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The synaptotagmin juxtamembrane domain is involved in neuroexocytosis

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ABSTRACT

Synaptotagmin is a synaptic vesicle membrane protein which changes conformation upon Ca^{2+} binding and triggers the fast neuroexocytosis that takes place at synapses. We have synthesized a series of peptides corresponding to the sequence of the cytosolic juxtamembrane domain of synaptotagmin, which is highly conserved among different isoforms and animal species, with or without either a hexyl hydrophobic chain or the hexyl group plus a fluorescein moiety. We show that these peptides inhibit neurotransmitter release, that they localize on the presynaptic membrane of the motor axon terminal at the neuromuscular junction and that they bind monophosphoinositides in a Ca^{2+}-independent manner. Based on these findings, we propose that the juxtamembrane cytosolic domain of synaptotagmin binds the cytosolic layer of the presynaptic membrane at rest. This binding brings synaptic vesicles and plasma membrane in a very close apposition, favouring the formation of hemifusion intermediates that enable rapid vesicle fusion.

1. Introduction

Neuroexocytosis is a central function of physiology and behavior and it consists of the Ca^{2+}-regulated fusion of cytosolic neurotransmitter/neuropeptide containing vesicles (SV) with the presynaptic membrane (PM) with release of its content into the synaptic cleft. Neuroexocytosis is mediated by a nanomachine that includes components located on the SV membrane, in the cytosol and on the PM cytosolic face [1–3]. SNAP-25 and syntaxin project their protein mass into the cytosol and can form a coiled–coil complex with a SV protein termed VAMP/synaptobrevin. This heterotrimeric oligomer is termed SNARE complex [4] and the determination of its structure has indicated that SNARE complex formation occurs via coil-coiling of a ~60 residues long SNARE domain present in the sequence of VAMP, SNAP-25 and syntaxin [5]. Other proteins play a major role in SV docking to the PM, but the SNARE complex and the SV Ca^{2+} binding protein synaptotagmin are those involved in the membrane fusion process which ultimately leads to the release of the SV content [6].

There is evidence that three or more SNARE complexes are required for neuroexocytosis to occur, i.e. a supercomplex of SNARE complexes is required [7–14]. The number of SNARE complexes present in such a nanomachine has not yet been determined with certainty and different events of exocytosis may require SNARE supercomplexes of different stoichiometries. Two cytosolic proteins, Munc-18 and complexin, are essential for the correct assembly of the neuroexocytosis nanomachine by regulating different stages of the formation of the SNARE complex and supercomplex [6]. A tentative arrangement of the supercomplex required for ultrafast neuroexocytosis has been proposed [3].

Synaptotagmins (Syt) form a large family of proteins which basically include a intravesicular domain of varying size, a transmembrane domain (TM) and a linker segment that connects the TM to two consecutive C2 domains that are exposed to the cytosol [15–17] (Fig. 1). Syt binds Ca^{2+} via the C2 domains each of which contains a conserved polycationic segment that binds anionic PM phospholipids, including phosphatidylserine and phosphatidylino

Abbreviations: SV, synaptic vesicles; PM, presynaptic membrane; α-BTX, alpha-bungarotoxin; Syt, synaptotagmin; TM, transmembrane; NMJ, neuromuscular junction; JMS, juxtamembrane segment; h-JMS, hexyl juxtamembrane segment; h-sJMS, hexyl scrambled juxtamembrane segment; h-FJMS, hexyl fluorescent juxtamembrane segment

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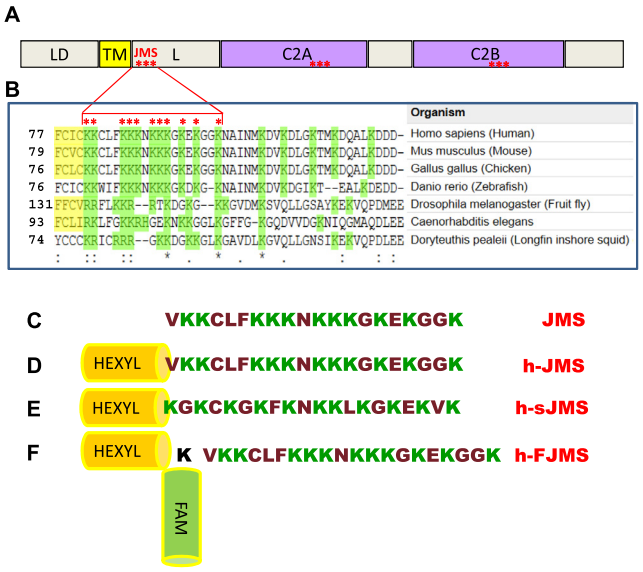

Fig. 1. Juxtamembrane polybasic peptide in synaptotagmin sequences. (A) Schematic representation of synaptotagmin domains. LD: lumenal domain; TM: transmembrane domain; L: linker domain; C2A and B: calcium binding domains; JMS: juxtamembrane Segment. The length of each box is proportional to the number of AA in the real sequence. Red asterisks indicate clusters of positive residues. (B) Sequence alignment of synaptotagmin-1 juxtamembrane segment in different species, obtained from www.uniprot.org; yellow indicates transmembrane domain, green basic residues. (C–F) Peptides used in this study. (C) Peptide corresponding to synaptotagmin polybasic segment (JMS, residues 80-98 in mouse sequence); (D) Modified peptide, with the hexyl moiety (h-JMS); (E) Scrambled peptide (h-sJMS); (F) Fluorescent peptide (h-FJMS).

sitol-4,5-biphosphate, in a Ca^{2+} dependent way, leading to membrane penetration and induction of a positive curvature in the PM [18–25]. This interaction leads to membrane penetration of segments of the C2 domain and to the bridging of the SV and PM membranes [26,27]. There is a growing consensus on the possibility that Ca^{2+} and anionic phospholipid binding to synaptotagmin triggers its rapid conformational change that is transmitted to the SNARE supercomplex leading to SV-PM fusion with the delivery of the vesicle content to the post-synaptic cell [2,3,6,17,18,21–23,28]. In the case of the neuromuscular junction synapse (NMJ), which is studied here, the released neurotransmitter is acetylcholine that migrates across the intersynaptic space to induce the opening of the acetylcholine receptor channel [29–31].

