Inhibition of Neurotransmission by Peptides Containing the Synaptic Protein Interaction Site of N-Type Ca^{2+} Channels

Sumiko Mochida,* Zu-Hang Sheng,† Carl Baker,†
Haruo Kobayashi,* and William A. Catterall†
*Department of Physiology
Tokyo Medical College
Tokyo 160
Japan
†Department of Pharmacology
University of Washington
Seattle, Washington 98195-7280

Summary

N-type Ca^{2+} channels bind directly to the synaptic core complex of VAMP/synaptobrevin, syntaxin, and SNAP-25. Peptides containing the synaptic protein interaction ("synprint") site caused dissociation of N-type Ca^{2+} channels from the synaptic core complex. Introduction of synprint peptides into presynaptic superior cervical ganglion neurons reversibly inhibited synaptic transmission. Fast EPSPs due to synchronous transmitter release were inhibited, while late EPSPs arising from asynchronous release following a train of action potentials were increased and paired-pulse facilitation was increased. The corresponding peptides from L-type Ca^{2+} channels had no effect, and the N-type peptides had no effect on Ca^{2+} currents through N-type Ca^{2+} channels. These results are consistent with the hypothesis that binding of the synaptic core complex to presynaptic N-type Ca^{2+} channels is required for Ca^{2+} influx to elicit rapid, synchronous neurotransmitter release.

Introduction

Arrival of the nerve impulse at a nerve terminal leads to opening of voltage-gated Ca^{2+} channels and rapid influx of Ca^{2+}. The increase in Ca^{2+} concentration at the active zone from a basal level of 100 nM to more than 200 μM triggers the fusion of docked synaptic vesicles, resulting in neurotransmitter release within 200 μs (Augustine and Neher, 1992; Zucker, 1993; Heidelberger et al., 1994; Barrett and Stevens, 1972; Llinas et al., 1981, 1992). N-type Ca^{2+} channels are localized in nerve terminals (Robitaille et al., 1990; Westenbroek et al., 1992) and participate in neurotransmitter release in central and peripheral synapses (Tsien et al., 1988; Wu and Saggau, 1994; Mintz et al., 1995). The synaptic plasma membrane proteins syntaxin (Bennett et al., 1992; Inoue et al., 1992; Yoshida et al., 1992) and synaptosome-associated protein of 25 kDa (SNAP-25) (Oyler et al., 1989) bind to the synaptic vesicle protein VAMP/synaptobrevin (Trimble et al., 1988) to form a stable synaptic core complex (Söllner et al., 1993; Calakos et al., 1994; O'Conner et al., 1993; Hayashi et al., 1994; Chapman et al., 1994). This complex binds to N-type Ca^{2+} channels (Bennett et al., 1992; Yoshida et al., 1992; Saisu et al., 1991; Lévèque et al., 1994), and physiological experiments suggest a close association of N-type Ca^{2+} channels

with sites of transmitter release (Stanley, 1993). N-type Ca^{2+} channels bind to the synaptic core complex through a site in the intracellular loop connecting domains II and III (Sheng et al., 1994; Rettig et al., 1996) of their α_{1B} subunits (Dubel et al., 1992). Their binding has a biphasic dependence on the concentration of Ca^{2+} with maximum binding in the range of 10 μM to 30 μM (Sheng et al., 1996). These results suggest a direct role for presynaptic N-type Ca^{2+} channels in docking and fusion of synaptic vesicles, but no experimental evidence for a requirement for binding of presynaptic Ca^{2+} channels to the synaptic core complex during neurotransmitter release has been presented. One prediction of the hypothesis that interaction with N-type Ca^{2+} channels is required for efficient release of neurotransmitters is that peptides containing the synaptic protein interaction ("synprint") site would inhibit synaptic transmission by binding to syntaxin and SNAP-25 and thereby preventing their binding to presynaptic Ca^{2+} channels. In this paper, we describe experiments that test this prediction by analysis of nerve impulse–evoked transmission between pairs of cultured superior cervical ganglion neurons in which synprint peptides have been introduced into the presynaptic partner.

Results

Dissociation of Immunoprecipitated Complexes of N-Type Ca^{2+} Channels and Synaptic Proteins by Synprint Peptides

Binding of N-type Ca^{2+} channels to recombinant syntaxin in vitro is prevented by fusion proteins containing the synprint site from the intracellular loop between domains II and III (L$_{II-III}$) of the α_{1B} subunit, which competes with the native channel for binding to syntaxin (Sheng et al., 1994). However, it is not known whether synprint peptides can cause dissociation of preformed complexes of syntaxin and N-type Ca^{2+} channels. We used purified recombinant fusion proteins containing synprint sites and purified complexes of syntaxin and N-type Ca^{2+} channels isolated from brain to test whether such fusion proteins can disrupt the interactions between the endogenous synaptic core complex and N-type Ca^{2+} channels (Figure 1). Fusion proteins containing overlapping segments of the synprint site of α_{1B} (Sheng et al., 1994) and the corresponding segments of the α_1 subunit of an L-type Ca^{2+} channel (α_{1S}) were expressed, purified, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Sheng et al., 1996; Figure 1A). We extracted N-type Ca^{2+} channels from brain membranes in association with the synaptic core complex, partially purified them by chromatography on wheat germ agglutinin–Sepharose, and analyzed them by coimmunoprecipitation with a specific anti-α_{1B} antibody as described previously (Westenbroek et al., 1992; Sheng et al., 1994). CNB3, an antibody against the amino acid sequence of the carboxyl terminus of α_{1B}, coimmunoprecipitated syntaxin (Figure 1B). This coimmunoprecipitation was efficiently blocked by incubation with 5.5 μM recombinant peptide L$_{II-III}$(718–963) containing the synprint site
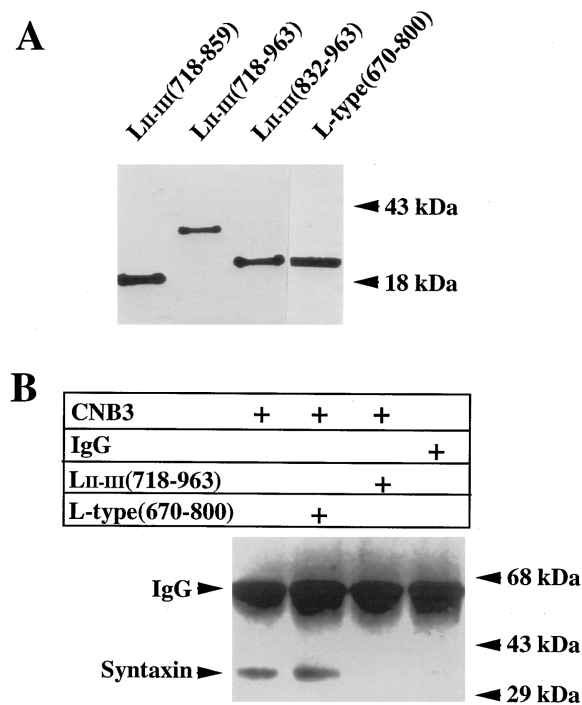
A

