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Physical Link and Functional Coupling of Presynaptic Calcium Channels and the Synaptic Vesicle Docking/ Fusion Machinery

Zu-Hang Sheng,[1] Ruth E. Westenbroek,[2] and William A. Catterall[2]

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N- and P/Q-type calcium channels are localized in high density in presynaptic nerve terminals and are crucial elements in neuronal excitation–secretion coupling. In addition to mediating Ca^{2+} entry to initiate transmitter release, they are thought to interact directly with proteins of the synaptic vesicle docking/fusion machinery. As outlined in the preceding article, these calcium channels can be purified from brain as a complex with SNARE proteins which are involved in exocytosis. In addition, N-type and P/Q-type calcium channels are co-localized with syntaxin in high-density clusters in nerve terminals. Here we review the role of the synaptic protein interaction (synprint) sites in the intracellular loop II–III (L_{II-III}) of both α_{1B} and α_{1A} subunits of N-type and P/Q-type calcium channels, which bind to syntaxin, SNAP-25, and synaptotagmin. Calcium has a biphasic effect on the interactions of N-type calcium channels with SNARE complexes, stimulating optimal binding in the range of 10–20 μM. PKC or CaM KII phosphorylation of the N-type synprint peptide inhibits interactions with native brain SNARE complexes containing syntaxin and SNAP-25. Introduction of the synprint peptides into presynaptic superior cervical ganglion neurons reversibly inhibits EPSPs from synchronous transmitter release by 42%. At physiological Ca^{2+} concentrations, synprint peptides cause an approximate 25% reduction in transmitter release of injected frog neuromuscular junction in cultures, consistent with detachment of 70% of the docked vesicles from calcium channels based on a theoretical model. Together, these studies suggest that presynaptic calcium channels not only provide the calcium signal required by the exocytotic machinery, but also contain structural elements that are integral to vesicle docking, priming, and fusion processes.

KEY WORDS: Presynaptic calcium channels; synaptic vesicle; fusion; synprint.

INTRODUCTION

Neurotransmitter release is initiated by influx of Ca^{2+} through voltage-dependent calcium channels within 200 μs of the action potential arriving at the synaptic terminal (Robitaille *et al.*, 1990; Berrett and Stevens, 1972), where clusters of presynaptic calcium channels are thought to supply Ca^{2+} to initiate release (Pumplin *et al.*, 1981; Pumplin, 1983; Zucker, 1993). Exocytosis of synaptic vesicles requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half-maximal activation at 190 μM (Heidelberger *et al.*, 1994; von Gersdorff and Matthews, 1994). The brief rise in Ca^{2+} concentration to the level necessary for exocytosis likely occurs only in proximity to the calcium channels (Cope and Mendell, 1982; Llinás *et al.*, 1991; Stanley, 1993), since intracellular calcium concentration falls off steeply as a function of distance away from the calcium channels. This limits the distance from Ca^{2+} entry site to Ca^{2+}-binding sites.

[1] Synaptic Function Unit, NINDS, NIH, Building 36, Rm. 5A23, Bethesda, MD 20892.

[2] Department of Pharmacology, Box 357280, University of Washington, Seattle, Washington 98195-7280.

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Increasing evidence supports the notion that the maintenance of such a critical intermolecular distance must involve a physical link between the release mechanism and the presynaptic calcium channels.

Major progress has been made toward understanding the molecular mechansims that underlie Ca^{2+}-dependent exocytosis by identifying proteins that are involved in the vesicle docking/fusion process at presynaptic nerve terminals and analyzing their interactions (reviewed by Bajjalieh and Scheller, 1995; Südhof, 1995). Vesicle docking and fusion are mediated by a stable core-complex of proteins including the synaptic vesicle protein VAMP/synaptobrevin (Trimble *et al.*, 1988) and the plasmalemmal proteins syntaxin and SNAP-25 (Bennett *et al.*, 1992; Yoshida *et al.*, 1992; Oyler *et al.*, 1989; Söllner *et al.*, 1993; Calakos *et al.*, 1994; O'Conner *et al.*, 1993; Hayashi *et al.*, 1994; Chapman *et al.*, 1994). Synaptotagmin (Matthew *et al.*, 1981), a synaptic vesicle protein, binds Ca^{2+} (Perin *et al.*, 1990; Li *et al.*, 1995a), and interacts with syntaxin in a Ca^{2+}-dependent manner (Li *et al.*, 1995b; Chapman *et al.*, 1995). It is thought to serve as a Ca^{2+} sensor for fast, Ca^{2+}-dependent neurotransmitter release (Elferink *et al.*, 1993; Bommert *et al.*, 1993; Geppert *et al.*, 1994; Broadie *et al.*, 1994; Littleton *et al.*, 1993; Nonet *et al.*, 1993).

Recent immunochemical studies in several laboratories have indicated a tight association of syntaxin and synaptotagmin with both N- and P/Q-type calcium channels (Bennett *et al.*, 1992; Lévêque *et al.*, 1992, 1994; Yoshida *et al.*, 1992; O'Conner *et al.*, 1993, El Far *et al.*, 1995; see previous article), implicating both types of calcium channels as components of synaptic vesicle docking/fusion machinery. Detailed structural characterization of the interactions among the neuronal calcium channels and different components of the exocytotic apparatus and how they are regulated by Ca^{2+} or modified through second messenger-activated pathways will shed light on the molecular mechanisms of neurotransmitter release. This review article is focused on our recent results on the localization of specific calcium channel types in nerve terminals and on the binding site in presynaptic calcium channels that is responsible for their interaction with SNARE proteins.

