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Interactions between Presynaptic Calcium Channels and Proteins Implicated in Synaptic Vesicle Trafficking and Exocytosis

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Monoclonal antibodies were generated by immunizing mice with chick brain synaptic membranes and screening for immunoprecipitation of solubilized ω conotoxin GVIA receptors (N-type calcium channels). Antibodies against two synaptic proteins (p35—syntaxin 1 and p58—synaptotagmin) were produced and used to purify and characterize a ternary complex containing N-type channels associated with these two proteins. These results provided the first evidence for a specific interaction between presynaptic calcium channels and SNARE proteins involved in synaptic vesicle docking and calcium-dependent exocytosis. Immunoprecipitation experiments supported the conclusion that syntaxin 1/SNAP-25/VAMP/synaptotagmin I or II complexes associate with N-type, P/Q-type, but not L-type calcium channels from rat brain nerve terminals. Immunofluorescent confocal microscopy at the frog neuromuscular junction was consistent with the co-localization of syntaxin 1, SNAP-25, and calcium channels, all of which are predominantly expressed at active zones of the presynaptic plasma membrane facing post-synaptic folds rich in acetylcholine receptors. The interaction of proteins implicated in calcium-dependent exocytosis with presynaptic calcium channels may locate the sensor(s) that trigger vesicle fusion within a microdomain of calcium entry.

KEY WORDS: Calcium channels; SNARE complexes; syntaxin; synaptotagmin; SNAP-25; VAMP; synaptic vesicles; exocytosis; neurotransmitter release.

INTRODUCTION

Calcium channels in excitable cells play a crucial role in coupling electrical signals propagated along the plasma membrane to a variety of intracellular processes, including the synaptic release of neurotransmitters. At the nerve terminal, calcium influx through channels located at the active zone triggers exocytosis of the classical rapid neurotransmitters (glutamate, acetylcholine, GABA) contained in small clear synaptic vesicles. An understanding of this process at a molecular level is essential to gain insights into the initiation of synaptic transmission, presynaptic modulation in neuronal plasticity, and defaults in synaptic transmission in neurological disease.

Synaptic vesicles are thought to transit through a series of defined stages that culminate in exocytosis. These stages include loading with neurotransmitter, targeted transport of vesicles to the plasma membrane, docking, priming, calcium-dependent triggering, and membrane fusion, and are followed by the recycling of vesicle membrane proteins by endocytosis. The steps between docking and fusion appear to require the assembly and maturation of multi-molecular complexes which are formed at the interface between the plasma membrane and docked synaptic vesicles and are composed of interacting proteins from both com-

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partments. A series of distinct experimental approaches in many laboratories has recently converged on the identification of the proteins involved and the elaboration of molecular models (reviewed by Bennett and Scheller 1993; Burgoyne and Morgan 1995; Sudhof, 1995).

Two key players are synaptotagmin (Südhof and Rizo, 1996) and syntaxin (Bennett *et al.*, 1993) which were cloned independently in several laboratories in the early 1990s. Compelling evidence now indicates that these proteins are involved in synaptic vesicle docking and fusion. However, in certain cases they were initially identified as calcium channel-associated proteins (Leveque *et al.*; 1992, Morita *et al.*, 1992; Yoshida *et al.*, 1992,). These observations provided the first evidence that protein–protein interactions couple calcium channels to the machinery of exocytosis. The aim of this article is to review the data indicating that native voltage-gated calcium channels from nerve terminals can associate with components of the exocytotic complex and to discuss the functional implications. It will also hopefully provide a useful introduction to the following article in which Zu-Hang Sheng and Bill Catterall focus on the identification and properties of the "synprint" site.

PURIFICATION STRATEGY

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 of membrane protein, which made purification a challenging task. Attempts to purify presynaptic N-type calcium channels from rat brain membranes were initiated in a collaboration between our two laboratories 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 performed by selecting hybridomas producing immunoglobulins that immunoprecipitated N-type (^{125}I-ω conotoxin GVIA-prelabeled receptors) but not L-type calcium channels (3H-PN200-110-prelabeled receptors) from solubilized brain membranes (Takahashi *et al.*, 1991). A panel of monoclonal antibodies was generated in this way which immunoprecipitated varying amounts of N-type channels and in immunoblots reacted with protein bands of diverse molecular sizes most notably p35, p58, and p105. Disappointingly at the time, none of the mAbs recognized a 220–250-kDa band, which would correspond to the ω conotoxin GVIA binding protein detected by photoaffinity labeling. Furthermore the apparent high abundance of p35, p58, and p105 appeared to be incompatible with that of supposedly low-density calcium channel subunits.