CNB3 column labels: L_{II-III}(718-859), L_{II-III}(718-963), L_{II-III}(832-963), L-type(670-800)

◀ 43 kDa

◀ 18 kDa

B

CNB3	+	+	+
IgG			+
L_{II-III}(718-963)		+	
L-type(670-800)	+		

IgG ▶ ◀ 68 kDa

◀ 43 kDa

Syntaxin ▶ ◀ 29 kDa

Figure 1. Recombinant α_{1B} Synprint Fusion Protein Blocks the Interaction of Native N-Type Ca^{2+} Channel with Syntaxin

(A) Coomassie blue–stained gel of the purified His-tagged N- and L-type Ca^{2+} channel fusion proteins. The His-tagged fusion proteins were purified using a Ni^{2+}-charged affinity resin and analyzed by SDS–PAGE.

(B) Effects of recombinant N- and L-type fusion proteins on coimmunoprecipitation of syntaxin 1 with N-type Ca^{2+} channels. A complex of syntaxin with N-type Ca^{2+} channels was purified from a detergent extract of rat brain membranes by affinity chromatography on wheat germ agglutinin–Sepharose and coimmunoprecipitated by CNB3, an antibody against the carboxyl terminus of the α_{1B} subunit of N-type Ca^{2+} channels, in the presence or absence of 5.5 μM recombinant L_{II-III}(718–963) of α_{1B} or 5.5 μM recombinant L_{II-III}(670–800) of α_{1S} as indicated. The immunoprecipitates were analyzed by SDS–PAGE, labeled with anti-syntaxin 1 monoclonal antibody, and visualized with rabbit anti-mouse coupled to horseradish peroxidase and enhanced chemiluminescence detection. The migration positions of syntaxin and mouse IgG added in the immunoprecipitation are indicated. The ECL signal intensities were quantitated with ImageQuant software (Molecular Dynamics), and the pixel values from three experiments were averaged. The recombinant N-type Ca^{2+} channel peptide L_{II-III}(718–963) reduced coimmunoprecipitation of syntaxin by 86% $\pm$ 3%, while L-type control fusion protein L_{II-III}(670–800) did not show any apparent effects (5% $\pm$ 2% reduction) (data not shown). In control experiments, the N-type and L-type peptides had no effects on immunoprecipitation of α_{1B} as determined by SDS–PAGE on a more porous gel system and immunoblotting of α_{1B} with the CNB3 antibody.

from the intracellular loop connecting domains II and III of α_{1B}, while a control fusion protein, L_{II-III}(670–800) from the α_{1S} subunit of an L-type Ca^{2+} channel, did not block coprecipitation of syntaxin (Figure 1B). These results provide further support for the conclusion that syntaxin specifically associates with N-type Ca^{2+} channels through interaction with a site in L_{II-III} of α_{1B} (Sheng et al., 1994, 1996) and show that this interaction can be disrupted specifically by excess fusion protein containing the synprint site of α_{1B}.

Inhibition of Synaptic Transmission by Synprint Peptides

To determine the functional significance of the interaction of the synaptic core complex with N-type Ca^{2+} channels in neurotransmission, we studied the effects of fusion proteins containing the synprint site of N-type Ca^{2+} channels on synaptic transmission at cholinergic synapses formed between superior cervical ganglion neurons (SCGNs) in culture (O'Lague et al., 1974). Cultures of SCGNs are favorable for these experiments because proteins can be introduced into the relatively large (30–40 μm) presynaptic cell bodies by microinjection, the injected proteins can rapidly diffuse to nerve terminals forming synapses with adjacent neurons, and the effects on stimulated release of acetylcholine (ACh) can be accurately monitored by recording the excitatory postsynaptic potentials (EPSPs) evoked by action potentials in the presynaptic neurons (Mochida et al., 1994, 1995). Synaptic transmission was monitored between closely spaced (<5 μm) pairs of neurons for 20–30 min, and then fusion proteins containing the synprint site from L_{II-III} of the α_{1B} subunit of N-type Ca^{2+} channels were allowed to diffuse into the presynaptic neurons from a suction pipette for 2–3 min. During this time, the protein concentration inside the presynaptic cell body approached approximately 2.5% of the concentration in the pipet, as estimated from the color intensity of the coinjected dye Fast Green FCF and correction for the effect of molecular mass on diffusion (Pusch and Neher, 1988). 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. After a stable period of control recordings, injection of the fusion protein L_{II-III}(718–963) from α_{1B} at t = 0 gradually decreased EPSP amplitude over a period of 10 min (Figure 2). The inhibitory effect was dependent on the injected concentration of L_{II-III}(718–963). With an injection pipette containing 65 μM L_{II-III}(718–963), which produced a maximum concentration of 1.6 μM in the cell soma, the inhibition of synaptic transmission was rapidly reversible (Figures 2A and 2D). The maximum decrease in EPSP amplitude, −24% $\pm$ 4.2% (n = 7, mean $\pm$ SEM, Table 1), was observed 10–15 min after starting the injection, and the EPSPs recovered to the control amplitude by 30–40 min after injection (Figure 2D). We assume that recovery follows proteolytic degradation of the intracellular synprint peptide in the cell body and presynaptic terminal. Nearly 2-fold greater inhibition was observed when a 2-fold higher pipette concentration (130 μM) was used (Figures 2B and 2D), indicating that the effect of the peptide was not near saturation. The maximum decrease in EPSP amplitude, −42% $\pm$ 5.1% (n = 7), was observed 30–40 min after starting injection (Figure 2D; Table 1). By 60 min after injection, the amplitude of the EPSPs had begun to recover to control levels in three cells but not in three other cells. L_{II-III} from the α_{1S} subunit of an L-type Ca^{2+} channel does not interact with syntaxin or SNAP-25 (Sheng et al., 1994). Injection of 140 μM of the fusion protein L_{II-III}(670–800) from α_{1S} produced no significant decrease in EPSP amplitude (−3% $\pm$ 2.8% at 30 min after the start of injection, n = 5; Figures 2C and 2D), indicating that the inhibitory effect of L_{II-III}(718–963) from the α_{1B} subunit of N-type Ca^{2+} channels is specific.
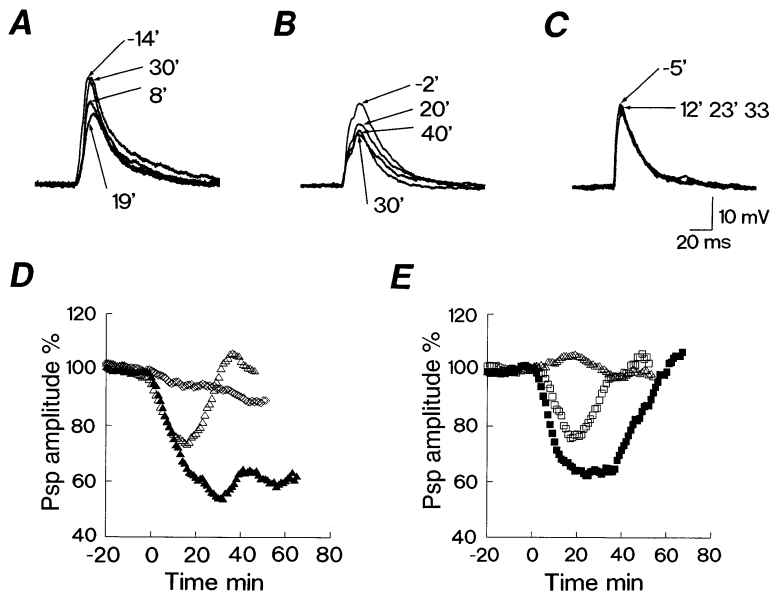
Figure 2. Effects of N-Type Ca²⁺ Channel Synprint Fusion Proteins on Synaptic Transmission of SCGNs in Culture