LOCALIZATION OF CALCIUM CHANNELS IN PRESYNAPTIC TERMINALS

Calcium channels are a complex of five subunits: a principal pore-forming α_1 subunit of 190–250 kDa in association with a disulfide linked $\alpha_2\delta$ dimer of 170 kDa, an intracellular β subunit of 55–72 kDa, and, in skeletal muscle, a transmembrane γ subunit of 33 kDa (Takahashi and Momiyama, 1987; Catterall, 1995). The primary structures of five distinct classes of neuronal calcium channel α_1 subunits have been cloned and characterized, termed classes A, B, C, D, and E. Cloned neuronal α_{1B} and α_{1A} encode N-type and P/Q-type calcium channels, respectively (Dubel *et al.*, 1992; Mori *et al.*, 1991; Sather *et al.*, 1993; Starr *et al.*, 1991; Stea *et al.*, 1993; Westenbroek *et al.*, 1992; Williams *et al.*, 1992a; Zhang *et al.*, 1993), whereas the α_{1C} and α_{1D} subunits are components of L-type channels. Both ω-conotoxin GVIA-sensitive N-type and ω-agatoxin IVA- and ω-conotoxin MVIIC-sensitive P/Q-type calcium channels participate in neurotransmitter release at central and peripheral synapses (Tsien *et al.*, 1988; Wu and Saggau, 1994; Mintz *et al.*, 1995). Consistent with this idea, the α_{1B} subunits of N-type calcium channels (Westenbroek *et al.*, 1992, 1995) and the α_{1A} subunits of P/Q-type calcium channels (Westenbroek *et al.*, 1995) are localized at low density in dendrites and at high density in presynaptic nerve terminals of many neurons (see cover photo). Calcium channels are localized along the dendrites of cerebellar Purkinje cells and in high density in synapses formed on them (cover photo, Fig. 1A, C). The SNARE protein syntaxin is localized in high-density clusters in the same area (Fig. 1B, D). The merged images reveal co-localization of the clusters of syntaxin and calcium channels as yellow dots (Fig. 1C, F). The high-density clusters of P/Q-type calcium channels in presynaptic nerve terminals are precisely co-localized with high-density clusters of the SNARE protein syntaxin (Fig. 1, Westenbroek *et al.*, 1995). Similar results are obtained with antibodies against α_{1B} (Westenbroek *et al.*, 1995). Thus, the α_{1A} and α_{1B} subunits are specialized for neurotransmitter release and co-localized with the SNARE proteins which carry out synaptic vesicle docking and exocytosis. What are the molecular specializations which allow these channels to bind SNARE proteins and efficiently initiate transmitter release and how important is their association with SNARE proteins in the release process?

THE SYNAPTIC PROTEIN INTERACTION (SYNPRINT) SITE ON N-TYPE CALCIUM CHANNELS

The amino acid sequences of all cloned α_1 subunits share overall structural features of four hydropho-

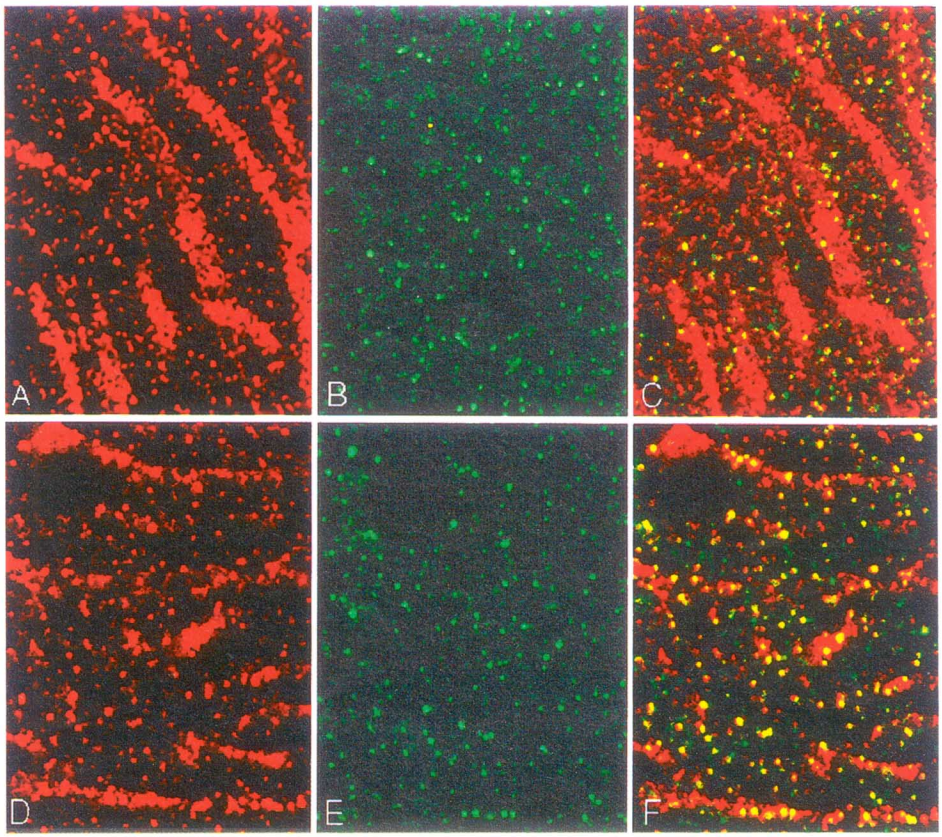

Fig. 1. Co-localization of P/Q-type calcium channels and syntaxin. Saggital sections were cut through the cerebellum of the adult rat brain and stained with anti-CNA1 antibodies against the α_{1A} subunits of P/Q-type calcium channels and monoclonal antibody 10H5 against rat syntaxin as described previously (Westenbroek *et al.*, 1995). The stained sections were viewed with a BioRad M60 confocal microscope. Dendrites of Purkinje cells and the nerve terminals innervating them are shown. (A, D) Molecular layer of the cerebellum stained with anti-CNA1 antibodies against P/Q-type calcium channels and visualized with Texas red-conjugated second antibody. (B, E) Same section stained with 10H5 anti-syntaxin antibodies and visualized with fluorescein-conjugated second antibody. (C, F) Merged image of panels A, B and D, E. Yellow dots reveal co-localization of the P/Q-type calcium channels and syntaxin in active zones in presynaptic terminals.

bic homologous transmembrane domains (I–IV) that are linked by intracellular hydrophilic loops of various lengths (Fig. 2). The homologous domains exhibit a high degree of sequence conservation between subtypes, but the cytoplasmic loops linking the domains are highly divergent. The most attractive candidates for synaptic protein interactions are the intracellular linkers and the cytoplasmic N- and C-terminals. The sequence variations of these cytoplasmic segments may reflect the capacity for distinct, functionally significant interactions of both N- and P/Q-type channels with cytoplasmic domains of synaptic proteins involved in vesicle docking/fusion processes.