Work was then pursued in two directions. Firstly one of the anti-p35 antibodies (mAb10H5) immunoprecipitated a large fraction (about 60%) of ^{125}I-ω conotoxin GVIA-prelabeled 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. Secondly p35, p58, and p105 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 sections.

PURIFICATION AND POLYPEPTIDE COMPOSITION OF N-TYPE CALCIUM CHANNELS

Purification Procedure

Rat brain membranes were washed with urea and potassium phosphate to remove extrinsic proteins and then extracted with CHAPS. Membranes were prelabeled with ^{125}I-ω conotoxin GVIA at a concentration calculated to give 0.5% receptor occupancy, thus providing a tracer which allowed subsequent assay of channel density throughout the procedure. The solubilized proteins were sequentially fractionated over three columns. N-type channels were bound to Chelating Sepharose Fast Flow loaded with cobalt ions. The resin was washed with buffers containing imidazole and eluted by chelating the immobilized cobalt ions with EDTA. The eluate was loaded onto a heparin–Ultrogel column, washed with 0.4 M NaCl, and recovered with 0.7 M NaCl. Pooled fractions were applied to mAb10H5-Sepharose and, following a 1 M NaCl wash,

proteins were eluted by a step to pH 10.5. After these three steps 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 analyzed 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 (Leveque *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).

Alpha$_1$ Subunits

The protein band migrating at 250 kDa was identified as the α_1B subunit. Photoaffinity labeling 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, which are 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-terminal 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 about 80% of ^{3}H-PN200-110 binding sites from brain membrane extracts, indicating that they recognize a significant fraction of neuronal 1, 4-dihydropyridine-sensitive calcium channels. These antibodies did not react with Western blots of purified N-type channels, which is 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) although surprisingly this protein was not affinity labeled by photoreactive ω conotoxin GVIA derivatives, suggesting that it does not contain the pharmacological site considered to be diagnostic for N-type calcium channels.

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 (Leveque *et al.*, 1994). It may thus constitute a truncated form of the N-type channel lacking carboxy terminal domains. In Western blots of brain homogenates prepared in conditions which minimize artefactual proteolysis the 100-kDa band appears to be the major immunoreactive species detected with BINt antibodies (C. Leveque, M. Seagar, and M. Takahashi, unpublished observations). A transcript with an internal deletion defining a calcium channel α_1 subunit with only two of the usual four domains has been characterized in skeletal muscle (Malouf *et al.*, 1992). More recently Campbell and coworkers have identified the 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 (K. Campbell, personal communication). It may be interesting to determine whether truncated α_1 subunits lacking domains III and IV can assemble noncovalently to form functional homodimeric channels with novel properties.

Auxiliary Subunits

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 dihydropyridine receptor. α_2 subunits from brain and skeletal, smooth, and cardiac muscle all display a shift in electrophoretic mobility upon disulfide reduction due to dissociation of the 30kDa δ polypeptide. We have been unable to demonstrate analogous behavior of the 140-kDa protein associated with the N-type calcium channel. However, Witcher and colleagues have reported that antibodies raised against the 140 kDa protein cross-react with skeletal muscle subunits (Witcher *et. al.*, 1993).

The 70- and 58-kDa protein bands may contain different β subunit isoforms. Scott *et al.* (1996) have recently examined in detail the question as to whether certain types of β subunits are specifically associated with N-type channels. Their analysis clearly points to a surprising heterogeneity with channels associated with either β1b, β3, and β4 subunits. β1b runs at about 72 kDa, whereas β3 and β4 subunits have similar molecular weight comigrating in SDS PAGE at about 57 kDa.

Associated Proteins

Proteins of p105, p58, and p35, initially identified in Western blots of brain membranes as antigens recognized by monoclonal antibodies selected for their ability to immunoprecipitate N-type channels, were also detected in purified channel preparations. However, the stability of their association with calcium channel subunits was variable as ascertained by their differing degrees of dissociation during the purification procedure.

p105—Na/K ATPase α Subunit

mAb9A7 was selected as an antibody that maximally immunoprecipitated about 20% of solubilized ^{125}I-ω conotoxin GVIA receptors, indicating that only a small proportion of N-type channels are associated with this protein. MAb9A7 recognizes a diffuse 105-kDa band in Western blots of brain membranes and other tissues (heart, skeletal muscle, kidney, etc.). The antigen was purified from brain membranes by immunoaffinity chromatography on mAb9A7-Sepharose and SDS PAGE. Sequencing of tryptic peptides identified this protein as the α subunit of the Na/K ATPase. The α subunit of the Na/K ATPase has also been identified (Choi *et al.*, 1997) in preparations of N-type channel purified by another procedure (McEnery, 1991). The functional significance of interactions between the voltage-gated calcium channels and the Na/K ATPase α subunit is unknown.