(A) L_{II-III}(718–963) of α_{1B} was introduced into the presynaptic neuron by diffusion from a suction pipette beginning when the membrane was disrupted by applying suction at t = 0. The pipette concentration of L_{II-III}(718–963) was 65 μM (n = 7). Postsynaptic potentials from one representative experiment recorded 14 min before injection and 8, 19, and 30 min after injection are illustrated.

(B) Injection of 130 μM L_{II-III}(718–963) of α_{1B} (n = 7). Postsynaptic potentials recorded 2 min before injection and 20, 30, and 40 min after injection are illustrated.

(C) As a control for the experiments of (A) and (B), 140 μM L_{II-III}(670–800) of the L-type Ca²⁺ channel subunit α_{1S}, was injected (n = 5). Postsynaptic potentials recorded 5 min before injection and 12, 23, and 33 min after injection are illustrated.

(D) Normalized average postsynaptic potentials are plotted from seven experiments with 65 μM L_{II-III}(718–963) of α_{1B} like the one illustrated in (A) (open triangles), seven experiments with 130 μM L_{II-III}(718–963) of α_{1B} like the one illustrated in (B) (closed triangles), and five experiments with 140 μM L_{II-III}(670–800) of α_{1S} like the one illustrated in (C) (open squares).

(E) Normalized average postsynaptic potentials following injection of L_{II-III}(718–859) (240 μM, n = 7, open squares), L_{II-III}(832–963) (200 μM, n = 7, closed squares), and control carrier solution (n = 7, open triangles).

Two nearby regions of L_{II-III} of the α_{1B} subunit of the N-type Ca²⁺ channel, which are both contained within the fusion protein L_{II-III}(718–963), are involved in interaction with syntaxin and SNAP-25 (Rettig et al., 1996). The shorter fusion protein L_{II-III}(718–859), containing only the first of these two regions, reversibly inhibited synaptic transmission, but higher concentrations were required for comparable inhibition (Figure 2E), as expected from the lower affinity interaction of this peptide (Rettig et al., 1996). The maximum decrease in EPSP amplitude, −23% ± 3.5% (n = 7), was observed 15–20 min after starting injection with a pipette containing 240 μM peptide, which produced a concentration approaching 6 μM in the cell soma. By 40 min after injection, the EPSPs had recovered to the amplitude observed before injection. The fusion protein L_{II-III}(832–963), containing the second region of interaction with syntaxin and SNAP-25, inhibited synaptic transmission more strongly at a concentration of 200 μM than L_{II-III}(718–859) did at 240 μM (Figure 2E). The maximum decrease in EPSP amplitude, −38% ± 5.1%, was observed 20–30 min after

starting injection, and the EPSPs recovered to control amplitude by 60 min after injection. As a further control, normal rabbit IgG at the same total protein concentration (4 mg/ml in the pipette) produced no significant decrease in EPSP amplitude (−5.0% ± 3.1%, n = 6, at 30 min after injection, Table 1), indicating that introduction of large protein molecules does not interfere with transmitter release. Similarly, injection of carrier solution also showed no significant inhibition of synaptic transmission (−3.0% ± 2.4%, n = 7, at 30 min after injection; Figure 2E).

The decrease in EPSP amplitude caused by introduction of 65 μM L_{II-III}(718–963) was not dependent on the rate of presynaptic activity. The time course of inhibition and recovery of the EPSP amplitude was identical when synapses were stimulated at 0.2 Hz, 0.05 Hz, or 0.01 Hz (n = 4–5). Inhibition of the release of transmitters is expected to be unaffected by stimulation frequency in this range, while inhibition of vesicle endocytosis and recycling should be accelerated by more rapid stimulation rates (e.g., Mochida et al., 1996). Thus, it is likely

Table 1. Effects of Microinjected L_{II-III} Fusion Proteins on Evoked EPSPs

Peptide Sequence	Concentration in Pipette	Number of Experiments	Change in Amplitude (%)	Change in Time Constant[a] (%)
α_{1B}(718–963)	2 mg/ml (65 μM)	7	−24 ± 4.2[b]	+17 ± 8.7
α_{1B}(718–963)	4 mg/ml (130 μM)	7	−42 ± 5.1[c]	+16 ± 7.4
α_{1B}(718–859)	4 mg/ml (240 μM)	7	−23 ± 3.5[d]	+27 ± 8.8
α_{1B}(832–963)	4 mg/ml (200 μM)	7	−38 ± 5.1[e]	+17 ± 7.0
α_{1S}(670–800)	2 mg/ml (140 μM)	5	−3 ± 2.8[f]	+3.2 ± 8.8
Rabbit IgG	4 mg/ml	6	−5 ± 3.1[f]	+3.5 ± 4.2
Carrier solution		7	−3 ± 2.4[f]	+0.2 ± 4.3

[a] Time constant of EPSPs was measured when the maximum increase was observed from 40 to 60 min after starting injection.

[b-e] EPSPs were measured when maximum inhibitory effect of fusion proteins was observed ([b] 10–15 min, [c] 30–40 min, [d] 15–20 min, and [e] 20–30 min after starting injection) and divided by preinjection value to yield the percentage change in the amplitude (mean ± SEM).

[f] EPSPs were measured at 30 min after starting injection of fusion protein, IgG, or carrier solution.

that synprint peptides act on the transmitter release process itself rather than by inhibition of vesicle recycling. In addition, the reduction of EPSP amplitude was maximal during the first stimulation in a train of three action potentials delivered at 100 Hz (see Figure 3). These results suggest that the synprint peptides reduce the number of docked and primed vesicles that are released during a single depolarization by acting prior to the action potential stimulus and do not require repetitive activation of the release process to act.

