**Communication**

Interaction of SNARE Complexes with P/Q-type Calcium Channels in Rat Cerebellar Synaptosomes*

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P- and Q-type calcium channels, which trigger rapid neurotransmitter release at many mammalian synapses, are blocked by ω-conotoxin MVIIC. ω-Conotoxin MVIIC binding to rat cerebellar synaptosomes was not displaced by ω-conotoxins GVIA or MVIIA (K, >1 μM), which are selective for N-type calcium channels. Solubilized 125I-ω-conotoxin MVIIC receptors were specifically recognized by antibodies directed against α1A calcium channel subunits, proteins known to constitute a pore with P/Q-like channel properties. Antibodies against syntaxin 1, SNAP 25, and VAMP 2 (synaptobrevin) each immunoprecipitated a similar fraction (20–40%) of ω-conotoxin MVIIC receptors. Immunoprecipitation was not additive, suggesting that heterotrimeric (SNARE) complexes containing these three proteins interact with P/Q-type calcium channels. Immobilized monoclonal anti-syntaxin antibodies retained α1A calcium channel subunits of 220, 180 and 160 kDa monitored by immunoblotting with site-directed antibodies. Synaptotagmin was detected in channel-associated complexes, but not synaptoophysin, Rab 3A nor rat cysteine string protein. Trimeric SNARE complexes are implicated in calcium-dependent exocytosis, a process thought to be regulated by synaptotagmin. Our results indicate that these proteins interact with P/Q-type calcium channels, which may optimize their location within domains of calcium influx.

Neuronal calcium channels are heteromeric proteins constituted by an α1 subunit, which forms the voltage-gated transmembrane pore, associated with auxiliary α2,β and β subunits. Five genes encoding homologous α1 subunits (α1A–E) with different channel properties are expressed in the rat brain (reviewed by Snutch and Reiner (1992) and Birnbaumer et al. (1994)). α1C and α1D subunits each form 1,4-dihydropyridine-sensitive L-type channels, whereas α1B subunits constitute N-type channels that are specifically blocked by ω-conotoxins GVIA or MVIIA (ωGVIA, ωMVIIA).1 Heterologously expressed α1A subunits induce currents that are inhibited by ω-agatoxin IVA (ωAgalVA) and ω-conotoxin MVIIIC (ωMVIIIC).2 In contrast, associations between α1A or α1B subunits are preferentially coupled to the exocytosis of rapid neurotransmitters.

Neurotransmitter release from axon terminals is triggered by calcium entry through voltage-gated channels in the presynaptic plasma membrane. The sub-millisecond delay between calcium influx and exocytosis of the contents of synaptic vesicles implies that the calcium channels involved are in close proximity to release sites (Llinas et al., 1981; Adler et al., 1991). Synaptic transmission in mammals is generally insensitive to the antagonists that act at L-type calcium channels, but is inhibited by peptide neurotoxins that block N- or P/Q-type calcium channels (Takahashi and Momiyama, 1993; Wheeler et al., 1994; Mintz et al., 1995; Turner and Dunlap, 1995). Pharmacological evidence therefore suggests that channels containing α1A or α1B subunits are preferentially coupled to the exocytosis of rapid neurotransmitters.

Recent biochemical evidence suggests that direct interactions between N-type calcium channel proteins and the exocytotic machinery may contribute to excitation-secretion coupling. N-type calcium channels can associate with syntaxin and synaptotagmin (Leveque et al., 1992; Yoshida et al., 1992; Bennett et al., 1992; Leveque et al., 1994), two key proteins that contribute to the targeting and calcium-dependent fusion of synaptic vesicles at the plasma membrane (reviewed by Scheller and Bennett (1994) and Südhof (1995)). This interaction involves binding of syntaxin to a cytoplasmic loop of the α1B subunit (Sheng et al., 1994). In contrast, associations between syntaxin and L-type channels have not been detected (Yoshida et al., 1992; El Far et al., 1995).

ωAgalVA and ωMVIIIC inhibit a predominant component of transmitter release at most rapid mammalian synapses, by blocking calcium influx through presynaptic P/Q-type calcium channels (Takahashi and Momiyama, 1993; Wheeler et al., 1994; Mintz et al., 1995). However, biochemical evidence for an interaction between P/Q-type channels and the exocytotic complex has not been reported. We have therefore used 125I-ωMVIIIC to label calcium channels containing α1A subunits solubilized from rat cerebellar synaptosomes, and to explore their association with proteins of the secretory pathway. Our results indicate that a population of calcium channels containing α1A subunits can interact with a trimeric SNARE complex (Söllner et al., 1993a) composed of syntaxin 1, SNAP 25, and VAMP 2 (synaptobrevin).