p58—Synaptotagmin

Chronologically the first mAb produced by immunization with synaptic membranes and screening for ^{125}I-ω conotoxin GVIA receptor immunoprecipitation 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 (Leveque *et al.*, 1992). Sequencing of the selected clones and microsequencing of the immunoaffinity-purifed protein revealed that p58 was identical to a protein cloned by Südhof and colleagues, and named synaptotagmin (Perin *et al.*, 1990), although it is still occasionally refered to as p65, from its initial description as an antigen in synaptic and neurosecretory vesicles (Matthew *et al.*, 1981). Synaptotagmin has a short intravesicular N-glycosylated amino-terminal domain, a single transmembrane region (TMR), and a larger carboxy terminal cytoplasmic tail which 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 are neuron specific and others ubiquitous (reviewed by Sudhof and 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 (unpublished observations).

Synaptotagmin is associated with about 50% of freshly solubilized N-type channels (Takahashi *et al.*, 1991; Leveque *et al.*, 1992). Interaction is, however, relatively labile and significant dissociation occurred during purification (Leveque *et al.*, 1994).

p35—Syntaxin

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 about 50% of solubilized ^{125}I-ω conotoxin GVIA receptors. When the two types of antibody were added together, immunoprecipitation was not additive, suggesting that about 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), of which two variants are known (1A and 1B), is now generally used. Syntaxin 1 is predominantly expressed in the plasma membrane where it is anchored by a C-terminal TMR. The cytoplasmically oriented N-terminal region contains three heptad repeats, which are characteristic of proteins forming coiled-coil structures. These regions may also form amphipathic α-helixes, which are thought to be crucial in protein–protein interactions. Five syntaxins (1–5) have now been characterized in the rat. Syntaxins 1A and B are specifically neuronal, while syntaxins 2–5 are ubiquitous (Bennett *et al.*, 1993).

Like synaptotagmin, syntaxin 1 is a relatively major constituent of the nerve terminal. Consequently only a small fraction of the total syntaxin 1 can be associated with N-type calcium channels. Interaction of syntaxin 1 with the N-type calcium channel was found to be very stable. Washing (1 M NaCl) and elution (pH 10.5) of N-type calcium channels bound to mAb10H5-Sepharose columns using relatively harsh physicochemical conditions did not dissociate the binary syntaxin *1* channel complex although significant amounts of synaptotagmin were found to leak from the column. This observation, together with reports that purified recombinant synaptotagmin and syntaxin bind to each other in solution (Bennett *et al.*, 1992), lead us to propose that syntaxin may provide an indirect link between synaptotagmins and the N-type calcium channel (Leveque *et al.*, 1994). More recent work (reviewed by Sheng and Catterall in this volume) suggests that this preliminary conclusion was incorrect as both syntaxin and synaptotagmin can interact directly with calcium channel α_1A or α_1B subunits.

THE SNARE HYPOTHESIS AND CALCIUM-DEPENDENT EXOCYTOSIS

Retrospectively, by far the most exciting finding that resulted from our attempts to purifiy and characterize native N-type calcium channel complexes from rat brain was the totally unsuspected association between channels, syntaxin and synaptotagmin. These two latter proteins were discovered as calcium channel-associated antigens concomittantly with their independent characterization as key components in the exocytosis of synaptic vesicles.

The pioneering work of Rothman and collaborators lead in the early 1990's to formulation of the SNARE hypothesis, which provides a general model for targeted vesicular transport (reviewed by Rothman, 1994). Briefly transport vesicles (e.g., synaptic vesicles) convey cargo (e.g., neurotransmitters) to a target compartment (e.g., the synaptic cleft). Interaction between the transport vesicle and the appropriate target membrane requires formation of a molecular complex involving membrane-anchored proteins from both compartments: v-SNAREs in the vesicle and t-SNAREs in the target. [Glossary: SNAREs (*SN*ap *RE*ceptors) bind SNAPs (*S*oluble *N*sf *A*ttachment *P*rotein*s*) which are adaptors for NSF (*N*-ethylmaleimide *S*ensitive *F*usion protein). Following association with the SNARE complex via SNAPs, NSF hydrolyzes ATP and promotes fusion between vesicle and target membranes, although the precise mechanisms await clarification.]