Prolonged Postsynaptic Potentials in Response to Repetitive Stimuli in the Presence of Synprint Peptides

In addition to effects on the peak EPSPs, synprint peptides also reproducibly slowed the decay of EPSPs elicited by single action potentials by 16% to 27% (Table 1). This effect was much more prominent with repetitive action potential stimuli. Although single action potentials always induced postsynaptic potentials that decayed exponentially, repetitive action potentials in the presence of 5.1 mM Ca^{2+} usually caused a rapid EPSP due to synchronous transmitter release followed by late EPSPs due to asynchronous release at synapses on SCG neurons that had been in cell culture for more than one month (Figure 3A). Asynchronous release likely depends on diffusion of residual Ca^{2+} from repetitive action potentials away from active zones and induction of transmitter release from synaptic vesicles that are not fully primed for release or from synaptic vesicles that are not optimally positioned to respond rapidly to Ca^{2+} influx. Introduction of $L_{II-III}(718–963)$ at 65 μM into these presynaptic neurons (n = 5) reduced the peak of the EPSPs in response to three repetitive action potentials delivered at 100 Hz every 90 s comparably to the reduction of EPSPs induced by single action potentials (Figure 3A, compare rows b and c). In addition, $L_{II-III}(718–963)$ substantially slowed the decay of EPSPs and increased the number of late EPSPs due to asynchronous release following stimulation with trains of three action potentials (Figure 3A). The increase in late EPSPs due to asynchronous release was not as evident when the extracellular concentration of Ca^{2+} was reduced to 1 mM (Figure 3D, n = 4), consistent with the idea that late EPSPs due to asynchronous release depend on residual Ca^{2+} that remains after multiple action potentials in the presence of 5.1 mM Ca^{2+}. Altogether, our results with single and triple action potential stimuli indicate that synprint peptides inhibit synchronous release and concomitantly increase asynchronous release, suggesting that the effect of these peptides is to shift synaptic vesicles from a pool primed for synchronous release to a pool that is not optimally primed or positioned for synchronous release.

Increased Paired-Pulse Facilitation in the Presence of Synprint Peptides

When two action potentials are generated in rapid succession, the postsynaptic response to the second is often larger due to paired-pulse facilitation. This facilitation is thought to result from increased residual Ca^{2+}
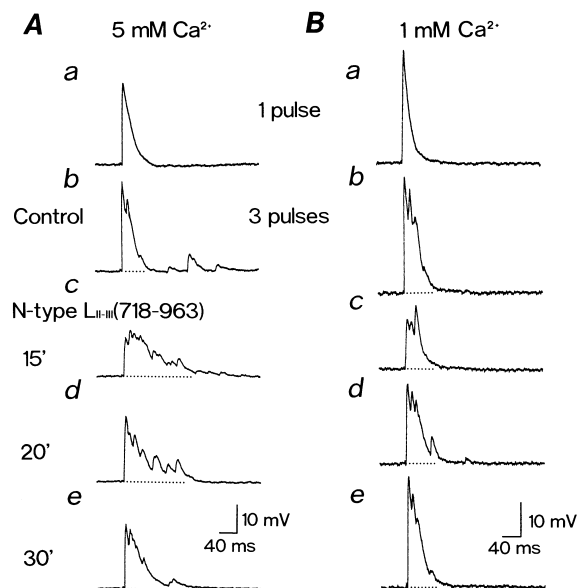

Figure 3. Increase of Late, Asynchronous EPSPs in the Presence of Synprint Peptides

$L_{II-III}(718–963)$ was introduced into the presynaptic cell body at t = 0 from a pipette containing 65 μM peptide.

(A) Synaptic responses were recorded from one pair of SCGN cultured for 36 days in Krebs' solution containing 5.1 mM Ca^{2+} in response to a single action potential (a) or three action potentials (b–e) at the indicated times after introduction of the synprint peptide. Presynaptic action potentials were elicited by one or three presynaptic current pulses (3 nA) of 5 ms at 100 Hz every 90 s. The results presented are typical of five pairs of cells studied under similar conditions.

(B) EPSPs recorded in Krebs' solution containing 1 mM Ca^{2+} after 35 days in culture in response to one action potential (a) or three action potentials (b–e) at the indicated times after introduction of the synprint peptide. The membrane potential of the postsynaptic cell was held at −80 mV to prevent generation of action potentials.

remaining in the presynaptic terminal from the first action potential, but other mechanisms may also contribute (Zucker, 1993). Although paired-pulse facilitation is not observed reproducibly at 5.1 mM Ca^{2+} in our experiments, it is observed at lower Ca^{2+} concentrations (Figure 4). At 2.5 mM Ca^{2+}, pairing of pulses resulted in an insignificant increase in the second pulse (102% ± 6.5% of control; n = 4). However, 10 min after introduction of the synprint peptide $L_{II-III}(718–963)$ at 65 μM, the size of the first EPSP was reduced to 67% ± 8.7% of the prepeptide control, and the EPSP in response to the paired second pulse was facilitated by 1.48-fold to 99% ± 7.4% (n = 4) of the prepeptide control EPSP (Figure 4A). At 1 mM Ca^{2+}, pairing of pulses increased the size of the second EPSP to 141% ± 11% of control (n = 4). Moreover, 10 min after introduction of the synprint peptide, the size of the first EPSP was reduced to 62% ± 14% of the prepeptide control, and the EPSP evoked by the paired second pulse was facilitated by 1.74-fold to 108% ± 4.6% of the prepeptide control value (Figure 4B). Thus, the synprint peptide not only increases asynchronous EPSPs in responses to trains of three action potentials (Figure 3), but also increases the size of the second synchronous EPSP relative to the first in paired-pulse facilitation.

A

2.5 mM Ca²⁺

a

Pre-AP

B

1 mM Ca²⁺

a

|50 mV

b

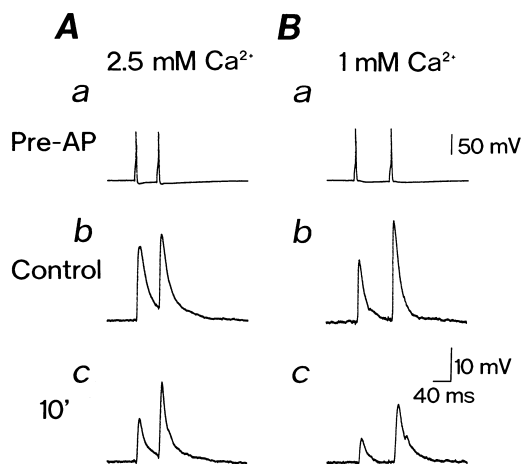Control

b

c

10'

c

|10 mV
40 ms

Figure 4. Increased Paired-Pulse Facilitation in the Presence of Synprint Peptides

EPSPs were recorded in Krebs' medium containing 2.5 mM Ca²⁺ (A) or 1 mM Ca²⁺ (B). The membrane potential of the postsynaptic cell was held at −80 mV, and EPSPs were evoked once every 20 s. Paired pulses were delivered after a 50 ms interval in (A) or after an 80 ms interval in (B), as required for optimal facilitation at these Ca²⁺ concentrations. (a) Presynaptic action potentials ([A], 2 nA, 5 ms pulses; [B], 1 nA, 5 ms pulses). (b) EPSPs evoked by a paired pulse protocol. (c) EPSPs evoked by a paired pulse protocol 10 min after injection of 65 μM L$_{II-III}$(718–963).

