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Interactions between proteins implicated in exocytosis and voltage-gated calcium channels

Michael Seagar[1]*, Christian Lévêque[1], Nathalie Charvin[1], Beatrice Marquèze[1], Nicole Martin-Moutot[1], Jeanne Andrée Boudier[1], Jean-Louis Boudier[1], Yoko Shoji-Kasai[2], Kazuki Sato[2] and Masami Takahashi[2]

[1]*INSERM Unité 464, Institut Jean Roche, Faculté de Médecine Secteur Nord, Bd Pierre Dramard, 13916, Marseille cedex 20, France*
[2]*Mitsubishi Kasei Institute of Life Science, Machida, Tokyo 194, Japan*

Neurotransmitter release from synaptic vesicles is triggered by voltage-gated calcium influx through P/Q-type or N-type calcium channels. Purification of N-type channels from rat brain synaptosomes initially suggested molecular interactions between calcium channels and two key proteins implicated in exocytosis: synaptotagmin I and syntaxin 1. Co-immunoprecipitation experiments were consistent with the hypothesis that both N- and P/Q-type calcium channels, but not L-type channels, are associated with the 7S complex containing syntaxin 1, SNAP-25, VAMP and synaptotagmin I or II. Immunofluorescence confocal microscopy at the frog neuromuscular junction confirmed that calcium channels, syntaxin 1 and SNAP-25 are co-localized at active zones of the presynaptic plasma membrane where transmitter release occurs. Experiments with recombinant proteins were performed to map synaptic protein interaction sites on the α_1A subunit, which forms the pore of the P/Q-type calcium channel. *In vitro*-translated ^{35}S-synaptotagmin I bound to a site located on the cytoplasmic loop linking homologous domains II and III of the α_1A subunit. This direct link would target synaptotagmin, a putative calcium sensor for exocytosis, to a microdomain of calcium influx close to the channel mouth. Cysteine string proteins (CSPs) contain a J-domain characteristic of molecular chaperones that cooperate with Hsp70. They are located on synaptic vesicles and thought to be involved in modulating the activity of presynaptic calcium channels. CSPs were found to bind to the same domain of the calcium channel as synaptotagmin, and also to associate with VAMP. CSPs may act as molecular chaperones in association with Hsp70 to direct assembly or dissociation of multi-protein complexes at the calcium channel.

Keywords: synaptotagmin; syntaxin; SNAP-25; cysteine string protein; synaptic vesicle; SNARE complex

1. LOCAL CALCIUM INFLUX AND EXOCYTOSIS AT THE NERVE TERMINAL

At the nerve terminal, calcium influx through channels located at the active zone triggers exocytosis of the classical rapid neurotransmitters (glutamate, acetylcholine, GABA), which are contained in small clear synaptic vesicles. Synaptic vesicle fusion requires relatively high (50–100 μM) internal calcium concentrations, which are only attained close to the inner mouth of the activated calcium channel (reviewed by Matthews 1996). Microdomains of high calcium occur at the pore exit because calcium ions enter the channel faster than they can diffuse into the surrounding cytoplasm. Calcium hotspots have been visualized in squid giant synaptic terminals injected with the low-affinity calcium-sensor photoprotein n-aequorin-J, in which action potentials elicit punctate signals exceeding 100 μM (Llinas *et al*. 1992). The injection into giant nerve terminals of fast calcium chelators such as BAPTA reduces synaptic responses, whereas slower buffers such as EGTA are much less efficient (Adler 1991; von Gersdorff & Matthews 1994). Modelling of ion diffusion and chelator-binding kinetics predict that BAPTA but not EGTA can significantly buffer calcium close to the channel mouth. The presynaptic effects of chelators are thus consistent with the idea that the calcium sensors that trigger exocytosis are located close to the cytoplasmic opening of channel pores (Matthews *et al*. 1996).

2. CALCIUM-CHANNEL SUBTYPES AND SYNAPTIC TRANSMITTER RELEASE

Which calcium-channel subtypes are involved in coupling electrical excitation to synaptic vesicle fusion? Patch-clamp methods and the use of specific drugs and toxins indicate that single neurons can express multiple types of voltage-gated calcium channels. Calcium channels were thus initially classified according to their biophysical properties (activation threshold, inactivation range, single channel conductance, etc.) and their sensitivity to pharmacological agents. At a molecular level,

*Author for correspondence (seagar.m@jean-roche.univ-mrs.fr).

Table 1. *Multiple types of voltage-gated calcium channels*

pore	current	pharmacology	tissues
α_1A	P/Q-type	ω-agatoxin IVA ω-conotoxin MVIIC	neurons
α_1B	N-type	ω-conotoxin GVIA ω-conotoxin MVIIA	neurons, neuroendocrine
α_1C, α_1D	L-type	1,4-dihydropyridines	neurons, neuroendocrine cardiovascular
α_1E	R-type	?	neurons
α_1G, α_1H	T-type	?	neurons, cardiovascular
α_1S	L-type	1,4-dihydropyridines	skeletal muscle

high-threshold calcium channels have been more thoroughly dissected. They are heteromeric proteins composed of an α_1 subunit, which forms the transmembrane ion-conducting pore and carries the binding sites for drugs that modulate channel activity. This central subunit is associated with at least two auxiliary subunits: α2δ and β that regulate the properties of the pore.

