Direct interaction of the calcium sensor protein synaptotagmin I with a cytoplasmic domain of the α_1A subunit of the P/Q-type calcium channel

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Synaptotagmins are synaptic vesicle proteins containing two calcium-binding C2 domains which are involved in coupling calcium influx through voltage-gated channels to vesicle fusion and exocytosis of neurotransmitters. The interaction of synaptotagmins with native P/Q-type calcium channels was studied in solubilized synaptosomes from rat cerebellum. Antibodies against synaptotagmins I and II, but not IV co-immunoprecipitated [¹²⁵I] ω -conotoxin MVIIIC-labelled calcium channels. Direct interactions were studied between *in vitro*-translated [³⁵S]synaptotagmin I and fusion proteins containing cytoplasmic loops of the α_1A subunit (BI isoform). Gel overlay revealed the association of synaptotagmin I with a single region (residues 780–969) located in the intracellular loop connecting homologous domains II and III. Saturable calcium-independent binding occurred with equilibrium dissociation constants of 70 nM and 340 nM at 4°C and pH 7.4, and association was blocked by addition of excess recombinant synaptotagmin I. Direct synaptotagmin binding to the pore-forming subunit of the P/Q-type channel may optimally locate the calcium-binding sites that initiate exocytosis within a zone of voltage-gated calcium entry.
Keywords: α_1A subunit/calcium channel/ ω -conotoxin MVIIIC/synaptotagmins

Introduction

Transmitter release from nerve terminals is controlled by calcium influx through voltage-gated channels. The consequent cytoplasmic calcium elevation triggers the fusion of synaptic vesicles at the active zone and the exocytosis of their contents into the synaptic cleft. Much recent effort has been directed at identifying nerve terminal proteins which couple calcium transients to exocytosis, and compelling mutational evidence indicates a key role for synaptotagmins as calcium sensor proteins in regulated secretion (reviewed by Littleton and Bellen, 1995; Südhof and Rizo, 1996).

Ten synaptotagmin genes have been identified in the rat

(Craxton and Goedert, 1995; Li *et al.*, 1995; Vician *et al.*, 1995; Thompson, 1996), of which some appear to be specifically expressed in distinct patterns in neural and neuroendocrine tissues (Ullrich *et al.*, 1994; Marquèze *et al.*, 1995; Berton *et al.*, 1997). Synaptotagmin I, a major membrane glycoprotein of both synaptic vesicles and neurosecretory granules has been the most extensively studied. It contains a short intravesicular N-terminal segment, a single transmembrane region and a cytoplasmic tail containing two regulatory C2 domains which bind calcium and mediate Ca^{2+} -dependent processes including phospholipid binding, self-association (Chapman *et al.*, 1996) and interaction with syntaxin (Li *et al.*, 1995).

Synaptotagmin and synaptic core complexes containing syntaxin, SNAP25 and VAMP can also associate with the voltage-dependent calcium channels that are involved in triggering exocytosis, including N-type (Lévêque *et al.*, 1992, 1994; Yoshida *et al.*, 1992; El Far *et al.*, 1995) and P/Q-type (Martin-Moutot *et al.*, 1996), but not L-type calcium channels (El Far *et al.*, 1995). Furthermore, co-expression of syntaxin with calcium channels induces a modification of current gating properties displaying a similar specificity for N- or P/Q-type channels (Bezprozvanny *et al.*, 1995). These findings are consistent with observations that neurotransmitter release at many central synapses is blocked by antagonists of N- or P/Q-type calcium channels, but is insensitive to inhibitors of L-type channels (Takahashi and Momiyama, 1993; Wheeler *et al.*, 1994). Neuronal calcium channels are heteromeric proteins constituted by an α_1 subunit which forms the transmembrane pore, associated with auxiliary $\alpha_2\delta$ and β subunits (Birnbaumer *et al.*, 1994). Association with core complexes involves syntaxin and SNAP25 binding to α_1 subunits on the cytoplasmic loop that links homologous domains II and III (Sheng *et al.*, 1994, 1996; Rettig *et al.*, 1996).