Inhibition of Ca²⁺ influx into presynaptic terminals by elevation of extracellular Mg²⁺ reduces EPSPs and increases paired-pulse facilitation at the neuromuscular junction (Mallart and Martin, 1968). However, a concentration of Mg²⁺ that produced 38% reduction of the EPSP caused only a 1.1-fold increase in paired-pulse facilitation, much less than caused by a comparable inhibition of synaptic transmission by synprint peptides in our experiments. To make this comparison in our SCGN preparation, extracellular Mg²⁺ was increased to 10 mM and paired-pulse facilitation was measured. EPSPs were reduced by 48%, but paired-pulse facilitation was increased by only 1.1-fold as observed by Mallart and Martin (1968). These results indicate that the effect of the synprint peptides on paired-pulse facilitation cannot be caused primarily by reduced Ca²⁺ influx.

Effects of Synprint Peptides on N-Type Ca²⁺ Currents in SCGNs

Synchronous transmitter release is thought to have a steep, power-law dependence on the local concentration of Ca²⁺ near the intracellular mouth of presynaptic Ca²⁺ channels in the 200 μs following arrival of the action potential (Augustine and Neher, 1992; Zucker, 1993; Heidelberger et al., 1994; Barrett and Stevens, 1972; Llinas et al., 1981, 1992). The concentration of Ca²⁺ near the intracellular mouth of Ca²⁺ channels in this short time interval is primarily determined by the rate of influx through the channel (Augustine and Neher, 1992; Zucker, 1993). Because the synprint peptides are derived from the Ca²⁺ channel, they are not expected to bind to N-type Ca²⁺ channels or affect their function. Moreover, the Ca²⁺-dependent increase in asynchronous release and in paired-pulse facilitation caused by

synprint peptides argues that they do not reduce Ca²⁺ influx. Nevertheless, it is important to verify that the synprint peptides do not affect Ca²⁺ channel function. Therefore, we measured the effects of the synprint peptides on Ca²⁺ currents through Ca²⁺ channels to assess their possible influence on rapid presynaptic Ca²⁺ transients in active zones.

Synaptic transmission between SCGNs is inhibited by block of presynaptic N-type Ca²⁺ channels (Mochida et al., 1995), but direct measurement of Ca²⁺ currents in presynaptic terminals of SCGNs is technically difficult. Therefore, to examine the effect of the L$_{II-III}$ peptides on N-type Ca²⁺ channel activity, we measured Ca²⁺ currents in the cell body by whole-cell patch clamp recording during introduction of L$_{II-III}$(718–963) from α$_{1B}$ into the cell through a low resistance patch pipette (Mochida et al., 1995). These currents were blocked 83% by 1.7 μM ω-conotoxin GVIA and therefore are primarily N-type (data not shown). This level of inhibition is comparable to the inhibition of synaptic transmission by ω-conotoxin GVIA (Mochida et al., 1995), indicating that N-type Ca²⁺ channels contribute the majority of Ca²⁺ current in both cell bodies and presynaptic terminals. Pipette concentrations of 6.5 μM L$_{II-III}$(718–963) and 12 μM L$_{II-III}$(718–859) were tested. Since those concentrations would equilibrate rapidly with the intracellular compartment in the whole-cell patch clamp configuration, the intracellular concentrations of peptides equalled or exceeded the highest concentrations of these peptides achieved in the synaptic transmission experiments (2 μM and 6 μM at the cell soma, respectively; less at the nerve terminal). The amplitude (Figure 5A), current–voltage relationship, and steady-state inactivation (Figure 5B) of Ca²⁺ currents were not significantly different from the Ca²⁺ current recordings with a patch pipette containing carrier solution or with a patch pipette containing L$_{II-III}$(670–800) from α$_{1S}$ at 7 μM. These results indicate that the fusion protein L$_{II-III}$(718–963) does not detectably affect Ca²⁺ influx and therefore support the conclusion that the inhibition of synaptic transmission produced by L$_{II-III}$(718–963) is due to reduction in the synchrony and efficiency of neurotransmitter release in response to Ca²⁺ channel activation.

Discussion

Our experiments show that fusion proteins containing the synprint site in L$_{II-III}$ from the α$_{1B}$ subunit disrupt the interaction of N-type channels to syntaxin (Figure 1) and inhibit synaptic transmission (Figure 2; Table 1) without effect on Ca²⁺ currents (Figure 5). The inhibition of rapid, synchronous synaptic transmission is accompanied by increased late, asynchronous EPSPs and increased paired-pulse facilitation, consistent with the conclusion that synaptic vesicles are shifted from a primed, ready-to-release pool to a pool that is not ready for rapid, synchronous release (Figures 3 and 4). The relative efficiency for inhibition of transmitter release by three different fusion proteins, L$_{II-III}$(718–963) > L$_{II-III}$(832–963) ≥ L$_{II-III}$(718–859), was consistent with their rank order of affinity in binding recombinant syntaxin in vitro (Rettig et al., 1996). These results provide direct evidence that
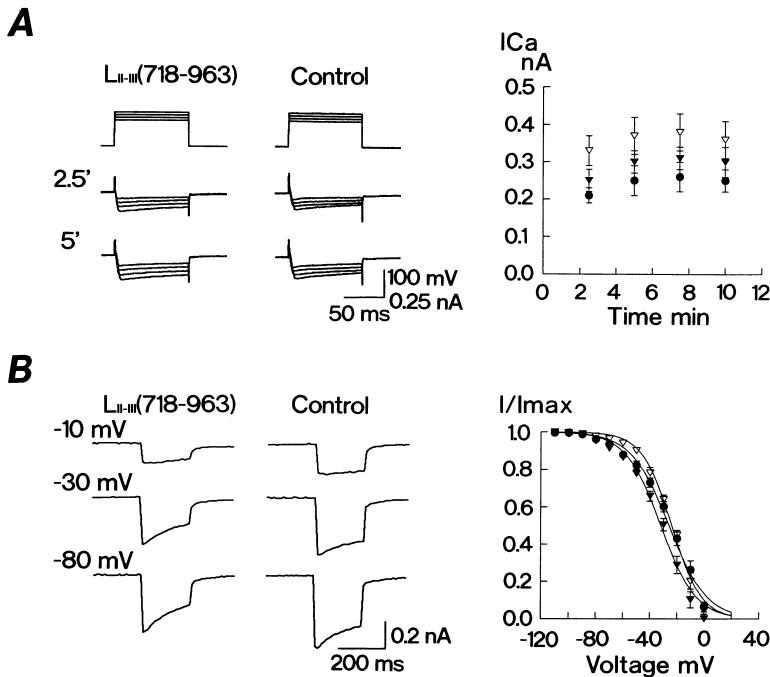
A