Calcium channel α_1 subunits display a structure similar to that of the α subunit of the voltage-dependent sodium channel, containing four homologous domains (I–IV), each constituted by six helical transmembrane segments. α2δ has a single transmembrane segment membrane and an extensively glycosylated extracellular domain. The β subunits do not contain transmembrane sequence and bind tightly to the cytoplasmic loop that links homologous domains I and II of the α_1 subunit. Gene families encoding homologous α_1 subunits (α_1A–H and α_1S) and β subunits (β1–4), plus a single gene encoding α2δ, have been cloned. Additional molecular diversity is provided by alternative mRNA splicing. The electrophysiological and pharmacological characteristics of different subunit combinations have been examined by heterologous expression and voltage clamp recording. These studies demonstrated that the nature of the α_1 subunit is the major determinant of channel properties (table 1). β subunits also play a role in modulating the activity of high-threshold calcium channels, but do not seem to be associated with the low-threshold T-type channels.

The small size of mammalian nerve terminals has generally precluded the direct recording of presynaptic calcium currents. However, electrophysiological analysis of synaptic transmission in numerous preparations and the use of the drugs and toxins listed above has been used to address the question as to which types of calcium channel trigger the synaptic release of neurotransmitters. In most synaptic fields of the central nervous system, transmission is partially blocked by the toxins active on N or P/Q-type channels, and often totally blocked by a combination of the two. At the mammalian neuromuscular junction P/Q-type channels predominate in the control of acetylcholine release. In contrast, L-channel agonists or antagonists generally have no effect on evoked transmitter release. Consequently, pharmacological evidence suggests that P/Q- and N-type channels, containing α_1A and α_1B subunits, respectively, are preferentially coupled to the exocytosis of the classical rapid neurotransmitters. However, L-type channels are involved in slower neurosecretory processes and contribute to triggering the exocytosis of catecholamines and neuropeptides contained in secretory granules or large dense-core vesicles.

3. PURIFICATION OF N-TYPE CALCIUM CHANNELS

The first evidence that specific molecular interactions couple calcium channels to the molecular machinery of exocytosis was provided by attempts to purify presynaptic N-type channels, which indicated that synaptotagmin I and syntaxin 1 are calcium-channel-associated proteins (Lévêque *et al*. 1992; Yoshida *et al*. 1992). The density of N-type calcium channels estimated by ^{125}I-ω-conotoxin GVIA (Olivera *et al*. 1994) binding assays in rat brain membrane preparations is about 1 pmol mg^{-1} of membrane protein. We set out to purify N-type calcium channels from crude nerve terminal preparations by combining immunoaffinity chromatography with more classical membrane protein purification procedures. Immunoaffinity chromatography required monoclonal antibodies (mAbs) specific for N-type calcium channels at a time when the sequence of the pore-forming alpha 1B subunit was still unknown, and a specific immunogen was thus unavailable.

The strategy chosen to produce mAbs involved immunizing SJ mice with a crude synaptic membrane preparation from chick brain, preparing hybridomas, and then screening with a highly specific assay based on the immunoprecipitation of channel–radioligand complexes. Cell cloning was perfomed by selecting hybridomas producing immunoglobulins that immunoprecipitated N-type (^{125}I-ω-conotoxin GVIA-prelabelled receptors), but not L-type calcium channels (^{3}H-PN200-110-prelabelled receptors) from solubilized brain membranes (Takahashi *et al*. 1991). A panel of monoclonal antibodies was generated that immunoprecipitated varying amounts of N-type channels and in immunoblots reacted with protein bands of diverse molecular sizes, most notably p35 and p58. Disappointingly, none of the mAbs recognized a 220–250 kDa band, which would correspond to the ω-conotoxin GVIA-binding protein detected by photoaffinity labelling. Furthermore, the apparent high abundance of p35 and p58 appeared to be incompatible with that of supposedly low-density calcium-channel subunits.

Work was then pursued in two directions. First, one of the anti-p35 antibodies (mAb10H5) immunoprecipitated a large fraction (*ca*. 60%) of ^{125}I-ω-conotoxin GVIA-prelabelled receptors (Yoshida *et al*. 1992). It was therefore

covalently coupled to Sepharose beads and incorporated into a multi-step N-type calcium-channel purification scheme, which eventually proved to be successful. Second, p35 and p58 were identified both by using the monoclonal antibodies to immunoscreen a rat brain λgt11 expression library, and by microsequencing peptides from immunoaffinity purified antigens. These two aspects will be discussed in the following paragraphs.