To identify and characterize the cytoplasmic loops of the N-type channels interacting with synaptic proteins, a series of hexahistidine-tagged (His)-fusion proteins expressing various cytoplasmic segments of α_{1B} and recombinant syntaxin 1A, SNAP-25, or VAMP-2 fused to glutathione S-transferase (GST) were generated. The recombinant GST-fusion proteins coupled to glutathione Sepharose beads were used as an affinity matrix to screen His-fusion proteins for specific binding. The *in vitro* binding studies showed that both syntaxin 1A and SNAP-25, but not VAMP-2, specifically interact with the cytoplasmic loop (L_{II-III}) between homologous domains II and III of the α_{1B} subunit of the class B N-type calcium channels from rat brain (Sheng *et al.*, 1994, 1996). These interactions are mediated by a specific segment of amino acid sequence (residues 718–963). We suggest the term

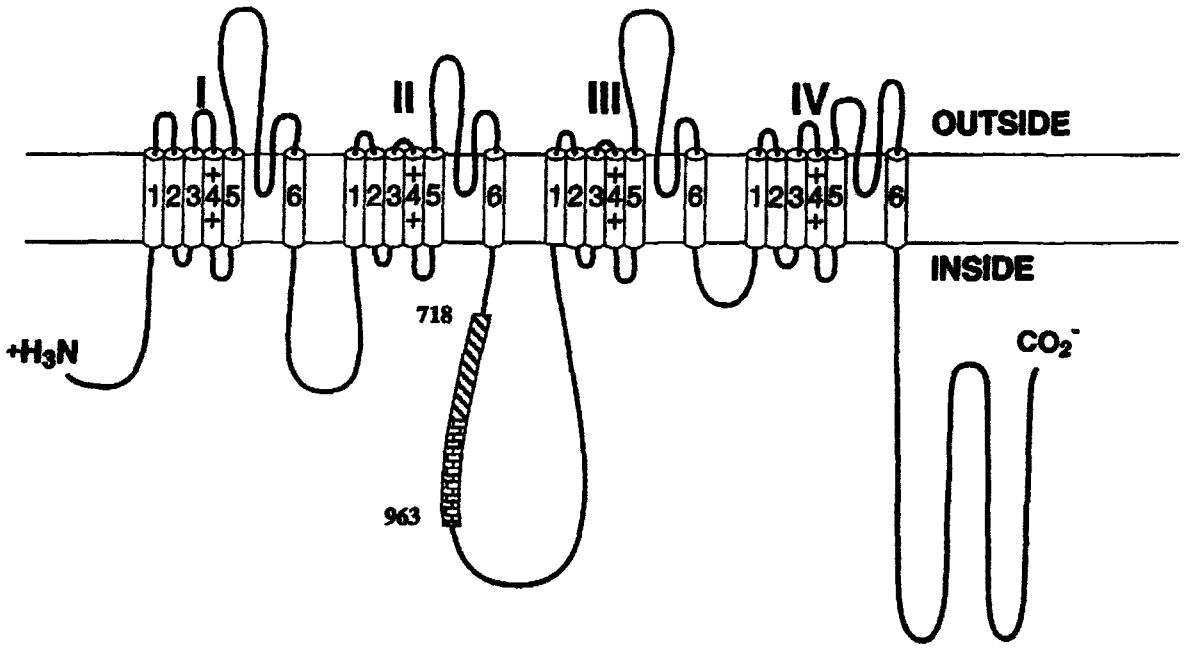

Fig. 2. The synaptic protein interaction (synprint) sites on both N- and P/Q-type calcium channels. Predicted topological structure of the α_1 subunits of class B N-type and class A P/Q-type calcium channels with synprint site in the intracellular loop between homologous domains II and III (L_{II-III}) indicated by the rectangle boxes. Amino acid positions of N- and P/Q-type synprint sites are defined in the regions between 718–963 and 722–1036, respectively.

"synprint" for this *syn*aptic *p*rotein *int*eraction site on the calcium channels (Fig. 2). The synprint site of N-type calcium channels contains two adjacent binding regions, with weak binding to the first subsite and tighter binding to the second subsite. The synprint peptide can specifically block co-immunoprecipitation of native N-type calcium channels with syntaxin 1, indicating that this binding site is required for stable interaction of these two proteins. This interaction takes place with the C-terminal one-third of syntaxin (181–288), suggesting that neuronal calcium channels bind to syntaxin 1A at a C-terminal site near the intracellular surface of the plasma membrane (Sheng *et al.*, 1994; Rettig *et al.*, 1996).

SPECIFIC INTERACTIONS OF SYNPRINT SITES FROM THE ISOFORMS OF α_{1A} WITH SNARE PROTEINS

Based on studies of the effects of calcium channel blockers on synaptic transmission, it is generally agreed that both ω-CTx-GVIA-sensitive N-type and ω-Aga-IVA-sensitive P/Q-type calcium channels play a role in controlling synaptic transmission in the mammalian central nervous system (Hirning *et al.*, 1988; Mintz *et al.*, 1992a, 1992b; Uchitel *et al.*, 1992; Luebke *et al.*, 1993; Takahashi and Momiyama, 1993; Turner *et al.*, 1993; Wheeler *et al.*, 1994). In contrast to N-type calcium channels, the corresponding synprint segment of L_{II-III} from the rbA isoform of α_{1A} (Starr *et al.*, 1991) does not bind to syntaxin (Sheng *et al.*, 1994). This result raises the possibility that P/Q-type calcium channels might have a distinct synprint site that is unable to interact with syntaxin in the transmitter release process.