It has been suggested that interactions between synaptic protein complexes and calcium channels may optimally locate the calcium sensor synaptotagmin within domains of elevated calcium concentration generated close to the channel (Lévêque *et al.*, 1992; Neher and Penner, 1994). However, it is not known whether synaptotagmin binds directly to the calcium channel or is linked indirectly via its association with a component of the core complex such as syntaxin. We have therefore used radiolabelled synaptotagmin I as a probe to detect and characterize binding sites on fusion proteins containing cytoplasmic loops of the α_1A (BI) subunit that constitutes the pore of the P/Q-type calcium channel.

Results

Interaction of synaptotagmins with P/Q-type calcium channels in cerebellar membranes

Antibodies were prepared against synthetic peptides specific to the sequences of synaptotagmins I, II and IV,

Fig. 1. Specificity of anti-synaptotagmin antibodies. (A) Purified MBP-synaptotagmin I, II and IV fusion proteins (MBP-Syt I, -II and -IV) were analysed by SDS-PAGE followed by Coomassie blue staining. (B) ELISA was performed with MBP-synaptotagmin fusion proteins immobilized in 96-well microplates, and anti-peptide antibodies to synaptotagmin I (mAb3F10A, a monoclonal antibody raised against residues 1–20 of syt I), synaptotagmin II (a polyclonal antibody to residues 1–20 of syt II) and synaptotagmin IV (a polyclonal antibody against residues 123–138 of syt IV). Immunoreactivity was quantified using a secondary antibody coupled to alkaline phosphatase, by monitoring the cleavage of *p*-nitrophenylphosphate.

which are neuronal synaptotagmin isoforms expressed in cerebellum (Marquze *et al.*, 1995; Berton *et al.*, 1997). Maltose binding protein (MBP) fusion proteins containing the entire coding sequences of synaptotagmins I, II and IV were prepared (Figure 1A) and the specificity of the antibodies was confirmed by ELISA (Figure 1B). Immunoprecipitation and Western blotting experiments (not shown) confirmed that antibodies coupled to protein A–Sepharose beads were able to trap their respective antigens from detergent extracts of cerebellar membranes. These antibodies were then used to detect synaptotagmin association with native P/Q-type calcium channels. Cerebellar P/Q-type channels were specifically labelled with 125 I-labelled ω -conotoxin MVIIC (ω MVIIC), solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) (Martin-Moutot *et al.*, 1996) and the ability of different anti-synaptotagmin antibodies to capture protein complexes containing 125 I ω MVIIC receptors was assayed. Antibodies directed against calcium channel α_1A

Fig. 2. Association of synaptotagmins I and II with P/Q-type calcium channels in cerebellar synaptosomes. (A) Cerebellar synaptosomes were labelled with 125 I ω MVIIC, solubilized in CHAPS and incubated with either anti- α_1A subunit antibodies, anti-synaptotagmin antibodies, or non-immune rabbit IgG (control), in the presence (hatched) or in the absence (shaded) of the peptide used to raise the antibodies. The radioactivity in immune complexes was counted (mean values, three experiments). (B) Cerebellar and cortical synaptosomes were analysed by SDS-PAGE followed by immunoblotting for synaptotagmins I and II. The positions of molecular mass standards are indicated.

subunits and synaptotagmins I and II immunoprecipitated significant fractions of P/Q-type calcium channels, whereas anti-synaptotagmin IV antibodies did not (Figure 2A). These antibodies reacted with protein bands of 65 and 67 kDa in Western blots of rat brain cerebellar membranes, corresponding to synaptotagmins I and II respectively (Figure 2B). In contrast, synaptotagmin I was strongly stained in membrane preparations from rat cortex, whereas synaptotagmin II was not detected. The expression pattern of synaptotagmins I and II, particularly the very low density of synaptotagmin II in the cortex, is in good agreement with the localization of their mRNAs by *in situ* hybridization (Marquze *et al.*, 1995). In all cases, immunoreactivity in Western blots was blocked by including an excess of the peptides used to raise the antibodies.