B

Figure 5. Effects of Synprint Fusion Proteins on Ca^{2+} Current Measured by Whole-Cell Patch Clamp Recording

(A) Peak Ca^{2+} currents. (Left) Ca^{2+} current records at 2.5 min and 5 min after beginning injection of $L_{II-III}(718-963)$ of α_{1B} from a pipette containing 6.5 μM. Control indicates current records with a patch pipette containing carrier solution. Depolarizations of 90 ms duration were applied from a holding potential of -80 mV. (Right) Averaged peak amplitudes of Ca^{2+} currents of four experiments were plotted against time after applying the whole-cell patch electrode. Patch pipette was filled with 6.5 μM $L_{II-III}(718-963)$ (open triangles), 12 μM $L_{II-III}(718-859)$ (closed triangles), or carrier solution (closed circles).

(B) Inactivation of Ca^{2+} currents. (Left) Current records evoked with 200 ms test pulses to $V_{test} = +10$ mV following 2 s at $V_h = -80$ mV, -30 mV, and -10 mV with a pipette filled with 6.5 μM $L_{II-III}(718-963)$. Control indicates current records with a patch pipette containing carrier solution. (Right) Voltage-dependent inactivation of Ca^{2+} currents. The amplitude of peak current was normalized to the maximal amplitude at $V_h = -110$ mV and plotted against the holding potential. Data points were fitted with a smooth curve derived from the Boltzmann relation, $I/I_{max} = [1 + exp((V - V_{1/2})/k)]^{-1}$ where $V_{1/2} = -24$ mV and k = 11 mV, $V_{1/2} = -32$ mV and k = 13 mV and $V_{1/2} = -26$ mV and k = 14 mV for 10 min after applying patch with a pipette containing 6.5 μM $L_{II-III}(718-963)$ from α_{1B} (open triangles, n = 7), 7 μM $L_{II-III}(670-800)$ from α_{1S} (closed triangles, n = 5), and carrier solution (closed circles, n = 5). No significant shift of the inactivation curve by $L_{II-III}(718-963)$ was seen.

synprint peptides interact with synaptic membrane proteins in vivo, as we have previously demonstrated in vitro, and that they inhibit synaptic transmission through these interactions. This fulfills one prediction of the hypothesis that interaction of the synprint in L_{II-III} of the α_{1B} subunit of N-type Ca^{2+} channels with the synaptic core complex containing syntaxin and SNAP-25 is required for efficient transmitter release triggered by presynaptic action potentials.

Only a partial inhibition of synaptic transmission, ranging from 23% to 42% for different synprint peptides, was observed at injected concentrations in the 65 μM to 240 μM range (Table 1). For $L_{II-III}(718-963)$, the estimated maximum concentration of 4 μM achieved in the cell body in our experiments exceeded the concentration required for half-maximal binding to syntaxin in vitro (0.4 μM at optimal Ca^{2+} concentration of 10–30 μM, Sheng et al., 1996) by approximately 10-fold and caused 42% inhibition of synaptic transmission. However, in the in vivo experiments, there is a lower resting concentration of Ca^{2+} and competition with a high local concentration of Ca^{2+} channels, so a requirement for higher concentrations of peptide competitors is expected. Moreover, peptides that are thought to block the action of other key synaptic proteins also cause partial inhibition of synaptic transmission, even at much higher injected peptide concentrations. Thus, peptides from the C2 domains of synaptotagmin inhibited transmission by ">50%" at 20 mM (Bommert et al., 1993), peptides from α-SNAP inhibited by 72% to 84% at 20 mM (DeBello et al., 1995), fusion proteins from synaptobrevin inhibited by 20% during one injection at 190 μM and by 60% following two injections (Hunt et al., 1994), and peptides from synaptobrevin inhibited 36% to 45% following injection of 1 mM concentrations (Cornille et al., 1995). It

is likely that these partial effects and requirements for high concentrations reflect the limited concentration of peptides that can be introduced into the cell body (Cornille et al., 1995) or nerve terminal (Bommert et al., 1993; DeBello et al., 1995; Hunt et al., 1994) and the barriers to diffusion of the peptides to all of the presynaptic active zones that contribute to transmitter release, rather than inherent inability of these reagents to fully block fast synaptic transmission. Similarly, in our experiments, it is likely that the full effect of the synprint peptides is prevented by limitations of concentration and access to all of the active zones in the presynaptic terminals of the cultured SCGNs that form an extensive network of varicosities (Wakshull et al., 1979).

The simplest interpretation of our results is that inhibition of transmitter release by synprint peptides is due to competitive block of binding of the synaptic core complex to Ca^{2+} channels. Interaction of N-type Ca^{2+} channels with the synaptic core complex has a biphasic dependence on Ca^{2+} concentration with maximal binding at approximately 20 μM free Ca^{2+} (Sheng et al., 1996), a concentration similar to the threshold for initiation of transmitter release. Those in vitro binding data and the results of this functional study suggest that interaction of presynaptic Ca^{2+} channels with the synaptic core complex may play a key role in docking, priming, or early events in Ca^{2+}-dependent fusion of synaptic vesicles.

While synprint peptides have been shown to interrupt interactions of N-type Ca^{2+} channels with the synaptic core complex in vitro (e.g., Figure 1), it is possible that they also have effects on other interactions among synaptic proteins by competitively occupying critical binding regions of syntaxin and/or SNAP-25. In fact, recent experiments have shown that synprint peptides can bind to the C2b domain of synaptotagmin in vitro as well as

to syntaxin and SNAP-25, and Ca²⁺-dependent competition between the synprint peptide and syntaxin for binding to synaptotagmin is observed under these conditions (Z.-H. S. and W. A. C., unpublished data). These results suggest that the synprint site on N-type Ca²⁺ channels may undergo a complex series of binding interactions with multiple presynaptic proteins during the process of transmitter release. Our results provide direct evidence for these protein–protein interactions in vivo and for their importance in transmitter release. Inhibition of interactions of syntaxin or synaptotagmin with proteins other than the N-type Ca²⁺ channel by synprint peptides may also contribute to the effects of these peptides on synaptic transmission.