Here, we have attempted to determine the possible role in neuroexocytosis of the highly cationic juxtamembrane segment of Syt by testing the effect of corresponding peptides on neurotransmitter release at the mouse and *Drosophila melanogaster* NMJ. Our approach is based on the known property of highly cationic peptides to cross the plasma membrane of cells [32–34]. We found that these peptides induce a rapid neuroparalysis, bind phosphoinositides in a Ca^{2+} independent mode and largely localize on the presynaptic membrane. Based on these findings, we propose a possible activity of the juxtamembrane segment of Syt in neuroexocytosis.

2. Results

2.1. Juxtamembrane polybasic peptides of synaptotagmin are powerful inhibitors of neuroexocytosis in the insect and mammalian synapses

Fig. 1 shows that the juxtamembrane segment of Syt 1 contains a set of positively charged residues that has the potential to interact with anionic phospholipids. This aminoacid sequence is highly conserved among the Syts involved in neuroexocytosis and among different species suggesting that it plays an important role in neuroexocytosis [16]. The NMJ also expresses Syt2 [35], but the juxtamembrane segment of this Syt isoform contains three cysteine residues that generate all kind of artifacts owing to the possibility of generating intra and inter disulfide bonds.

The peptide corresponding to the juxtamembrane segment (JMS) of the mouse neuronal Syt isoform 1 (segment 80–98 of the mouse sequence) was synthesized with or without an hexyl chain at its N-terminus to mimic the TM binding to the lipid bilayer (lower part of Fig. 1), also on the basis of a previous study where the TM domains of VAMP/synaptobrevin and of syntaxin where replaced by hydrocarbon chains [36]. A peptide with a scrambled distribution of the same residues along the sequence was also prepared to be used as a control.

The effect of the peptides was first tested in the mouse phrenic hemidiaphragm nerve preparation, a well established model of *ex vivo* neuromuscular junction (NMJ). Both the JMS peptide and its hexyl derivative (h-JMS) are able to inhibit the nerve-stimulated contraction of the muscle. The JMS peptide is a less effective inhibitor of muscle twitching that its hexylated counterpart, as expected on the basis of the higher membrane partition of the latter compound (Fig. 2A). We have used up to 0.2 mM concentration only in the hemidiaphragm NMJ; this preparations is >99% muscle volume and only a minor fraction is nerve terminals. In fact, the end plate is less than 0.1% of the total muscle surface [37]. Accordingly, the predominant muscle will take up peptide and therefore the effective amount of peptide available for the nerve terminal is greatly reduced. At the end of each experiment the integrity of the muscle was tested and the peptides were found to have no effect on muscle contraction.

The hexyl scrambled peptide (h-sJMS) has a residual effect, which can be attributed to the high number of cationic residues that inevitably makes it partially similar to the native segment in terms of charge distribution. This finding indicates that electrostatic interactions play an important, but not unique, role in the inhibitory effect. However, at lower concentrations (below 100 μM), h-sJMS is without effect (black line in Fig. 2B), whereas the native sequence still induces a defined reduction of muscular twitch. The scrambled peptide was designed by the software 'PepControls' (http://bioware.ucd.ie) and retains the same charge as the sequence from the synaptotagmin one. It seems very unlikely that the relative positions of the charged residue in the sequence of h-JMS and h-sJMS would alter their permeability to membranes. In fact the peptide chain and its lateral chains are flexible and the membrane permeating species is a complex among the cationic peptide and the anionic plasma membrane lipid. Thus it is safe to assume that the relative lack of effects of the control peptide h-sJMS are not a consequence of it having an unexpected low rate of entry into cells. Likewise, h-JMS and h-sJMS will have the same general effect on membrane surface potential as the total number of electric charges is the same.

In order to have a different read-out of the effect of the h-JMS peptide on neuroexocytosis, another set of experiments was performed using an intracellular electrode to record the evoked junctional potentials (EJP) from the mouse diaphragm. Fig. 2C shows that h-JMS inhibits very rapidly the EJPat the mouse NMJ.

As shown in Fig. 1, the juxtamembrane segment is conserved among species and, therefore, we tested the effect of the h-JMS on the *D. melanogaster* third instar larva NMJ. Fig. 2D shows that this peptide is a highly effective inhibitor of neurotransmitter (glutamate) release at the insect NMJ reinforcing the suggestion that the JMS of Syt plays a major role in neuroexocytosis.

This process can be assayed directly by imaging the release of appropriate fluorescent dyes. Using rat primary spinal cord motor neurons loaded with FM 1–43, we compared the release of the dye in the presence or absence of the h-JMS peptide. As schematized in Fig. 3A, for each experiment, regions corresponding to the synaptic