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1 The abbreviations used are: ωGVIA, ω-conotoxin GVIA; ωMVIIA, ω-conotoxin MVIIA; ωAgalVA, ω-agatoxin IVA; ωMVIIIC, ω-conotoxin MVIIIC; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonic acid.
EXPERIMENTAL PROCEDURES

Materials—[\text{Nle}^{125}]\text{MVIIC}, \text{aMVIIC}, and \text{aMVIIA} were synthesized on an Applied Biosystems 431A apparatus. \text{aGVIA} was purchased from the Peptide Institute (Osaka, Japan), and Na\text{[Nle]}^{25} from DuPont NEN. Mono\text{[125]}\text{Jodo[Nle]}^{25}\text{MVIIC} (2210 Ci/mmol), hereafter designated as \text{125}\text{I-MVIIC} was prepared according to Martin-Moutot et al. (1995) and purified by high performance liquid chromatography on an analytical C18 column (Beckman). Monoclonal antibodies to synaptotagmin (mAb 1D12), syntaxin (mAb 10H5), and SNAP 2 (mAb BR05) and polyclonal antibodies against VAMP2 and calcium channel \text{a}1\text{A} subunits (rba-1 and rba-2) were prepared as described previously (Yoshida et al., 1992; El Far et al., 1995; Martin-Moutot et al., 1995). Monoclonal anti-synaptophysin (mAb 17IB5) antibodies were provided by Dr. S. Fujita, Mitsubishi Kasei Institute. Polyclonal antibodies were raised in rabbits against the carboxyl-terminal peptide (C)GPDLDQAPPHQD of the rat Rab 3A sequence.

Binding Assays—10 \mu g rat cerebellar P2 membranes in 0.1 ml of 25 mM Tris, 150 mM NaCl, 0.1% bovine serum albumin adjusted to pH 7.4 with HCl (TBSA) were incubated with 0.5 nm \text{125}\text{I-MVIIC} for 1 h at 30 \degree C. Membrane-bound radioactivity was measured after rapid filtration and washing on GF/C (Whatman) filters treated with 0.3% polyethyleneimine to trap and measure total \text{125}\text{I-ligand/receptor complex.} After 2 h at 4 \degree C, immune complexes were recovered by mixing for 1 h with Protein A-Sepharose 4BCL. Beads were recovered by centrifugation and washed in TBSA, and the immunoprecipitated radioactivity was quantified. Aliquots of the initial extract were filtered through GF/B filters (Whatman) treated with 0.3% polyethyleneimine to trap and measure total \text{125}\text{I-ligand/receptor complex.}

Anti-syntaxin antibodies (mAb 10H5) were covalently coupled to Protein A-Sepharose (Leveque et al., 1994). 4 ml of a CHAPS extract of cerebellar synaptosomes were batch-incubated overnight with 1 ml of immunoaffinity resin. Beads were loaded into a column, washed with 20 ml of 10 mM Tris, 0.2 mM sucrose, adjusted to pH 7.4 with HCl, and eluted with 0.1 M triethylamine, 0.2 M NaCl, 0.4% CHAPS adjusted to pH 10.5 with HCl. All steps were performed at 4 \degree C in buffers containing Complete protease inhibitor mixture (Boehringer Mannheim). SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (Martin-Moutot et al., 1995), using Protein A-peroxidase and an ECL detection kit (Amersham Corp.).

RESULTS AND DISCUSSION

\text{aConotoxins provide useful probes for the biochemical assay of voltage-dependent calcium channels.} \text{aGVIA and \text{aConotoxins (aMVIIC) are selective antagonists of N-type channels in which the transmembrane pore is constituted by an \text{a}1\text{B} subunit. In contrast,} \text{aMVIIC, which is highly homologous to \text{aMVIIC, blocks P, Q, and N-type calcium channels in neurons (Hillyard et al., 1992; Randall and Tsien, 1995, and inhibits currents induced by the heterologous expression of calcium channels containing \text{a}1\text{A} or \text{a}2\text{B} subunits (Sather et al., 1993; Grantham et al., 1994). Although micromolar concentrations of \text{aMVIIC display little selectivity and block both P/Q- and N-type channels, subnanomolar concentrations of \text{125}\text{I-MVIIC reveal binding sites pharmacologically distinct from those associated with} N\text{-type channels (Hillyard et al., 1992; Kristipati et al., 1994), and which are therefore likely to be constituted by P/Q-type channels (Martin-Moutot et al., 1995). \text{125}\text{I-MVIIC displayed specific high affinity binding to rat cerebellar synaptosomes (Fig. 1A), which was displaced by native \text{aMVIIC with a Ki of about 1 nM, but not by the selective N-type calcium channel antagonists} \text{aGVIA or aMVIIA (Ki > 1 \mu M). Conversely, using \text{125}\text{I-GVIA as a probe, significant inhibition was only obtained with relatively high concentrations of \text{aMVIIC (Ki > 30 nM; data not shown), presumably due to aMVIIC competing with \text{aGVIA at the same binding site on N-type calcium channels.} Nevertheless these data suggest that at sub-nanomolar concentrations, \text{125}\text{I-MVIIC labels binding sites that are distinct from those associated with \text{a}1\text{B subunits.}}}