Examination of the amino acid sequence differences between the BI and rbA isoforms of α_{1A} (Mori *et al.*, 1991; Starr *et al.*, 1991) shows considerably lower identity (78%) in L_{II-III} loop than in the remainder of the protein (>98%). The distinctly different levels of amino acid sequence identity in these regions suggest that this loop may be subject to alternative splicing. In support of this, Sakurai *et al.* (1995) have found that α_{1A} isoforms with amino acid sequences characteristic of L_{II-III} from rbA and BI are present in both rat and rabbit brain using site-directed antibodies. Thus, the synprint region of the α_{1A} subunit is subject to alternative splicing to yield at least two isoforms.

Binding data with fusion proteins containing the intracellular loop L_{II-III} of these two α_{1A} isoforms demonstrates binding with different affinities to the presyn-

aptic proteins syntaxin and SNAP-25 (Rettig *et al.*, 1996). The BI isoform has higher affinity for binding to both syntaxin and SNAP-25 than the rbA isoform. Under *in vitro* binding conditions, binding of rbA to SNAP-25 is clearly detected, while binding to syntaxin is not (Rettig *et al.*, 1996). If these binding interactions are required for efficient coupling of Ca^{2+} influx with synaptic vesicle fusion, these data may imply that a neuron could modulate the efficiency of synaptic transmission by regulating the expression of different isoforms of a single class A calcium channel gene. Consistent with this idea, the BI and rbA isoforms of α_{1A} subunits are differentially distributed in rat brain (Sakurai *et al.*, 1995).

Similar to the synprint site of N-type channels, the synprint site from the BI isoform of α_{1A} involves two adjacent segments of the intracellular loop connecting domains II and III between amino acid residues 722 and 1036 (Fig. 2), and it binds specifically to the C-terminal one-third of syntaxin 1A (amino acid 181–288), whereas no binding to the N-terminal two-thirds of syntaxin 1A occurs (Rettig *et al.*, 1995). These interactions of the BI synprint peptide with both syntaxin and SNAP-25 are competitively blocked by the corresponding synprint region of the N-type channels, indicating that these two channels bind to overlapping or identical regions of syntaxin as well as SNAP-25 (Rettig *et al.*, 1996). Immunoprecipitation studies show that an antibody against a fusion protein with the amino acid sequence of L_{II-III} of the rbA isoform of α_{1A} coimmunoprecipitates SNAP-25 and syntaxin. This immnoprecipitation was blocked by preincubation of the antibody with the corresponding fusion protein, supporting specific associations of syntaxin and SNAP-25 with class A calcium channels by interactions with a site in L_{II-III} of α_{1A} (Rettig *et al.*, 1996). Collectively, these results provide a molecular basis for a physical coupling of neuronal N-type and P/Q-type calcium channels with synaptic vesicle docking/fusion complexes, enabling tight structural and functional association of calcium entry sites and neurotransmitter release sites.

INTERACTIONS OF THE SYNPRINT SITE WITH SYNAPTOTAGMIN

The vesicle SNARE protein synaptotagmin is thought to serve as the calcium sensor for fast neurotransmitter release. Immunochemical studies show that it is associated with purified N-type and P/Q-type calcium channels, similar to syntaxin and SNAP-25 (Leveque *et al.*, 1992, 1994). The interaction of synaptotagmin with the synprint sites of N-type and P/Q-type calcium channels was measured using similar methods as described above (Sheng *et al.*, 1997; Charvin *et al.*, 1997). Synaptotagmin forms a specific complex with synprint sites from both α_{1A} and α_{1B}, and these two synprint peptides compete for binding to synaptotagmin. Moreover, binding of synaptotagmin to the synprint site of α_{1B} prevents binding of synaptotagmin to syntaxin, an interaction that is thought to be critical for initiation of exocytosis (Sheng *et al.*, 1997). Thus, syntaxin may bind sequentially to calcium channels and then to synaptotagmin during the release process. The different calcium dependence of these binding interactions (see below) may serve to order them in the release pathway.

PHYSIOLOGICAL SIGNIFICANCE OF THE INTERACTION OF N-TYPE CALCIUM CHANNELS WITH SNARE PROTEINS IN SYNAPTIC TRANSMISSION

The structural and functional coupling between calcium entry sites and release sites of docked synaptic vesicles would ensure that neurotransmitter release is triggered rapidly when the action potential invades the nerve terminal (Pumplin *et al.*, 1981; Pumplin, 1983; Stanley, 1993). The Ca^{2+} influx rapidly elevates the calcium concentration to 200–300 μM in microdomains of approximately 0.3 μm^2 in the presynaptic terminal near release sites (Llinás *et al.*, 1992; Zucker, 1993). This transient calcium influx triggers rapid vesicle fusion and neurotransmitter release through Ca^{2+} binding to receptor(s) on the exocytotic apparatus (Fig. 3). Following calcium channel closure, diffusion and sequestration rapidly decrease the calcium concentration at the release site to subthreshold levels, terminating the exocytotic process. Although biochemical data support the hypothesis that there is a tight association between calcium channels and exocytotic apparatus, which is consistent with current knowledge about synaptic proteins and calcium channels, the functional roles of the synaptic protein–calcium channel interactions in neuronal calcium-activated exocytosis remain to be determined.