In view of these results, the possibility of a direct interaction between synaptotagmin I and the α_1A subunit of the P/Q-type calcium channel was examined using recombinant proteins.

Fig. 3. Interaction of synaptotagmin I with a domain of the P/Q-type calcium channel. (A) [^{35}S]synaptotagmin I was prepared by *in vitro* translation and analysed by SDS-PAGE and autoradiography. (B) Purified GST fusion proteins containing cytoplasmic loops of the α_{1A} subunit (I-II, II-III and III-IV) and GST were analysed by SDS-PAGE followed by Coomassie blue staining or (C) transferred to a PVDF membrane, probed with [^{35}S]synaptotagmin I and analysed by autoradiography.

Interaction between synaptotagmin I and a cytoplasmic domain of the α_{1A} subunit

^{35}S -labelled synaptotagmin I was prepared by *in vitro* translation in the presence of [^{35}S]methionine (Figure 3A). Glutathione S-transferase (GST) fusion proteins containing sequences from cytoplasmic loops I-II, II-III and III-IV linking homologous domains of the α_{1A} subunit were purified, separated by SDS-PAGE and detected by Coomassie blue staining (Figure 3B). Proteins from a separate gel were transferred to PVDF membranes and probed with [^{35}S]synaptotagmin I. Autoradiography of the overlay revealed binding of [^{35}S]synaptotagmin I to proteins containing loop II-III (Figure 3C), but not to other loops nor to GST, suggesting a specific interaction.

These data were quantified by performing binding assays in solution, then recovering fusion protein-[^{35}S]synaptotagmin I complexes on glutathione-Sepharose beads. Significant interaction was detected with the fusion protein containing residues 780-969 of the BI isoform of the α_{1A} subunit (Figure 4A), but not with other intracellular domains, nor the equivalent II-III region of the α_{1C} subunit of the L-type calcium channel. The ability of this region to associate with native synaptotagmin was examined by incubating GST- α_{1A} ₇₈₀₋₉₆₉ with solubilized

Fig. 4. Interaction of recombinant and native synaptotagmin I with the II-III linker region of the P/Q-type calcium channel. (A) GST fusion proteins were bound to glutathione-agarose beads and incubated with [^{35}S]synaptotagmin I. After washing, the binding of [^{35}S]synaptotagmin I was quantified by scintillation counting. The ability of fusion proteins to bind [^{35}S]synaptotagmin I was compared with that of the anti-synaptotagmin antibody mAb1D12 (SytAb), bound to protein A-Sepharose beads (mean $\pm$ SD, $n = 3$). (B) GST fusion proteins were bound to glutathione-agarose beads and incubated with solubilized synaptosomes (P₂) or the post-P₂ supernatant (S₂) from rat brain. Beads were washed and bound proteins were analysed by SDS-PAGE followed by immunoblotting with anti-synaptotagmin antibody mAb1D12. In the control lane 10 μg of rat brain synaptosomes were directly analysed by Western blotting with mAb1D12. Arrows indicate the apparent molecular masses of synaptotagmin and its proteolytic fragments.

cerebellar membranes. Protein complexes were trapped on glutathione-Sepharose and analysed by SDS-PAGE and Western blotting. Immunoreactive bands containing synaptotagmin were identified (Figure 4B). These results indicate that both recombinant and native synaptotagmins can associate with an intracellular domain of the P/Q-type calcium channel.

Characterization of the interaction between synaptotagmin I and GST- α_{1A} ₇₈₀₋₉₆₉

Incubation of [^{35}S]synaptotagmin I with increasing concentrations of GST- α_{1A} ₇₈₀₋₉₆₉ revealed saturable binding (Figure 5A). This interaction was reversible and did not occur with GST alone, nor when a large excess of MBP-synaptotagmin I fusion protein was included in the assay (Figure 5C). Analysis of the saturation curve (Figure 5B)