The starting material for N-type channel purification was a membrane fraction from homogenized rat brains. Proteins were solubilized in the detergent CHAPS and then sequentially fractionated over three columns: Chelating Sepharose Fast Flow loaded with cobalt ions; Heparin-Ultrogel column; and mAb10H5-Sepharose. About 15% of the ^{125}I-ω-conotoxin GVIA receptors in the initial extract were recovered, and a 1000-fold purification was achieved. Proteins were then fractionated by velocity sedimentation and analysed by SDS-PAGE and silver staining of proteins. Five major polypeptides of apparent molecular masses of 250, 140, 70, 58 and 35 kDa consistently comigrated with the peak of ^{125}I-ω-conotoxin GVIA-binding activity (Lévêque *et al*. 1994). Four of these displayed similar electrophoretic mobility to polypeptides in N-type calcium-channel preparations reported by other laboratories using totally different purification strategies (McEnery *et al*. 1991; Witcher *et al*. 1993).

(a) *Channel subunits*

The protein band migrating at 250 kDa was identified as the α_1B subunit. Photoaffinity labelling of purified preparations confirmed that the 250 kDa protein carries the binding site for the selective N-type channel antagonist ω-conotoxin GVIA, consistent with the conclusion that it forms the calcium-conducting pore. Three sequence-directed anti-α_1 subunit antibodies were also used to probe Western blots of purified proteins: CNB2 antibodies (Westenbroek *et al*. 1992), raised against a peptide from the cytoplasmic loop linking homologous domains II–III, specific to the α_1B sequence; BINt antibodies directed against an N-terminal peptide, which is partially conserved between α_1A and α_1B subunits; and CR2 antibodies, which were raised against a fusion protein containing sequence from the C-teminal domain of the α_1C subunit. Both CNB2 and BINt antibodies immunoprecipitate ω-conotoxin GVIA binding sites from crude membrane extracts or purified preparations, and both strongly immunostained the purified 250 kDa protein. CR2 antibodies immunoprecipitated *ca*. 80% of 3H-PN200-110 binding sites from brain membrane extracts, indicating that they recognize a significant fraction of L-type calcium channels. These antibodies did not react with Western blots of purified N-type channels compatible with the interpretation that our preparation is not significantly contaminated with L-type channel proteins.

CNB2 and BINt antibodies also reacted with smaller protein bands, which are thus immunologically related to α_1 subunits and may represent either proteolytic fragments or proteins encoded by a short transcript. A 210 kDa form was detected with CNB2 antibodies in agreement with observations by Westenbroek *et al*. (1992). A 100 kDa form reacted with both CNB2 and BINt antibodies and thus contained both the N-terminus and a portion of the linker region between homologous domains II and III (Lévêque *et al*. 1994). It may thus constitute a truncated form of the N-type channel lacking carboxyterminal domains. More recently, Campbell and co-workers have identified a 95 kDa component in their purifed ω-conotoxin GVIA receptor preparation. Proteolytic cleavage and microsequencing demonstrated that this protein is a short form of the α_1A subunit, containing only the homologous domains I and II (Scott *et al*. 1998). It may be interesting to determine whether truncated α_1 subunits lacking domains III and IV can assemble non-covalently to form functional homodimeric channels with novel properties.

Auxiliary subunits have not been systematically characterized in our preparation. The 140 kDa protein has a molecular mass similar to that of the reduced α_2 subunit from the rabbit skeletal muscle L-type calcium channel. The 70 and 58 kDa protein bands may contain different β subunit isoforms. β1b runs at about 72 kDa, whereas β3 and β4 subunits have similar molecular weights, comigrating in SDS-PAGE at *ca*. 57 kDa (Scott *et al*. 1996).

(b) *Associated proteins*

Proteins p58 and p35, initially identified in Western blots of brain membranes as the antigens recognized by monoclonal antibodies selected for their ability to immunoprecipitate N-type channels, were also detected in purified channel preparations. Chronologically, the first mAb produced was designated mAb1D12 (Takahashi *et al*. 1991). MAb1D12 immunostained a 58 kDa protein in Western blots of chick or rat brain membranes. This protein was shown to be specific to neuronal or neuroendocrine tissues, and at the light microscope level was localized to central and peripheral nerve terminals. Analysis at the ultrastructural level gave intriguing results as this 'calcium-channel-associated protein' appeared to be predominantly distributed in synaptic vesicles, although immunoperoxidase staining was also detected at the presynaptic plasma membrane (Takahashi *et al*. 1991).