Our biochemical results predict that peptides containing synprint sites, if injected into neurons, would inhibit synaptic transmission by competitively binding to syntaxin and SNAP-25. This subsequently prevents their binding to presynaptic calcium channels, thus increasing the distance between docked vesicles and

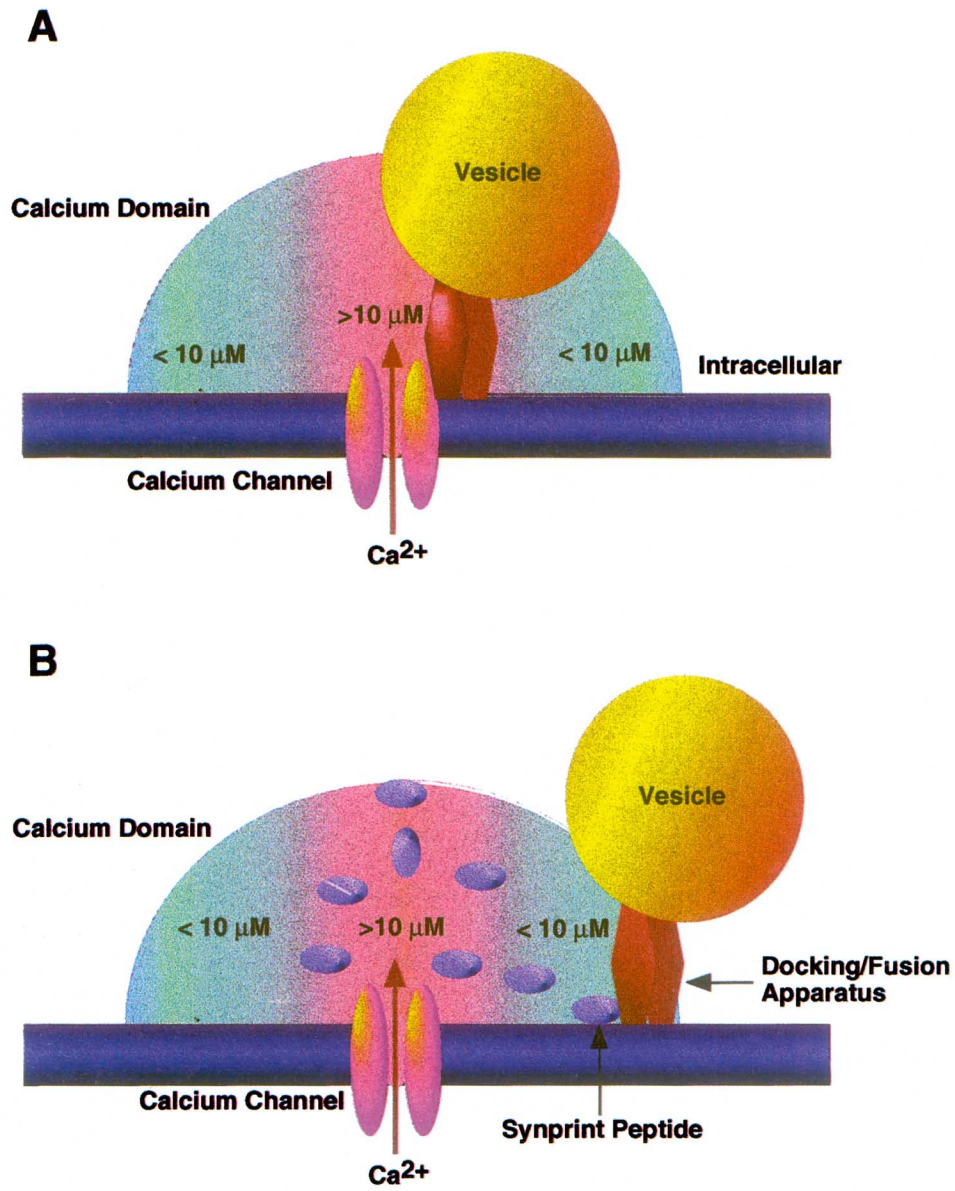

Fig. 3. The proposed inhibitory role of injected peptides containing N-type synprint site on neurotransmission of SCG neurons and neuromuscular junctions. (A) The physical link between N-type calcium channels and synaptic vesicle docking/fusion apparatus. Exocytosis of synaptic vesicles requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half-maximal activation at 190 μM (Heidelberger *et al.*, 1994; von Gersdorff and Matthew, 1994). The brief rise in Ca^{2+} concentration to the level necessary for exocytosis likely occurs only in proximity to the calcium channels (Llinás *et al.*, 1991; Cope and Mendell, 1982; Stanley, 1993), since the intracellular calcium concentration falls off steeply as a function of distance away from the calcium channels. Thus, when binding to the synprint site, synaptic vesicles are docked in proximity to Ca^{2+} entry sites. Upon Ca^{2+} influx, the fusion apparatus is activated for rapid, Ca^{2+}-dependent, synchronous synaptic transmission. (B) The peptides containing the synprint site competitively block the physical link between N-type calcium channels and synaptic vesicle docking/fusion apparatus, and subsequently remove pre-docked vesicles away from calcium entry sites. This decreases the degree of efficiency by shifting Ca^{2+}-dependence to higher values. Rapid, synchronous synaptic transmission is inhibited, while late, asynchronous EPSPs and paired-pulse facilitation are increased, consistent with the conclusion that synaptic vesicles are shifted from a pool primed for synchronous release to a pool not optimally primed or positioned for synchronous release.