Fig. 5. Saturation analysis of the interaction of synaptotagmin I with the II-III linker region of the P/Q-type calcium channel. (A) Increasing concentrations of GST- α_{1A} II-III (●) or GST (○) were bound to glutathione-agarose beads and incubated with [³⁵S]synaptotagmin I. Beads were washed and bound [³⁵S]synaptotagmin I was quantified by scintillation counting (mean $\pm$ SD, $n = 3$). (B) Specific binding was calculated by subtracting non-specific from total binding and was represented as a Scatchard plot. (C) Right panel: dissociation was initiated by dilution of the samples in an excess of binding buffer. Left panel: in competition experiments GST- α_{1A} II-III was bound to glutathione-agarose beads and incubated with [³⁵S]synaptotagmin I in the presence of MBP-synaptotagmin I fusion protein or MBP alone. Samples were centrifuged and radioactivity bound to the beads was evaluated by scintillation counting (mean values, three experiments).

revealed two sites, with equilibrium dissociation constants (K_D) of 70 and 340 nM at pH 7.4 and 4°C.

As synaptotagmin I contains calcium-binding sites, we examined the effects of increasing calcium concentrations in 10-fold increments over a range of 10 nM to 1 mM. No significant effects of calcium on [³⁵S]synaptotagmin I binding to GST- α_{1A} 780-969 nor to GST alone were revealed (Figure 6A). In positive control experiments (Figure 6B), MBP-synaptotagmin was found to bind to GST-syntaxin I, but not to GST. The interaction between GST-syntaxin I and MBP-synaptotagmin I, estimated by densitometric scanning of SDS-PAGE gels stained with Coomassie blue, was stimulated 2-fold by the presence of calcium, in agreement with previous reports (Li *et al.*, 1995).

Discussion

Neurotransmitter release from axonal terminals is triggered by calcium entry through voltage-gated channels in the presynaptic plasma membrane and compelling evidence suggests that certain synaptotagmins may act as calcium-

Fig. 6. Lack of effect of calcium on the interaction between synaptotagmin I and the II-III linker region of the P/Q-type calcium channel. (A) GST- α_{1A} II-III or GST were bound to glutathione-agarose beads and incubated with [³⁵S]synaptotagmin I, in the presence of the indicated concentrations of calculated free calcium, established using a calcium/EGTA buffer. Beads were washed and bound [³⁵S]synaptotagmin I was quantified by scintillation counting. (B) GST-syntaxin IA or GST were bound to glutathione-agarose beads and incubated with MBP-synaptotagmin I in the presence of 1 mM EGTA or 0.5 mM CaCl₂. Beads were washed and bound proteins were analysed by SDS-PAGE and Coomassie blue staining.

dependent regulators of exocytosis. Synaptotagmin displays calcium binding in the 100 μM range (reviewed by Südhof and Rizo, 1996), which parallels the affinity of the calcium sensor for exocytosis (reviewed by Matthews, 1996), and the introduction of synaptotagmin peptides or anti-synaptotagmin antibodies into neuronal cells disrupts exocytosis (Bommert *et al.*, 1993; Elferink *et al.*, 1993). Synaptic responses in hippocampal neurones from mice homozygous for a mutation in the synaptotagmin I gene display suppression of synchronous, but not asynchronous or spontaneous neurotransmitter release (Geppert *et al.*, 1994). Certain mutations in the synaptotagmin gene of *Drosophila melanogaster* affect the calcium dependence of evoked transmitter release at the neuromuscular junction, compatible with the view that synaptotagmin functions as a calcium receptor for initiating exocytosis (Littleton *et al.*, 1994).

The sub-millisecond delay between calcium influx and

neurotransmitter release implies that calcium channels at active zones are closely associated with the calcium sensors for exocytosis (Llinas *et al.*, 1981). We have therefore examined interactions between P/Q-type calcium channels, which control a predominant fraction of transmitter release at many synaptic fields in the mammalian brain (Takahashi and Momiyama 1993; Wheeler *et al.*, 1994) and the synaptotagmins.