In addition to the importance of the interaction of N-type Ca²⁺ channels with the synaptic core complex for neurotransmitter release as suggested by this work, coexpression of syntaxin 1A with N-type Ca²⁺ channels consisting of α_{1B}, $\alpha 2\delta$, and β_3 in Xenopus oocytes promotes inactivation, shifting the voltage dependence of steady-state inactivation 20 mV toward more negative holding potentials and slowing recovery from inactivation 3-fold (Bezprozvanny et al., 1995). These results raise the possibility that interaction with the synaptic core complex may modulate Ca²⁺ channel function as well as position synaptic vesicles near Ca²⁺ channels for efficient transmitter release. We did not observe effects on Ca²⁺ currents due to blocking the interaction of syntaxin with N-type Ca²⁺ channels in our experiments (Figure 5), but we recorded Ca²⁺ currents in cell bodies where syntaxin may not be bound to N-type channels. If presynaptic Ca²⁺ channels are inhibited by interaction with syntaxin in sympathetic neurons as N-type Ca²⁺ channels are in Xenopus oocytes, the synprint peptides would be expected to increase Ca²⁺ currents and thereby increase the efficiency of transmitter release. This effect would counteract the inhibition of synchronous transmitter release by synprint peptides, but may contribute to the asynchronous transmitter release following repetitive stimulation and to the increased paired-pulse facilitation that we have observed. Thus, if syntaxin has the reported inhibitory effect on N-type Ca²⁺ channels in presynaptic terminals in vivo, the inhibitory effects of the synprint peptides reported here are likely to underestimate the importance of interaction of N-type Ca²⁺ channels with synaptic proteins in transmitter release.

Experimental Procedures

Fusion Proteins
His–fusion proteins containing the syntaxin- and SNAP-25-binding region of N-type Ca²⁺ channel subunit α_{1B}, L$_{II–III}$(718–859), L$_{II–III}$(718–963), L$_{II–III}$(832–963), and as a control, the corresponding region of the L-type Ca²⁺ channel subunit α_{1S}, L$_{II–III}$(670–800), were expressed and purified as described previously (Sheng et al., 1994). All recombinant proteins were expressed in the Escherichia coli BL26 cells, a protease-deficient strain (Novagen). The fusion proteins were extracted by mild sonication in PBS (50 mM Na-phosphate [pH 8.0], 300 mM NaCl plus protease inhibitors), solubilized with 1% Triton X-100 and 0.5% Sarkosyl (N-lauryl sarkosine) for 20 min on ice, purified by binding to Ni²⁺-charged NTA–agarose column (QIAGEN), and eluted with 20 mM imidazole in PBS. For biochemical experiments, the eluates were concentrated with Centriprep-10 filtration units (Amicon) and dialyzed in a 10 K cutoff dialysis cassette (Pierce) against PBS with 0.1% Triton X-100 and 0.1% glycerol. For peptide

injection experiments, the eluates were dialyzed against 150 mM potassium acetate, 10 mM HEPES (pH 7.3), with no glycerol or Triton X-100.

Isolation of N-Type Ca²⁺ Channel Complexes by Immunoprecipitation
Immunoprecipitation was performed in TBS buffer with a Ca²⁺-buffering system (50 mM Tris–HCl, 140 mM NaCl, 50 mM HEPES, 5 mM HEDTA, 3.52 mM CaCl₂, and 0.3% Triton X-100 [pH 7.2]), which produced 10 μM free [Ca²⁺] as estimated using the Max Chelator software (version 6.63). After a 3 hr incubation, the precipitates were immobilized to protein A–Sepharose beads and washed three times with TBS-Ca²⁺ buffer. Proteins bound to the beads were solubilized with SDS sample buffer at 95°C for 5 min, analyzed by SDS–tricine gradient gel (10%–20%; Novex), and finally blotted with monoclonal antibody 10H5 (Yoshida et al., 1992) against syntaxin 1.

Synaptic Transmission between SCGNs
SCG cells from 7-day postnatal rats were prepared as described previously (Mochida et al., 1994, 1995). Conventional intracellular recordings were made from two neighboring neurons, cultured for 3–4 weeks, using microelectrodes filled with 1 M potassium acetate (40–70 MΩ). Neuron pairs were selected by the proximity of their cell bodies. Postsynaptic responses (EPSPs) were recorded from one of the neurons when action potentials were generated in the other neuron by passage of current through an intracellular recording electrode. Experiments were carried out at 32°C–34°C. Neurons were superfused with modified Krebs' solution consisting of 136 mM NaCl, 5.9 mM KCl, 5.1 mM CaCl₂, 1.2 mM MgCl₂, 11 mM glucose, and 3 mM Na–HEPES (pH 7.4). Intracellular injection of fusion proteins was performed as described previously (Mochida et al., 1994, 1995). Fusion proteins were dissolved in the suction pipette solution: 150 mM potassium acetate, 5 mM Mg²⁺–ATP, 10 mM HEPES (pH 7.4). Fusion proteins were introduced into the presynaptic cell body by diffusion from a suction glass pipette (17–20 MΩ tip resistance). Fast Green FCF (5%, Sigma) was included in the peptide solution to confirm their entry into the presynaptic cell body. EPSPs were recorded once every 20 s (0.05 Hz). Electrophysiological data were collected and analyzed using software written by Dr. L. Tauc (CNRS, France). For Figure 2, the peak amplitudes of EPSPs were measured and averaged. The resultant values were smoothed by an eight-point moving average algorithm and plotted against recording time (one point/min) with t = 0 indicating the beginning of the presynaptic injection.

Ca²⁺ Currents
Whole-cell patch clamp recordings were obtained with standard techniques, as described previously (Mochida et al., 1995). Cell currents were recorded with Nihon Koden CEZ-2351 whole-cell clamp amplifier and analyzed on a pCLAMP system (Axon Instruments) in the presence of 0.2 μM nifedipine to reduce L-type Ca²⁺ currents. Fusion proteins were dissolved in the internal solution containing 130 mM cesium acetate, 5 mM Mg–ATP, 10 mM HEPES, and 10 mM EGTA (pH 7.4). The external solution contained 136 mM TEA, 5.9 mM CsCl, 5.1 mM CaCl₂, 11 mM glucose, and 3 mM Na–HEPES. Pipette resistances were 1.5–2 MΩ. For accurate determination of Ca²⁺ current, 5 μM La³⁺ was added and La³⁺-insensitive currents were subtracted digitally.

References

Augustine, G.J., and Neher, E. (1992). Neuronal Ca^{2+} signaling takes the local route. Curr. Opin. Neurobiol. *2*, 302–307.

Barrett, E.F., and Stevens, C.F. (1972). The kinetics of transmitter release at the frog neuromuscular junction. J. Physiol. (Lond.) *227*, 691–708.

Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science *257*, 255–259.