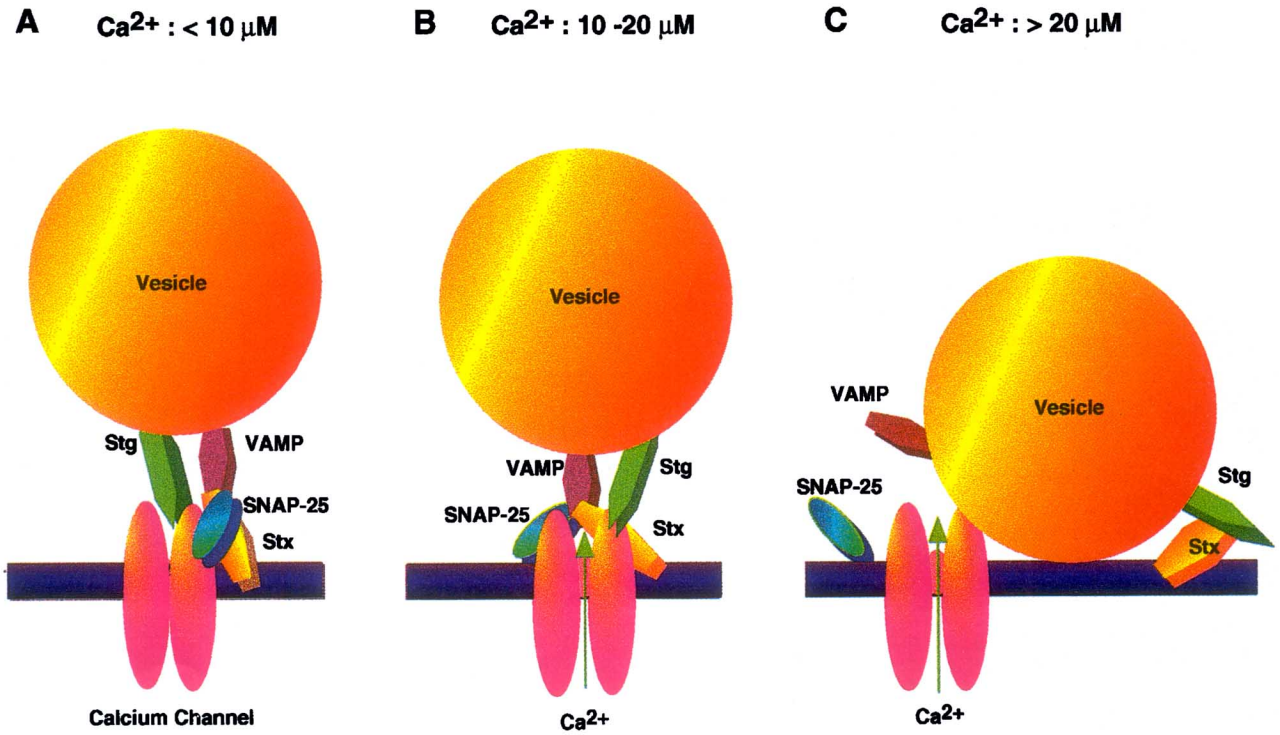

Fig. 4. The proposed model for the sequential Ca^{2+}-dependent interactions of multiple proteins with syntaxin during synaptic vesicle docking/fusion process. (A) The pre-docked vesicles form a low-affinity complex with the N-type calcium channels through binding to syntaxin and SNAP-25 at resting [Ca^{2+}] level (<10 μM). (B) The initial Ca^{2+} influx (10–20 μM) greatly increases the affinity of this coupling, so the binding energy of Ca^{2+} to this complex may contribute to the energetic driving force for the early priming steps of the fusion process. (C) Finally, as the free Ca^{2+} reaches the threshold for release (>20 μM), the binding affinity of this coupling is reduced, and syntaxin and SNAP-25 dissociate from the channels. Higher levels of Ca^{2+} (above 30 μM) may be needed to enable displacement of syntaxin from the calcium channels and efficient binding of synaptotagmin in order for fusion to proceed. Thus, sequential Ca^{2+}-dependent interactions of multiple proteins with syntaxin may serve to order the biochemical events leading to membrane fusion.

calcium channels and increasing the requirement for calcium influx to initiate transmitter release. To test this hypothesis, we investigated the physiological relevance of this interaction by injecting competing peptides into the presynaptic cells of both sympathetic ganglion neuron synapses and *Xenopus* embryonic neuromuscular junctions in culture (Mochida *et al.*, 1996; Rettig *et al.*, 1997).

Cultures of superior cervical ganglion neurons (SCGNs) are favorable for functional testing for several reasons. Peptides can be introduced into the relatively large (30–40 μM) presynaptic cell bodies by microinjection, the injected peptides can rapidly diffuse down short axons to nerve terminals forming synapses with adjacent neurons, the effects on stimulated release of acetylcholine (Ach) can be accurately monitored by recording the excitatory postsynaptic potentials (EPSPs) evoked by action potentials in presynaptic neurons, and only N-type calcium channels control Ach release at these synapses so a homogeneous population of channels can be studied. Synaptic transmission was monitored between closely spaced pairs of neurons for 20–30 min, and then peptides containing synprint site were allowed to diffuse into the presynaptic neurons from a suction pipette for 2–3 min. EPSPs were evoked by action potentials elicited by current pulses applied to the presynaptic cell through a recording microelectrode and were recorded with a second microelectrode in the nearby postsynaptic cell (Mochida *et al.*, 1994, 1995). Peptides containing the synprint site from the α_{1B} subunit disrupt the interaction of native N-type channels to syntaxin and reduce synaptic transmission in SCGN synapses by 23–42% for different synprint peptides, without any effect on Ca^{2+} currents (Mochida *et al.*, 1996). Rapid, synchronous synaptic transmission is inhibited, while late, asynchronous EPSPs and paired-pulse facilitation are increased, consistent with the conclusion that syn-

aptic vesicles are shifted from a pool primed for synchronous release to a pool that is not optimally primed or positioned for synchronous release (Fig. 3). The corresponding peptides from L-type calcium channels have no effect on EPSPs. The relative efficiency for inhibition of transmitter release by three different peptides, L_{II-III} (718–963) > L_{II-III} (832–963) > L_{II-III} (718–859), was consistent with their rank order of affinity for *in vitro* binding with syntaxin (Mochida *et al.*, 1996; Rettig *et al.*, 1996). These results provide direct evidence that binding of presynaptic calcium channels to the synaptic docking/fusion complex is required for rapid, synchronous neurotransmitter release.