Immunoprecipitation assays demonstrated that antibodies against synaptotagmins I or II but not synaptotagmin IV captured native P/Q-type channels. This is consistent with the high degree of homology between synaptotagmins I and II. In contrast, synaptotagmin IV (Vician *et al.*, 1995) has relatively low homology to synaptotagmins I and II, and does not bind syntaxin or phospholipids in a Ca^{2+} -dependent manner (Ullrich *et al.*, 1994), although some controversy surrounds the latter issue (Fukuda *et al.*, 1995; Südhof and Rizo, 1996). However, we cannot eliminate the possibility that our results are a reflection of the relatively low expression of synaptotagmin IV. Given the large number of synaptotagmin isoforms identified to date, it will be interesting to investigate differences in their capacity to interact with P/Q-type calcium channels more thoroughly.

Although these results demonstrate the association of synaptotagmins with calcium channels *in vitro*, they cannot inform us as to whether association occurs *in vivo* and, if so, in which subcellular compartments. Synaptotagmins are predominantly distributed in synaptic vesicles, but the fact that they have also been localized to the presynaptic plasma membrane should be taken into account (Takahashi *et al.*, 1991).

Immunoprecipitation of complexes containing synaptotagmin and P/Q-type calcium channels does not demonstrate direct interaction between the two proteins, and may occur because both proteins can bind simultaneously to a third partner. Synaptic vesicle exocytosis is thought to require formation of a trimeric synaptic core complex containing two abundant plasma membrane proteins, syntaxin and SNAP25, and a synaptic vesicle membrane protein, VAMP. Multiple synaptic proteins can bind to the core complex, suggesting that it constitutes the hub of a precise sequence of protein–protein interactions that prepare vesicle fusion (reviewed by Südhof, 1995). P/Q-type calcium channels associate with synaptic core complexes (Martin-Moutot *et al.*, 1996)—an interaction that involves syntaxin and SNAP25 binding to the cytoplasmic loop connecting homologous domains II and III of the $\alpha_1\text{A}$ (BI) subunit (Rettig *et al.*, 1996). Synaptotagmin I can bind to syntaxin and trimeric core complexes, an interaction which could thus mediate an indirect association with the calcium channel. However, our experiments with recombinant synaptotagmin I confirmed direct interaction with residues 780–969 of the BI isoform of the $\alpha_1\text{A}$ subunit, with two K_D s of 70 and 350 nM. Detection of two binding sites could be due to the presence of proteolytic fragments of the GST $\alpha_1\text{A}_{780-969}$ fusion protein with different affinities for synaptotagmin I. Alternatively, although self-association is unlikely at the low [^{35}S]synaptotagmin I concentrations used in our assays, it may reflect distinct binding affinities of monomeric versus multimeric forms of synaptotagmin I.

The synaptotagmin interaction site is thus located within

the syntaxin and SNAP25 binding domain (residues 722–1036) reported by Rettig *et al.* (1996). This suggests that distinct sequential protein–protein interactions may occur in a restricted region of the calcium channel, possibly representing steps in the assembly of a fusion-competent complex. It will therefore be important to focus future investigations towards the precise mapping of binding sites for synaptotagmin, syntaxin and SNAP25 on the P/Q-type calcium channel, which may be independent or overlapping, possibly leading to allosteric or competitive interactions. Rigorous analysis of these interactions will however be challenging, given the ability of each component to bind to multiple partners in the complex.

The detection of a synaptotagmin binding site on the calcium channel has several implications for the mechanisms of transmitter release. Firstly, it provides a novel site for interaction between the synaptic vesicle and the plasma membrane that may be relevant to the docking process. Secondly, synaptotagmin alone can mediate a link which would localize calcium-binding C2 domains within a cloud of voltage-gated calcium entry close to the channel. Finally, synaptotagmin I binds to the $\alpha_1\text{A}$ subunit of the calcium channel, a protein which contains four charged helical S4 segments that sense the membrane electric field. This direct coupling of a calcium sensor to a voltage sensor may be relevant to the Ca^{2+} -voltage hypothesis (reviewed by Parnas and Parnas, 1994; see also discussion by Neher and Penner, 1994) which holds that transmitter release is regulated by both Ca^{2+} and membrane potential. In this context it is conceivable that the ability of synaptotagmin to trigger release may also be modulated by voltage-driven conformational transitions corresponding to channel gating.