p58 was identified by immunoscreening a rat brain expression library with mAb1D12 (Lévêque *et al*. 1992). Sequencing of the selected clones and microsequencing of the immunoaffinity purifed protein revealed that p58 was identical to synaptotagmin I (Perin *et al*. 1990). Synaptotagmin has a short intravesicular N-glycosylated amino-terminal domain, a single transmembrane region (TMR), and a larger carboxyterminal cytoplasmic tail that contains two C2 (C2A and B) domains, homologous to the C2 domains present in the calcium-dependent isoforms of protein kinase C. More recently, C2 domains have been characterized in other calcium-dependent enzymes: phospholipases C and A2; as well as in synaptic proteins implicated in vesicle trafficking (rabphilin-3A, DOC2 and Munc 13-1). At least ten distinct synaptotagmins have been described to date in the rat, some of which are neuron-specific and others ubiquitous (reviewed by Sudhof & Rizo 1996). MAb1D12 binds to two neuron-specific synaptotagmins, at a cytoplasmic epitope located between the TMR and the C2A domain, interacting with synaptotagmin I with high affinity but also recognizing synaptotagmin II, albeit much more

weakly (C. Lévêque and N. Charvin, unpublished observations). Synaptotagmin is associated with *ca.* 50% of freshly solubilized N-type channels (Takahashi *et al.* 1991; Lévêque *et al.* 1992). This interaction is, however, relatively labile, and significant dissociation occurred during purification (Lévêque *et al.* 1994).

A series of mAbs (10H5, 6D2, 6H1) were produced which immunoprecipitated N-type but not L-type calcium channels, and reacted in immunoblots with p35, a neuronal and neuroendocrine-specific protein, which migrated as a closely spaced doublet (Yoshida *et al.* 1992). Anti-p35 antibodies and anti-synaptotagmin antibodies (mAb1D12) each immunoprecipitated *ca.* 50% of solubilized ^{125}I-ω-conotoxin GVIA receptors. When the two types of antibody were included together, immunoprecipitation was not additive, suggesting that *ca.* 50% of N-type calcium channels form a ternary complex with synaptotagmin and p35. The fact that anti-p35 antibodies co-immunoprecipitated synaptotagmin and vice versa was also consistent with this hypothesis (Yoshida *et al.* 1992). A mixture of anti-p35 mAbs was used to screen a rat brain expression library, and a clone with a 2.1 kb insert was isolated and sequenced (Yoshida *et al.* 1992). p35 is identical to proteins independently cloned in several laboratories in the same period and designated as HPC-1 (Inoue *et al.* 1992); synaptocanalin (Morita *et al.* 1992); GR33 (Smirnova *et al.* 1993); although the name syntaxin 1 (Bennett *et al.* 1992) is now generally used. Syntaxin 1 is predominantly expressed in the plasma membrane where it is anchored by a C-terminal TMR. The cytoplasmically orientated N-terminal region contains three heptad repeats, which are characteristic of proteins forming coiled-coil structures.

4. PRESYNAPTIC CALCIUM CHANNELS ASSOCIATE WITH THE SYNAPTIC CORE COMPLEX

Thus the biochemical characterization of native N-type calcium-channel complexes from rat brain revealed an unsuspected association between channels, syntaxin and synaptotagmin. Synaptic vesicle fusion is thought to depend on the assembly of a v-SNARE: VAMP (vesicle-associated membrane protein or synaptobrevin); with two t-SNAREs, syntaxin 1 and SNAP-25 (Sollner *et al.* 1993), to form an extremely stable heterotrimeric synaptic core complex (Hayashi *et al.* 1994). Synaptotagmins, which interact with this trimeric complex, may mediate the calcium-dependency of the release process. Most, but not all synaptotagmin isoforms bind calcium ions in a phospholipid-dependent manner via their C2 domains (Brose *et al.* 1992; Li *et al.* 1995), and display calcium-dependent interactions with syntaxins (Li *et al.* 1995). Synaptotagmins may be thought of as auxiliary calcium-dependent v-SNAREs as they bind βSNAP and NSF and interact with the t-SNAREs syntaxin and SNAP-25 (Schiavo *et al.* 1995).

Genetic experiments have provided compelling evidence that synaptotagmins play an important role in neurotransmitter release. Deletion of the synaptotagmin I gene in mice is lethal before postnatal day 4 (Geppert *et al.* 1994). Analysis of synaptic transmission between hippocampal neurons cultured from these animals revealed that rapid synchronous glutamate release was severely impaired, although a delayed component of evoked release, and calcium-independent release induced by hyperosmotic stimulation were not affected by defiency in synptotagmin I. Analysis of synaptotagmin mutants in *Drosophila* has shown that with certain combinations of alleles, the calcium cooperativity of transmitter release at the neuromuscular junction is modified (Littleton *et al.* 1994), suggesting that synaptotagmin may indeed play a role in triggering calcium-dependent exocytosis.