Early work at the frog neuromuscular junction revealed that fast synaptic transmission is steeply dependent on the external calcium concentration $[Ca^{2+}]_e$, and the probability of acetylcholine release at the frog neuromuscular junction increases as the fourth power of $[Ca^{2+}]_e$ (Fatt and Katz, 1951). To determine whether the inhibition of synaptic transmission by synprint peptides might be due to displacement of the docked vesicle away from the calcium channels and resultant changes in the calcium dependence of transmission, we used *Xenopus* nerve-muscle co-cultures from developing embryos in which synaptic transmission is mainly dependent on N-type calcium channels (Yazejian *et al.*, 1997). This is an ideal preparation to study the effects of N-type synprint peptides on synaptic transmission because the calcium transients in the presynaptic terminal can be imaged in parallel with measurements of synaptic transmission. During the first days of development, the embryos undergo cell divisions without substantial growth. Injection of synprint peptides into early blastomeres leads to loading of all progeny cells, including spinal cord neurons and muscle cells. Following cell culture, synaptic transmission of peptide-loaded and control cells were compared by measuring postsynaptic responses under different external Ca^{2+} concentrations. The dependence of synaptic transmission on calcium concentration was shifted to higher concentrations so that, at physiological Ca^{2+} concentrations, approximately 50% reduction of transmitter release of injected neurons was observed. Analysis by a theoretical model indicated that at least 70% of the docked vesicles were detached from calcium channels under these conditions. High calcium concentrations can overcome this inhibition. Injection of the corresponding region of the L-type calcium channels had virtually no effect (Rettig *et al.*, 1997). These data suggest that disruption of the physical link between N-type calcium channels and synaptic vesicle docking/fusion apparatus displaces pre-docked vesicles from calcium entry sites, making neurotransmitter release less efficient by shifting its Ca^{2+}-dependence to higher values (Fig. 3). These findings are consistent with the functional data on rat superior cervical ganglion neurons (Mochida *et al.*, 1996) where a maximum of 42% inhibition of synaptic transmission was observed following diffusion of synprint peptides.

MODULATION OF THE INTERACTIONS BETWEEN N-TYPE CALCIUM CHANNELS AND THE SYNAPTIC DOCKING/FUSION CORE COMPLEX

An important unresolved issue in understanding neurotransmitter release is the mechanism of its Ca^{2+}-dependence. Neurotransmitter release is initiated by influx of Ca^{2+} through voltage-dependent calcium channels (Smith and Augustine, 1988; Robitaille *et al.*, 1990), within 200 μs of the action potential arriving at the synaptic terminals (Berrett and Stevens, 1972), as the Ca^{2+} concentration increases from 100 nM to >200 μM (Llinás *et al.*, 1992). Exocytosis requires high Ca^{2+} concentration, with a threshold of 20–50 μM and half maximal activation at 190 μM (Heidelberger *et al.*, 1994; von Gersdorff and Mattews, 1994). Synaptotagmin may be the low-affinity Ca^{2+}-sensor since it binds to syntaxin and phospholipid in a Ca^{2+}-dependent manner in the range of 10–50 μM and 100–300 μM Ca^{2+}, respectively (Perin *et al.*, 1990; Brose *et al.*, 1992; Davletov and Südhof, 1993; Chapman and Jahn, 1994; Geppert *et al.*, 1994). However, other Ca^{2+}-responsive proteins may also be involved in the docking/fusion process as residual neurotransmission persists in synaptotagmin-null mutants (DiAntonio *et al.*, 1993; Nonet *et al.*, 1993; Littleton *et al.*, 1993).

To find additional Ca^{2+}-responsive interactions among synaptic proteins and calcium channels, we measured the binding of these recombinant proteins *in vitro*. We found that the interaction of the N-type synprint peptide with recombinant syntaxin, SNAP-25, the syntaxin-SNAP-25 dimer, or the synaptic core complex of syntaxin-SNAP-25-VAMP/synaptobrevin has a biphasic dependence on Ca^{2+} concentration, with maximal binding at approximately 20 μM free Ca^{2+} (Sheng *et al.*, 1996). This Ca^{2+}-dependent interaction takes place in the same concentration range as the threshold for fast transmitter release. We also measured the effect of Ca^{2+} on binding of the native N-type

calcium channels specifically labeled with ω-CTx-GVIA to a complex of native syntaxin and SNAP-25 with GST-synaptobrevin bound to glutathione-Sepharose. Basal binding is observed in the absence of Ca^{2+}, maximal binding at 20 μM Ca^{2+}, and reduced binding at 100 μM Ca^{2+} (Sheng *et al.*, 1996). Thus, the direct interaction of presynaptic calcium channels with the synaptic fusion core-complex is a Ca^{2+}-sensitive process and may play a key role in docking and fusion of synaptic vesicles.

Using both immobilized recombinant proteins and native presynaptic membrane proteins, we found that the synprint peptide of N-type channels and synaptotagmin competitively interact with syntaxin (Sheng *et al.*, 1997). This competition is Ca^{2+}-dependent because of the Ca^{2+} dependence of the interactions between syntaxin and these two proteins. The affinity of N-type calcium channels for binding to syntaxin is modulated by Ca^{2+} concentration, with maximal binding at a range of 10–30 μM near the threshold for neurotransmitter release (Sheng *et al.*, 1996). In contrast, maximum binding of syntaxin to synaptotagmin I and II requires higher concentrations of Ca^{2+} in the range from 100 μM to 1 mM (Li *et al.*, 1995a, 1995b; Chapman *et al.*, 1995; Kee and Scheller, 1996). As the Ca^{2+} concentration increases beyond 30 μM, interaction of syntaxin with the synprint site will be weakened and interaction with synaptotagmin will be strengthened. Thus, these studies provide potential biochemical correlates for the sequence of events during synaptic vesicle exocytosis.