Materials and methods

Antibodies

A monoclonal antibody (mAb) directed against synaptotagmin I (mAb3F10A, raised against a peptide corresponding to residues 1–20), polyclonal antibodies against synaptotagmin II (residues 1–20) and a monoclonal antibody that recognizes both synaptotagmins I and II (mAb1D12), were prepared as previously described (Shoji-Kasai *et al.*, 1992). Polyclonal antibodies against synaptotagmin IV were raised in rabbits against peptide (C)PKLFPETKEAVSPES (residues 123–138) from the sequence of synaptotagmin IV.

Recombinant proteins

Rat synaptotagmins I, II and IV were bacterially expressed as MBP fusion proteins using the New England Biolabs Protein Fusion and Purification System. Plasmids encoding the entire coding sequence of synaptotagmins I, II and IV were constructed by PCR amplification of rat synaptotagmin cDNA using oligonucleotides containing appropriate flanking restriction sites for subcloning into the pMAL-C₂ vector (New England Biolabs). Recombinant proteins were purified by affinity chromatography on an amylose column (New England Biolabs) and stored at -80°C . GST fusion proteins from the rabbit brain calcium channel $\alpha_1\text{A}$ (BI-2, Mori *et al.*, 1991) subunit were prepared as described previously (De Waard *et al.*, 1997). GST fusion proteins containing the III–IV loop of $\alpha_1\text{A}$ and the II–III loop of $\alpha_1\text{C}$ (Wei *et al.*, 1991) were constructed by amplifying base pairs 4561–4725 and 2350–2757 of the respective genes and subcloning into pGEXkG and pGEX2Tk. A GST fusion protein containing full-length syntaxin 1A was constructed by cloning into pGEX2T. Fusion protein production was induced in cultures of protease-deficient *Escherichia coli* strain BL21, and purification from lysates was performed with glutathione–agarose. [^{35}S]methionine-labelled (>1000 Ci/mmol, Dupont) synaptotagmin I was synthesized *in vitro* by coupled transcription and translation using the TNTTM system (Promega).

Immunoassays

ELISAs were performed by immobilizing synaptotagmin fusion proteins in 96-well microplates, blocking with 1% bovine serum albumin in Tris 25 mM, NaCl 150 mM adjusted to pH 7.4 with HCl (Tris-buffered saline, TBS) and incubating with 1–10 µg/ml IgG for 2 h at 37°C. After washing and incubation with a secondary antibody coupled to alkaline phosphatase, immunoreactivity was quantified by monitoring the cleavage of *p*-nitrophenylphosphate. The preparation of [¹²⁵I]-labelled ω-conotoxin MVIIC (2200 Ci/mmol), SDS-PAGE, Western blotting and immunoprecipitation assays with solubilized [¹²⁵I]ω-conotoxin MVIIC receptors were performed as described by Martin-Moutot *et al.* (1996).

Binding assays

Following SDS-PAGE and electroblotting to PVDF membranes, overlay assays were performed by blocking the membrane with 5% non-fat milk and 5% BSA in TBS and incubation with 30 000 c.p.m./ml of [³⁵S]synaptotagmin I in the same buffer. After washing for 1 h in 5% BSA in TBS, membranes were exposed overnight to Kodak X-Omat film. Binding assays were performed in solution (De Waard *et al.*, 1995) by incubating GST fusion proteins with *in vitro*-translated [³⁵S]synaptotagmin I in 200 µl of EGTA-Ca²⁺ buffer (0.5% BSA, 0.1% Triton X-100, EGTA 50 µM and CaCl₂ to give the appropriate free Ca²⁺ concentration, in TBS) at 4°C in the presence of 50 µl glutathione-agarose beads. Beads were washed three times by centrifugation with the same buffer and [³⁵S]synaptotagmin I binding was quantified by scintillation counting.

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