If a synaptotagmin–syntaxin 1–SNAP-25–VAMP complex constitutes the molecular correlate of a synaptic vesicle docked at the plasma membrane and awaiting the calcium influx, all four proteins may be associated with N-type calcium channels. This hypothesis was extended to include the possible interaction of synaptic complexes with P/Q-calcium channels and tested in immunoprecipitation experiments. The association of complexes with native calcium channels can be quantitatively evaluated in immunoprecipitation experiments using detergent-solubilized calcium channels prelabelled with an appropriate radioligand. These methods have been employed to study the interaction of trimeric core complexes with N-type calcium channels labelled with ^{125}I-ω-conotoxin GVIA and P/Q-type calcium channels labelled with ^{125}I-ω-conotoxin MVIIC.

The use of ^{125}I-ω-conotoxin MVIIC as a biochemical marker for P/Q-type channels requires caution as electrophysiological data indicate that this toxin also blocks N-type channels. Radioligand-binding experiments at physiological salt concentrations suggest, however, that subnanomolar concentrations of ^{125}I-ω-conotoxin MVIIC label high-affinity sites (K_D=1 nM) that are presumably associated with P/Q-type channels, whereas substantially greater concentrations are required to occupy low-affinity sites associated with N-type channels (K_D=30 nM). This interpretation was confirmed by demonstrating that high-affinity binding sites were immunoprecipitated by specific antibodies raised against peptides from the sequence of α_1A subunits (P/Q-type channels), but not by antibodies against α_1B (N-type channels) or α_1C subunits (L-type channels) (Martin-Moutot *et al.* 1996).

As these findings are consistent with the conclusion that ^{125}I-ω-conotoxin MVIIC used at subnanomolar concentrations specifically labels P/Q-type calcium channels, we examined the capacity of a panel of antibodies directed against synaptic proteins implicated in vesicle trafficking to co-imunoprecipitate these channels. Antibodies against syntaxin 1, SNAP-25 or VAMP all immunoprecipitated P/Q-type calcium channels extracted from cerebellar synaptosomes. Immunoadsorption with increasing concentrations of antibodies reached a plateau level, indicating that a limited fraction (20–40%) of channels is associated with each SNARE protein. If each SNARE protein interacts with a different fraction of calcium channels, then immunoprecipitation by two antibodies combined should be additive. Alternatively, if all three SNARE proteins are associated with the same channel population, then immunoprecipitation should not be additive. Data were compatible with the second hypothesis, suggesting the interaction of a trimeric synaptic core complex with a

significant fraction of P/Q-type calcium channels. In control experiments, antibodies against synaptophysin, Rab3A or CSP did not capture more P/Q-type calcium channels than non-immune IgG (Martin-Moutot *et al*. 1996). Similar results were obtained when analogous immunoprecipitation experiments were performed with native N-type, but not L-type calcium channels (El Far *et al*. 1995; Pupier *et al*. 1997; M. Seagar and M. Takahashi, unpublished results).

Anti-synaptotagmin antibodies also immunoprecipitate a similar fraction of P/Q-type calcium channels. Experiments with antibodies specific for different synaptotagmins have shown that synaptotagmin I and II, but not synaptotagmin IV, can associate with P/Q-type channels (Charvin *et al*. 1997). This may be related to the fact that synaptotagmin IV neither binds calcium ions, nor displays calcium-dependent binding to syntaxins (Li *et al*. 1995).

Results using detergent-solubilized, native calcium-channel preparations are thus consistent with the interaction of a protein complex containing syntaxin 1, SNAP-25, VAMP, and synaptotagmin I or II with either N-type or P/Q-type but not L-type calcium channels. They are compatible with the idea that synaptic vesicles docked at the plasma membrane can associate with the classes of voltage-gated calcium channels that trigger neurotransmitter release. They are also consistent with reports that the co-expression of syntaxin with calcium channels in *Xenopus* oocytes leads to a modification of the gating properties of N-type and P/Q-type, but not L-type channels (Bezprozvanny *et al*. 1995).

5. CO-LOCALIZATION OF CALCIUM CHANNELS, SYNTAXIN 1 AND SNAP-25 AT ACTIVE ZONES

What is the morphological evidence that SNARE proteins and calcium channels are preferentially co-localized at active zones where neurotransmitter release occurs? In the central nervous system, although syntaxin 1 and SNAP-25 are expressed in the presynaptic plasma membrane, their distribution is certainly not limited to active zones and both proteins are also present in axonal plasma membranes (Garcia *et al*. 1995), synaptic vesicles, and clathrin-coated vesicles (Koh *et al*. 1993; Walch-Solimena *et al*. 1995). Confocal immunofluorescent microscopy on cerebellar sections has shown that both α_1A and α_1B subunits are found in synaptic terminals, although they also occur on dendritic shafts at about an eightfold lower density (Westenbroek *et al*. 1995). Furthermore, co-localization of α_1A and syntaxin 1 in the same synaptic terminals has been demonstrated (Westenbroek *et al*. 1995).

