al., 2003). In order to gain further insight into the nature of the Syp/Syb complex, we started to analyze the region of the molecules necessary for the interaction. Using clostridial neurotoxin cleavage and recombinant N- and C-terminally His-tagged synaptobrevin peptides, we show here that the C-terminal region of synaptobrevin selectively interacts with synaptophysin.

Material and methods

Antibodies

The following antibodies were generous gifts from R. Jahn, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany: Mouse monoclonal antibodies against synaptobrevin II (clone 69.1, Edelmann et al., 1995) and synaptophysin (clone 7.2, Jahn et al., 1985) and rabbit polyclonal antibodies against synaptobrevin I and II (Jahn et al., 1985). Monoclonal antibodies against syntaxin1A/B (clone HPC-1) and synaptotagmin were purchased from Synaptic Systems, Göttingen, Germany. A monoclonal antibody against SNAP25 was from Sternberger Monoclonals, Baltimore, MD/US.

Secondary antibodies for Western blot detection, horse anti-mouse and goat anti-rabbit conjugated with horseradish peroxidase were purchased from Vector Laboratories, Burlingame, CA, USA.

Toxins and recombinant synaptobrevin derivatives

The light chains of tetanus toxin (TeNt), botulinum neurotoxin B (BoNt/B) and D (BoNt/D) were produced as carboxyl-terminal His6 fusion proteins.

His-tagged C- or N-terminal synaptobrevins were expressed using either pQE30 or pET28a vectors. For protein expression, 1 ml of C-terminal His-tagged synaptobrevin plasmid transformed in M15pREP4 (Qiagen, Hilden, Germany) strain was inoculated in 100 ml of Luria-Bertani (LB) broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Similarly, 1 ml of C/N-terminal His-tagged synaptobrevin plasmid transformed in BL21 (Novagen) strain was cultivated in 100 ml of LB broth containing 50 μg/ml ampicillin. The respective cultures were grown at 37 °C overnight at 225 rpm. When the OD_{600} of the cultures reached 0.4–0.7, IPTG was added to a final concentration of 1 mM, and cultures were grown for a further 3 h. Cells were pelleted at 4000*g* for 25 min, resuspended in 1 ml of solubilization buffer (200 mM Tris/HCl, 150 mM NaCl, 1 mM PMSF, pH 7.4) and lysed with a Dounce homogenizer (20 strokes at 900 rpm). Cell lysates were further incubated with 1 mg/ml lysozyme and DNase (diluted 1:1000) on ice for 30 min and subsequently homogenized. The homogenate was extracted with 0.5% Triton X-100 on ice for 30 min and centrifuged at 10,000*g* for 25 min at 4 °C. Total amount of protein in the extracts was determined using the BCA (bicinchoninic acid; Sigma, St. Louis, MO, USA) method. One mg of protein extract was used for Ni-NTA purification.

Ni-NTA columns were washed once with washing buffer (20 mM Tris/HCl, 150 mM NaCl, 20 mM imidazole, 0.5% Triton X-100, pH 7.4) and centrifuged at 700*g* for 2 min. The equilibrated columns were incubated with 600 μl of the protein extract (corresponding to 1 mg of protein) for 60 min on ice followed by centrifugation at 700*g* for 2 min. Columns were washed twice with washing buffer and the flow-through from all the washes was stored in Laemmli sample buffer for final gel analysis. The columns were further incubated with 200 μl of elution buffer (20 mM Tris/HCl, 150 mM NaCl, 250 mM imidazole, 0.5% Triton X-100, pH 7.4)