Binding of the synprint peptides from the rbA and BI isoforms of α_{1A} shows different dependences on calcium concentration from the synprint peptide of α_{1B} (Kim and Catterall, 1997). The BI isoform of α_{1A} binds syntaxin, SNAP-25, and synaptotagmin in a calcium-independent manner. The rbA isoform of α_{1A} does not bind to syntaxin appreciably *in vitro* and binds SNAP-25 in a calcium-independent manner. However, binding of synaptotagmin to the rbA isoform of α_{1A} is calcium-dependent with maximum binding at 10–20 μM calcium, similar to the calcium dependence of binding of syntaxin and SNAP-25 to α_{1B}. The differences in calcium dependence of interaction of these synprint peptides with different SNARE proteins suggest that the calcium dependence of this interaction is not an essential element of the transmitter release pathway but serves a modulatory role which may confer different regulatory properties on transmitter release mediated by the different presynaptic calcium channels.

The efficiency of synaptic transmission could be regulated by modulating the interactions of calcium channels with synaptic protein complexes. Second-messenger activated regulation of neurotransmitter release via modulation of the interactions of proteins with the exocytotic apparatus has a potentially important role in synaptic plasticity. Several protein kinases including CaM KII, PKC, PKA, and PKG are expressed in presynaptic nerve terminals. Both N-type calcium channels and SNARE proteins are phosphorylated by one or more of these protein kinases (Hell *et al.*, 1994; Hirning and Scheller, 1996; Shimazaki *et al.*, 1996). *In vitro* biochemical studies showed that phosphorylation of the synprint peptide with PKC and CaM KII, but not PKA or PKG, strongly inhibited binding of recombinant syntaxin or SNAP-25, and also inhibited its interactions with native rat brain SNARE complexes containing syntaxin and SNAP-25 (Yokoyama *et al.*, 1997). These results suggest that phosphorylation of the synprint site by PKC or CaM KII may serve as a biochemical switch for interactions between N-type calcium channels and SNARE protein complexes, and is a candidate presynaptic mechanism for the regulation of neurotransmission.

CALCIUM CHANNEL INTERACTIONS WITH SNARE PROTEINS IN TRANSMITTER RELEASE

Understanding how presynaptic calcium channels interact with SNARE proteins in a Ca^{2+}-dependent manner should not only help to clarify their functions in synaptic vesicle docking/fusion process but can also provide a model for how syntaxin–synaptotagmin interaction involving calcium channels is regulated by Ca^{2+}. Several lines of evidence suggest that Ca^{2+} binding to synaptotagmin is part of the signal that initiates rapid exocytosis (Perin *et al.*, 1990; Brose *et al.*, 1992; Davletov and Südhof, 1993; Chapman and Jahn, 1994; Geppert *et al.*, 1994). Our studies show that the interaction of N-type calcium channels with synaptic vesicle docking/fusion core complex is also dependent on changes in $[Ca^{2+}]$ near the threshold level for initiation of transmitter release. We propose that docked vesicles form a low-affinity complex with the N-type calcium channels through binding to syntaxin and SNAP-25 at resting $[Ca^{2+}]$ level. Ca^{2+} influx greatly increases the affinity of this coupling, so the binding energy of Ca^{2+} to this complex may contribute to the energetic driving force for the early priming steps of the fusion process.

Finally, as the free Ca^{2+} reaches the threshold for release (20–50 μM), the binding affinity of this coupling is reduced, and syntaxin and SNAP-25 dissociate from the channels. Higher levels of Ca^{2+} (above 30 μM) may be needed to enable displacement of syntaxin from the calcium channels and efficient binding of synaptotagmin in order for fusion to proceed. Thus, sequential Ca^{2+}-dependent interactions of multiple proteins with syntaxin may serve to order the biochemical events leading to membrane fusion (Fig. 4).

For presynaptic calcium channels containing α_{1A} as their pore-forming subunit, different calcium-dependent interactions are observed *in vitro*. We propose that the sequence of interactions of syntaxin first with the synprint site of presynaptic calcium channels and then with synaptotagmin remains the same and is an essential element of the release pathway. For the BI isoform of α_{1A}, no calcium-dependent interactions are observed *in vitro*, so it is likely that synaptotagmin displaces the synprint site from syntaxin without the aid of a calcium-dependent decrease in binding affinity between the synprint site and syntaxin. This may require a higher concentration of calcium to stimulate the interaction of synaptotagmin and syntaxin and therefore increase the steepness and cooperativity of the calcium dependence of neurotransmitter release mediated by the BI isoform of α_{1A}. For the rbA isoform of α_{1A}, a biphasic dependence of binding to synaptotagmin is observed, but there is no calcium-dependent binding to SNAP-25 or syntaxin. In this case, calcium-dependent release of synaptotagmin from interaction with the synprint peptide may allow the interaction of synaptotagmin with syntaxin to occur more easily as calcium concentrations increase and thereby enhance the release process. The differences in calcium-dependent interactions of these synprint peptides may allow differential modulation of the release process by calcium and perhaps by other influences such as protein phosphorylation.

CONCLUSION

The interactions between the presynaptic calcium channels and synaptic proteins syntaxin, SNAP-25, and synaptotagmin are particularly interesting, first because these SNARE proteins are components of the synaptic core complex that has been proposed to mediate most types of intracellular membrane fusion, and second because the mode of binding in some cases is Ca^{2+}-dependent with maximal binding at 20 μM Ca^{2+} which is near threshold level for initiating neurotransmitter release. Our results described in this review provide a molecular basis for a physical link between Ca^{2+} influx into nerve terminals and subsequent exocytosis of neurotransmitters at synapses, and further indicate that presynaptic calcium channels not only provide the calcium signal required by the exocytotic apparatus, but also contain structural elements that are integral to vesicle docking, priming, and fusion, and possibly to regulation of these processes by protein phosphorylation (Figs. 3 and 4). Synaptic transmission is regulated by multiple receptor pathways which act through G proteins and protein phosphorylation. Further studies on the regulation of the interactions between calcium channels and presynaptic proteins in *in vitro* and *in vivo* systems should help to elucidate the regulatory mechanisms of synaptic transmission at the molecular level.

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