Experiments using confocal microscopy to determine the distribution of SNARE proteins and calcium channels at active zones were performed at the frog neuromuscular junction (Boudier *et al*. 1996), a classical physiological model of rapid transmitter release that has a particularly convenient structure. The presynaptic nerve terminal runs parallel to the muscle fibre and forms active zones of transmitter release in regularly spaced bands at 1 μM intervals. Active zones are situated directly across the synaptic cleft from the postsynaptic folds, which contain a high density of nicotinic acetylcholine receptors (nAChRs). The distribution of presynaptic exocytotic domains can thus be predicted to be the 'mirror image' of postsynaptic nAChRs labelled with rhodamine-tagged α-bungarotoxin (R-αBuTx). The distribution of presynaptic proteins was therefore studied using indirect immunofluorescence with FITC, in a double-labelling protocol with R-αBuTx.

Two different perpendicular orientations of the neuromuscular junction relative to the axis of observation were analysed. In both orientations syntaxin 1, SNAP-25 and calcium channels (labelled with antibodies that cross-react with mammalian α_1A and α_1B subunits) displayed apparent co-localization with nAChRs (Boudier *et al*. 1996). Apparent co-localization of pre-and postsynaptic proteins occurs because the width of the synaptic cleft (*ca*. 60 nm) is smaller than the limit of resolution of the confocal microscope. The fact that FITC labelling was truly presynaptic was verified by immunostaining and then pulling the collagenase-treated nerve terminal free of the muscle fibre. These proteins are thus essentially localized at the presynaptic plasma membrane facing the synaptic cleft, and appear as regular immunofluorescent bands at 1 μm intervals, i.e. predominantly at exocytotic active zones, perfectly co-incident with nAChRs.

In contrast to t-SNAREs and calcium channels, the v-SNARE VAMP was only partially co-localized with nAChRs and significant immunostaining extended back within the nerve terminal, often leaving unstained patches between active zones. This pattern indicates localization both in microvesicles docked at plasma membrane active zones and in clusters of microvesicles in the cytoplasm facing each active zone (Boudier *et al*. 1996).

The co-localization of t-SNAREs and calcium channels at the active zones where the calcium-dependent exocytosis of acetylcholine occurs is thus strikingly consistent with biochemical data indicating molecular interactions. The immunolocalization of calcium channels is in good agreement with data obtained using fluorescent derivatives of ω-conotoxin GVIA, which blocks transmitter release at the amphibian neuromuscular junction (Robitaille *et al*. 1990). Taken together these findings provide data complementary to the classic freeze-fracture studies of Heuser & Reese (1981) at the frog neuromuscular junction in which exocytotic fusion pores were visualized in close proximity to the parallel arrays of 10 nm membrane particles believed to represent presynaptic calcium channels.

6. DIRECT INTERACTIONS BETWEEN THE α_1A SUBUNIT OF THE P/Q-TYPE CALCIUM CHANNEL AND PROTEINS IMPLICATED IN TRANSMITTER RELEASE

(a) *Synaptotagmin I*

Experiments were performed with recombinant proteins to examine the possibility that synaptotagmins bind directly to the pore-forming α_1A subunit of the P/Q-type calcium channel (Charvin *et al*. 1997). Calcium-channel α_1 subunits contain four homologous domains (I–IV), each constituted by six helical transmembrane segments. The extensive cytoplasmic loops linking these homologous domains provide a likely site

for interaction with proteins in the cytoplasmic compartment. ^{35}S-labelled synaptotagmin I was generated by *in vitro* translation in the presence of ^{35}S-methionine. We then examined the ability of ^{35}S-synaptotagmin I to bind to bacterially expressed polypeptides containing the cytoplasmic domains of the α_1A subunit fused to GST. This approach identified a synaptotagmin binding site with an apparent $K_D = 70$ nM. The fact that synaptotagmin I is a calcium-binding protein prompted us to analyse the effects of calcium on binding to the α_1A subunit. However, varying the calcium concentration within the range of 1 μM to 1 mM did not significantly modulate binding. The synaptotagmin binding site is located in a segment of *ca*. 200 amino acids (BI isoform, residues 780–969) in the loop linking homologous domains II and III of the α_1A subunit. This site is thus located within the syntaxin 1 and SNAP-25 interaction domain (BI, residues 722–1036) reported by Rettig *et al*. (1996). Evidence in favour of a direct interaction of synaptotagmin I with α_1B subunits of the N-type calcium has been also obtained (Sheng *et al*. 1997; Wiser *et al*. 1997). Thus distinct sequential protein–protein interactions may occur in a restricted regions of P/Q- and N-type calcium channels, possibly representing steps in the assembly of a fusion-competent complex. However, precise analysis of competitive or allosteric interactions between synaptotagmin I, syntaxin 1, SNAP-25, and the cytoplasmic binding domain of the P/Q-type channel is difficult owing to the fact that every single protein in this list can bind directly to all the others.