Synaptic vesicle docking and fusion is thought to depend on the assembly of a v-SNARE [VAMP (vesicle-associated membrane protein or synaptobrevin)] and two t-SNAREs [syntaxin 1 and SNAP-25 (synaptosome-associated protein of 25 kDa, no relation to the SNAPs defined above) (Sollner *et al*, 1993a)] to form an extremely stable heterotrimeric synaptic core complex (Hayashi *et al.*, 1994). The primary importance of these proteins in synaptic exocytosis became evident when it was recognized that they are specific substrates for clostridial neurotoxins which are potent inhibitors of neurotransmitter release. Tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) light chains act as metalloproteases which cleave specific SNARE proteins. For example, the TeNT light chain

attacks VAMP, while light chains associated with different BoNT serotypes clip, for example, VAMP (BoNT B), syntaxin 1 (BoNT C1), or SNAP-25 (BoNT A) (reviewed by Niemann *et al.*, 1994). Toxin action on individual SNARE proteins compromises the stability of the subsequently formed core complex, presumably abolishing its ability to mediate exocytosis (Hayashi *et al.*, 1995; Pellegrini *et al.*, 1995). The general relevance of the SNARE hypothesis to vesicular transport is illustrated by the fact that homologues of these three proteins act at multiple subcellular sites (e.g., ER to Golgi transport), and throughout evolution (e.g., the secretory pathway in yeast involves syntaxin and VAMP homologues) (reviewed by Hay and Scheller, 1997).

While the SNARE hypothesis provides a basic framework for understanding constitutive exocytosis, regulated exocytosis apears to require more sophisticated machinery. Synaptic release of neurotransmitters involves a finely regulated exocytotic mechanism, which can be initiated, with a delay of the order of hundreds of microseconds, by highly localized calcium transients. Current evidence suggests that synaptotagmins 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). They associate with the synaptic core complex (Sollner *et al.*, 1993b) and display calcium-dependent interactions with syntaxins (Li *et al.*, 1995). Thus 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 post-natal 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 synaptotagmin 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.

However, other calcium-binding proteins are certainly involved in exocytosis. Overexpression of Doc2 and rabphilin3A stimulate potassium-evoked calcium-dependent release of co-expressed growth hormone from adrenal chromaffin and PC12 cells, and transfection of these genes in an antisense orientation inhibits secretion (Orita *et al.*, 1996; Chung *et al.*, 1995). More recently, a novel calcium-binding protein CAPS has also been shown to be required for calcium-dependent catecholamine release from PC12 cells (Ann *et al.*, 1997).

We summarize the two main points reviewed so far: (i) synaptotagmin and syntaxin appear to be specifically associated with N-type calcium channels and (ii) a protein complex containing synaptotagmin, syntaxin, SNAP-25, and VAMP is thought to constitute the molecular correlate of a synaptic vesicle docked at the plasma membrane and awaiting the calcium influx that will trigger fusion. It is thus tempting to postulate attachment of the fusion-competent complex at the appropriate source of calcium entry, which would require that SNAP-25 and VAMP be also associated with N-type calcium channels. This hypothesis was tested in immunoprecipitation experiments and extended to include the possible interaction of exocytotic complexes with P/Q-calcium channels.

P/Q-type calcium channels are blocked by ω agatoxin IVA and ω conotoxin MVIIC (Olivera *et al.*, 1994). Synaptic transmission in many areas of the central nervous system is only partially inhibited by concentrations of ω conotoxin GVIA which saturate presynaptic N-type calcium channels. Subsequent addition of either ω agatoxin IVA or ω conotoxin MVIIC then leads to significant additional inhibition, and in certain cases complete blockade of neurotransmission, whereas L-type calcium channel antagonists are not effective (Takahashi and Momiyama, 1993; Wheeler *et al.*, 1994). These observations imply that both N-type and P/Q-type, but not L-type, calcium channels are involved in triggering the fusion of synaptic vesicles. At some synapses, such as the parallel fiber/Purkinje cell synapse of the cerebellum or the mammalian neuromuscular junction, P/Q-type calcium channels clearly predominate.

ASSOCIATION OF SNARE COMPLEXES WITH P/Q AND N-TYPE CALCIUM CHANNELS

The association of complexes containing synaptic proteins implicated in exocytosis with native calcium

channels can be quantitatively evaluated in immunoprecipitation experiments using detergent-solubilized calcium channels prelabeled with an appropriate radioligand. These methods have been employed to study the interaction of trimeric core complexes with N-type calcium channels labeled with 125I-ω conotoxin GVIA and P/Q-type calcium channels labeled with 125I-ω conotoxin MVIIC.