Bezprozvanny, I., Scheller, R.H., and Tsien, R.W. (1995). Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature *378*, 623–626.

Bommert, K., Charlton, M.P., DeBello, W.M., Chin, G.J., Betz, H., and Augustine, G.J. (1993). Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. Nature *363*, 163–165.

Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994). Protein–protein interactions contributing to the specificity of intracellular vesicular trafficking. Science *263*, 1146–1149.

Chapman, E.R., An, S., Barton, N., and Jahn, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. J. Biol. Chem. *269*, 27427–27432.

Cornille, F., Deloye, F., Fournié-Zaluski, M.-C., Roques, B.P., and Poulain, B. (1995). Inhibition of neurotransmitter release by synthetic proline-rich peptides shows that the N-terminal domain of vesicle-associated membrane protein/synaptobrevin is critical for neuro-exocytosis. J. Biol. Chem. *270*, 16826–16832.

DeBello, W.M., O'Connor, V., Dresbach, T., Whiteheart, S.W., Wang, S.S.-H., Schweizer, F.E., Betz, H., Rothman, J.E., and Augustine, G.J. (1995). SNAP-mediated protein–protein interactions essential for neurotransmitter release. Nature *373*, 626–630.

Dubel, S.J., Starr, T.V.P., Hell, J., Ahlijanian, M.K., Enyeart, J.J., Catterall, W.A., and Snutch, T.P. (1992). Molecular cloning of the α1 subunit of an ω-conotoxin-sensitive calcium channel. Proc. Natl. Acad. Sci. USA *89*, 5058–5062.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J. *13*, 5051–5061.

Heidelberger, R., Heinemann, C., Neher, E., and Matthews, G. (1994). Calcium dependence of the rate of exocytosis in a synaptic terminal. Nature *371*, 513–515.

Hunt, J.M., Bommert, K., Charlton, M.P., Kistner, A., Haberman, E., Augustine, G.J., and Betz, H. (1994). A post-docking role for synaptobrevin in synaptic vesicle fusion. Neuron *12*, 1269–1279.

Inoue, A., Obata, K., and Akagawa, K. (1992). Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. J. Biol. Chem. *267*, 10613–10619.

Lévèque, C., El Far, O., Martin-Moutot, N., Sato, K., Kato, R., Takahashi, M., and Seagar, M.J. (1994). Purification of the N-type calcium channel associated with syntaxin and synaptotagmin: a complex implicated in synaptic vesicle exocytosis. J. Biol. Chem. *269*, 6306–6312.

Llinas, R.R., Steinberg, I.Z., and Walton, K. (1981). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys. J. *33*, 323–351.

Llinas, R.R., Sugimori, M., and Silver, R.B. (1992). Microdomains of high calcium concentration in a presynaptic terminal. Science *256*, 677–679.

Mallart, A., and Martin, A.R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. J. Physiol. *196*, 593–604.

Mintz, I.M., Sabatini, B.L., and Regehr, W.G. (1995). Calcium control of transmitter release at a cerebellar synapse. Neuron *15*, 675–688.

Mochida, S., Kobayashi, H., Matsuda, Y., Yuda, Y., Muramoto, K., and Nonomura, Y. (1994). Myosin II is involved in transmitter release at synapses formed between rat sympathetic neurons in culture. Neuron *13*, 1131–1142.

Mochida, S., Saisu, H., Kobayashi, H., and Abe, T. (1995). Impairment of syntaxin by botulinum neurotoxin C1 or antibodies inhibits acetylcholine release but not Ca^{2+} channel activity. Neuroscience *65*, 905–915.

Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H., and Mikoshiba, K. (1996). Role of synaptotagmin C2 domains in neurotransmitter secretion and inositol high polyphosphate binding at mammalian cholinergic synapses. Neuroscience, in press.

O'Conner, V.M., Shamotienko, O., Grishin, E., and Betz, H. (1993). On the structure of the "synaptosecretosome": evidence for a neurexin/synaptotagmin/syntaxin/Ca^{2+} channel complex. FEBS Lett. *326*, 255–260.

O'Lague, P.H., Obata, K., Claude, P., Furshpan, E.J., and Potter, D.D. (1974). Evidence for cholinergic synapses between dissociated rat sympathetic neurons in cell culture. Proc. Natl. Acad. Sci. USA *71*, 3602–3606.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J. Cell Biol. *109*, 3039–3052.

Pusch, M., and Neher, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. Pflügers Arch. *411*, 204–211.

Rettig, J., Sheng, Z.-H., Kim, D.K., Hodson, C.D., Snutch, T.P., and Catterall, W.A. (1996). Isoform-specific interaction of the α_{1A} subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. Proc. Natl. Acad. Sci. USA, *93*, 7363–7368.

Robitaille, R., Adler, E.M., and Charlton, M.P. (1990). Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. Neuron *5*, 773–779.

Saisu, H., Ibaraki, K., Yamaguchi, T., Sekine, Y., and Abe, T. (1991). Monoclonal antibodies immunoprecipitating ω-conotoxin-sensitive calcium channel molecules recognize two novel proteins localized in the nervous system. Biochem. Biophys. Res. Commun. *181*, 59–66.

Sheng, Z.-H., Rettig, J., Takahashi, M., and Catterall, W.A. (1994). Identification of a syntaxin-binding site on N-type calcium channels. Neuron *13*, 1303–1313.

Sheng, Z.-H., Rettig, J., Cook, T., and Catterall, W.A. (1996). Calcium-dependent interaction of N-type calcium channels with the synaptic core-complex. Nature *379*, 451–454.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–323.

Stanley, E.F. (1993). Single calcium channels and acetylcholine release at a presynaptic nerve terminal. Neuron *11*, 1007–1011.

Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc. Natl. Acad. Sci. USA *85*, 4538–4542.

Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R., and Fox, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci. *11*, 431–438.

Wakshull, E., Johnson, M.I., and Burton, H. (1979). Postnatal rat sympathetic neurons in culture. I. A comparison with embryonic neurons. J. Neurophysiol. *42*, 1410–1425.

Westenbroek, R.E., Hell, J.W., Warner, C., Dubel, S.J., Snutch, T.P., and Catterall, W.A. (1992). Biochemical properties and subcellular distribution of an N-type calcium channel α1 subunit. Neuron *9*, 1099–1115.

Wu, L.-G., and Saggau, P. (1994). Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3–CA1 synapses of the hippocampus. J. Neurosci. *14*, 5613–5622.

Yoshida, A., Oho, C., Omori, A., Kawahara, R., Ito, T., and Takahashi, M. (1992). HPC-1 is associated with synaptotagmin and ω-conotoxin receptor. J. Biol. Chem. *267*, 24925–24928.

Zucker, R.S. (1993). Calcium and transmitter release. J. Physiol. (Paris) *87*, 25–36.