(b) *Cysteine string proteins (CSPs)*

CSPs were initially discovered in *Drosophila* as neuronal antigens predominantly localized in synaptic regions (Zinsmaier *et al*. 1990). Subsequently, CSPs were identified in *Torpedo* (Gundersen & Umbach 1992) and mammals (Mastrogiacomo & Gundersen 1995), where they are associated with the cytoplasmic surface of secretory vesicles, including both small synaptic vesicles and large dense-core vesicles (Pupier *et al*. 1997). CSPs have a characteristic domain structure. The N-terminal region contains a J-domain with homology to bacterial Dna-J proteins that act as chaperones. Mammalian CSPs cooperate with the heat-shock protein (Hsc70), activating its ATPase activity and preventing aggregation of model protein substrates (Chamberlain & Burgoyne 1997). The J-domain is coupled via a conserved linker sequence to the cysteine-rich motif from which the name of these proteins is derived. In *Drosophila* this motif contains an uninterrupted string of 11 cysteine residues flanked on either side by another pair of cysteines. Palmitoylation of these cysteine residues probably provides the membrane anchor for CSPs.

The association of CSPs with synaptic vesicles suggests that they are involved in membrane trafficking and/or exocytosis of neurotransmitters. Deletion of the CSP gene in *Drosophila* causes temperature-sensitive failure of synaptic transmission, resulting in paralysis due to impaired excitation–secretion coupling at nerve terminals (Zinsmaier *et al*. 1994). Recent findings suggest that the default involves either a deficit in calcium entry or in the ability of calcium to trigger exocytosis (Umbach & Gundersen 1997). These data are thus consistent with earlier results indicating that CSPs act as positive modulators of ω-conotoxin GVIA-sensitive calcium channels from the *Torpedo* electric organ, heterologously expressed in *Xenopus* oocytes (Gundersen & Umbach 1992). As the mechanisms by which CSPs act on exocytosis and/or regulate calcium-channel activity are unknown. We examined the molecular interactions of CSPs with proteins of the synaptic core (SNARE) complex and P/Q-type calcium channels.

Co-immunoprecipitation suggested that CSPs occur in complexes containing synaptobrevin (VAMP), but not syntaxin 1, SNAP-25 nor P/Q-type calcium channels labelled with ^{125}I-ω-conotoxin MVIIC. However, binding experiments with ^{35}S-labelled CSP1 demonstrated an interaction (apparent $K_D = 700$ nM at pH 7.4 and 4 °C) with a fusion protein containing a segment of the cytoplasmic loop linking homologous domains II–III of the α_1A calcium-channel subunit (BI isoform, residues 780–969). Binding was specific as it was displaced by unlabelled CSP1, and no interactions were detected with fusion proteins containing other calcium-channel domains, syntaxin 1A, SNAP-25 or synaptotagmin I. The CSP binding site on the P/Q-type calcium channel is thus located within the same 200 residue synaptic protein interaction site that binds syntaxin I, SNAP-25 and synaptotagmin I. Surface plasmon resonance experiments using a Biacore apparatus to detect interactions between recombinant proteins also confirmed MBP–CSP1 binding to GST–II–IIIA immobilized on a sensor chip via covalently linked anti-GST antibodies. Binding monitored by this method was again specific for the II–III domain of the α_1A subunit, as control experiments did not reveal interactions between MBP and GST–II–IIIA, nor between MBP–CSP1 and other cytoplasmic domains of the α_1A subunit fused to GST.

GST–II–IIIA immobilized on agarose beads trapped native CSPs from solubilized rat brain nerve terminals. However, MBP–CSP1 beads did not bind native P/Q-type calium channels, suggesting that a limiting parameter may be the accessibility of the CSP binding site on the calcium channel. Syntaxin 1, SNAP-25 and synaptotagmin I bind to the same domain on the calcium channel as CSP, and these three proteins all display a higher affinity than CSP for binding to calcium channels. Furthermore, a large fraction of native P/Q-type channels are stably associated with complexes containing syntaxin I, SNAP-25, VAMP and synaptotagmin I or II (Martin-Moutot *et al*. 1996; Charvin *et al*. 1997). SNARE complexes associated with native calcium channels may therefore prevent interaction with endogenous or exogenous CSPs.

If CSPs promote calcium-channel opening by binding to the II–III linker region of the α_1A subunit, how may intrinsic chaperone activity and cooperation with Hsp70 be involved? Apart from their role in protein folding during translation, chaperones contribute to the assembly and dissociation of multi-protein complexes. It is thus possible that CSPs act as chaperones to promote assembly or disassembly of synaptic protein complexes associated with the II–III linker region of the calcium channel (figure 1). Co-expression of syntaxin 1 with Q-type calcium channels decreases their availability for opening