The use of 125I-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 125I-ω conotoxin MVIIC label high-affinity sites (K_D = 1 nM) which 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 125I-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 cysteine string protein did not capture more P/Q-type calcium channels than nonimmune IgG (Martin-Moutot *et al.*, 1996).

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 does not bind calcium ions nor display calcium-dependent binding to syntaxins (Li *et al.*, 1995). Although it was initially supposed that synaptotagmin interacted indirectly with the calcium channel, recent experiments with recombinant proteins indicate direct binding of synaptotagmins to "synprint" peptides from the cytoplasmic loops linking homologous domains II–III of α_1A (Charvin *et al.*, 1997) or α_1B (Sheng *et al.*, 1997; Wiser *et al.*, 1997) subunits (see discussion by Sheng and Catterall in this volume).

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, Seagar and Takahashi, unpublished results).

It is worth pointing out that anti-α_1A subunit (P/Q-type channel) antibodies do not co-immunoprecipitate detectable levels of 125I-ω conotoxin GVIA receptors (N-type channels), and that anti-α_1B subunit (N-type channel) antibodies do not capture 125I-ω conotoxin MVIIC receptors (P/Q-type channels), which argues against association of an N-type and a P/Q-type calcium channel in the same synaptic protein complex. Thus, although it has been suggested that N- and P/Q-type channels may be localized in proximity and cooperate to trigger release from a single exocytotic site (Wheeler *et al.*, 1994), our data do not provide any support for molecular interaction of the two channel types.

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).

CO-LOCALIZATION OF SNARES AND CHANNELS

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 which has a particularly convenient structure. The presynaptic nerve terminal runs parallel to the muscle fiber 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 post-synaptic 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 post-synaptic nAChRs labeled with rhodamine-tagged α-bungarotoxin (R-αBuTx). The distribution of presynaptic proteins was therefore studied using indirect immunofluorescence with FITC, in a double labeling protocol with R-αBuTx.

Two different perpendicular orientations of the neuromuscular junction relative to the axis of observation were analyzed. In both orientations syntaxin 1, SNAP-25, and calcium channels (labeled with BINT 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 (about 60 nm) is smaller than the limit of resolution of the confocal microscope. The fact that FITC labeling was truly presynaptic was verified by immunostaining and then pulling the collagenase-treated nerve terminal free of the muscle fiber. 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 and 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.

CONCLUSIONS

A first comment concerns the techniques which initially pointed to interactions between calcium channels and the proteins which constitute the exocytotic machinery. Attempts to raise mAbs against calcium channels, which are minor constituents of the synaptic membrane preparation used to immunize mice, did not completely achieve the primary objective. However, this approach did identify several major membrane proteins that interact with the chosen target and uncovered a potentially novel mechanism with important functional consequences. Research in the ion channel field is now increasingly directed at understanding how channels are targeted to, anchored at, and function in a defined membrane domain. In this context, a convenient specific marker for the channel exists, analogous methods may be generally applicable to screening for

and identifying components of the molecular environment in which our favorite proteins function.

Why do calcium channels interact with proteins thought to be located at the interface between a docked synaptic vesicle and the plasma membrane? Synaptic vesicle fusion requires relatively high (50–100 μM) internal calcium concentrations, that 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. These domains have in fact 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 (Llinás *et al.*, 1992). The injection of fast calcium chelators such as BAPTA into nerve terminals reduces synaptic responses, whereas slower buffers such as EGTA do not (Adler, 1991; von Gersdorff and Matthews, 1994). Modeling of ion diffusion and chelator binding kinetics in fact predict that BAPTA but not EGTA can significantly buffer calcium within microdomains at the channel mouth. The presynaptic effects of chelators are thus compatible with the idea that the calcium-binding protein that triggers exocytosis is located close to the cytoplasmic opening of the channel pore (Matthews *et al.*, 1996). Therefore 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. An alternative negative formulation would be that it would ensure that primed fusion-ready vesicles that are not linked to channels are much less likely to release their contents when an action potential invades the nerve terminal. It follows on from this hypothesis that regulatory processes which modulate interactions beween channels and synaptic core complexes could play an important role in the plasticity of presynaptic events.

Functional evidence is necessary to test these hypotheses. This requires detailed molecular analysis of the domains involved in calcium channel-synaptic protein interactions, and model synaptic systems in which these interactions can be specifically perturbed in order to evaluate their role in transmitter release.

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