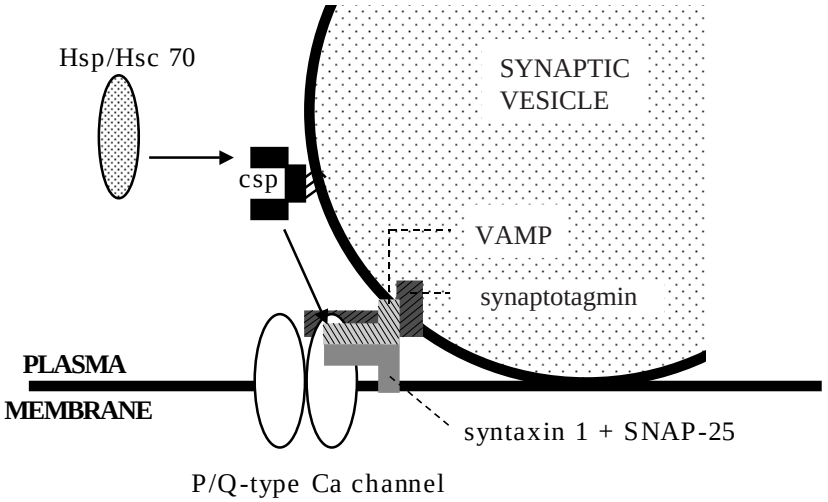

Figure 1. Proposed role for CSPs in calcium channel modulation and exocytosis. Trimeric SNARE complexes associated with synaptotagmin form a 7S complex at the interface between a docked synaptic vesicle and the presynaptic plasma membrane. The 7S complex is in turn tethered to P/Q-type calcium channels via three potential partners, syntaxin 1, SNAP-25 and synaptotagmin, which can each bind directly to a cytoplasmic loop of the pore-forming α_1A channel subunit. These interactions modulate the availability of channels to open in response to membrane depolarization. They may also allow conformational transitions of the channel driven by membrane potential to be transmitted to the exocytotic machinery. CSPs recruit Hsp/Hsc 70 to the calcium channel and these co-chaperones coordinate assembly or disassembly of multiprotein complexes. CSPs may thus favour channel opening by promoting the dissociation of inhibitory proteins such as syntaxin 1.

by stabilizing an inactivated state (Bezprozvanny *et al*. 1995). CSPs could in principle facilitate channel activation by promoting the dissociation of syntaxin. Our data indicate that CSPs can interact directly with the P/Q-type calcium channel and also suggest an association with VAMP. CSP may coordinate sequential protein–protein interactions between syntaxin and two of its binding partners, calcium channels and VAMP, and consequently modulate channel opening and calcium-dependent exocytosis. It is interesting to note that a strikingly similar pattern of evoked synaptic responses results from either the disruption of SNARE protein interaction with calcium channels in rat sympathetic neurons (Mochida *et al*. 1996), or the deletion of the CSP gene in motoneurons of *Drosophila* larvae (Heckmann *et al*. 1997). Both manipulations produced a reduction in synchronous transmitter release, while asynchronous release and paired pulse facilitation were increased. The regulation by CSP of SNARE protein interactions with calcium channels may thus be necessary to ensure synchronous synaptic transmission.

7. CONCLUSIONS

Why do calcium channels interact with proteins thought to be located at the interface between a docked synaptic vesicle and the plasma membrane? The most tempting hypothesis is that molecular links between calcium channels and the exocytotic machinery would guarantee that the calcium trigger initiating vesicle fusion is located within a restricted zone where the appropriate calcium concentration is attained upon channel activation. In addition, these interactions affect the ability of calcium channels to open in response to depolarization, which may economize calcium influx at inappropriate sites (Gundersen & Umbach 1992; Bezprozvanny *et al*. 1995). Regulatory processes affecting interactions beween channels and synaptic core complexes could thus play an important role in the plasticity of presynaptic events.

A striking parallel exists between excitation–exocytosis coupling in nerve terminals and excitation–contraction coupling in skeletal muscle. Both involve interactions between the cytoplasmic loop connecting domains II and III of calcium channel α_1 subunits and proteins located in an intracellular compartment. In the case of excitation–contraction coupling, the α_1S subunit acts as a voltage sensor transducing a conformational change induced by depolarization of the plasma membrane into gating of the ryanodine receptor in the sarcoplasmic reticulum, independently of calcium influx. By analogy, we can speculate that voltage-driven conformational transitions of α_1A subunits in nerve terminals could be transmitted to the II–III loop, and thus modulate synaptic protein interactions and neurotransmitter release.

Functional evidence is necessary to test these hypotheses, which are essentially based on *in vitro* analysis of protein binding. The general relevance of interactions between calcium channels and the exocytotic machinery to neurotransmission is supported by evidence that injection of the entire synaptic protein-binding domain from the α_1B subunit disrupts transmitter release in sympathetic neurons (Mochida *et al*. 1996), and at the amphibian neuromuscular junction (Rettig *et al*. 1997). Further progress will require detailed molecular analysis of the motifs involved in interactions between individual synaptic proteins and calcium channels, and model synaptic systems in which these interactions can be perturbed in order to evaluate their specific role in transmitter release.

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