Cerebellar membranes were labeled with \text{125}\text{I-aMVIIC, and radioligand-receptor complexes were solubilized using CHAPS. Approximately 50\% of these complexes were adsorbed by antibodies directed against a peptide specific to the \text{a}1\text{A sequence. Only 8\% were retained if antibodies were preincubated with the cognate \text{a}1\text{A peptide (Fig. 1B). \text{Anti-aGVIA antibodies reacted with specific 125I-aMVIIC binding sites as the amount of immunoprecipitated radioactivity was strongly reduced if labeling was performed in the presence of 0.3 \mu M \text{aMVIIC but not 0.3 \mu M aGVIA. No significant immunoprecipitation of 125I-aMVIIC binding sites was detected with antibodies directed against a1B (8 \pm 1\% or a1C (8 \pm 1\% subunits, although these antibodies recognize >50\% of 125I-aGVIA receptors (N-type channels) or [3H]PN200–110 receptors (L-type channels) respectively in analogous experiments (not shown).}

High affinity 125I-aMVIIC binding thus provides a specific assay for calcium channels containing \text{a}1\text{A subunits, and immunoprecipitation of solubilized receptors can be used to examine whether proteins implicated in exocytosis are associated with.
with these subunits. Antibodies against three proteins that form the core SNARE complex, syntaxin 1, SNAP 25, and VAMP2, each adsorbed 20–40% of solubilized 125I-ωMVIIC receptors, whereas non-immune IgG trapped <5% (Fig. 2A).

Recovery of 125I-ωMVIIC receptors by antibodies against other synaptic proteins implicated in the secretory pathway: synaptophysin, Rab 3A (Fig. 2A), and cysteine string protein (data not shown) was similar to that obtained with non-immune IgG, although these antibodies did immunoprecipitate their respective antigens (not shown).

Immunoadsorption with increasing concentrations of antibodies against syntaxin, SNAP 25, or VAMP reached a plateau level, indicating that a limited fraction of calcium channels is associated with each SNARE protein (Fig. 2A). If each SNARE protein interacts individually with a different fraction of calcium channels, immunoprecipitation by two antibodies combined should be additive. Alternatively, if all three SNARE proteins are associated with the same population of calcium channels, immunoprecipitation should not be additive. The results shown in Fig. 2B are consistent with the second hypothesis. When saturating amounts (20 μg) of two antibodies were combined in the same assay, the percentage of immunoprecipitation was similar to that obtained when anti-syntaxin antibodies were combined with control antibodies. These results suggest that SNAP 25 and VAMP are only associated with calcium channels that are bound to syntaxin, implying the interaction of a trimeric syntaxin-SNAP 25-VAMP complex with a significant fraction of 125I-ωMVIIC-labeled calcium channels.

The experiments illustrated in Fig. 2C were performed to verify that immunoprecipitation of 125I-ωMVIIC binding sites associated with N-type channels did not occur. In each case the amount of radioactivity adsorbed by the antibodies was strongly reduced by addition of 0.3 μM ωMVIIC, but not significantly affected by 0.3 μM ωGVIA, indicating that the detected interaction with SNARE complexes is specific for P/Q-type channels that contain α1A subunits. Synaptotagmin, a synaptic vesicle transmembrane protein that is thought to function as a calcium sensor in exocytosis, can associate with N-type calcium channels (Leveque et al., 1992, 1994; Yoshida et al., 1992) and SNARE complexes (Söllner et al., 1993b), by binding to syntaxin (Li et al., 1995). An antibody that recognizes synaptotagmin isoforms I and II also immunoprecipitated P/Q-type channels (Fig. 2C).

In order to confirm the association of α1A subunits with syntaxin, CHAPS extracts of cerebellar membranes were loaded onto an immunoaffinity matrix composed of a monoclonal anti-syntaxin antibody covalently coupled to Protein A-Sepharose 4BCL. After extensive washing, the column was eluted by a step to pH 10.5. Western blots of the recovered proteins were probed with anti-α1A antibodies, revealing several immunoreactive bands (Fig. 3, lane 1). These proteins were not detected when CHAPS extracts were loaded onto a control column (Fig. 3, lane 2) or when the antibodies were preincubated with an excess of the cognate α1A peptide (Fig. 3, lane 3). The 220-, 180-, and 160-kDa proteins have been detected in cerebellar homogenates using anti-α1A (rba-2) antibodies (Martin-Moutot et al., 1995), and similar size forms have also been reported by Westenbroek and colleagues (1995). Heterogeneity in the size of these α1A polypeptides expressed in the brain may result from alternative splicing or proteolytic processing. However, the 140-kDa protein was not detected in homogenates and is likely to be an α1A cleavage product, generated during affinity chromatography in spite of the presence of protease inhibitors. These results thus indicate that syntaxin interacts with α1A subunits of P/Q-type calcium channels.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Association of SNARE complexes with P/Q-type calcium channels. A, solubilized 125I-ωMVIIC receptor complexes were incubated with anti-syntaxin 1 (squares), anti-SNAP 25 (diamonds), anti-VAMP 2 (circles), anti-Rab 3A (triangles), and anti-synaptophysin (asterisks) antibodies or non-immune IgG (crosses), and the radioactivity recovered in immune complexes was counted (means ± S.D., n = 3) B, additivity was examined with 20 μg of each of the two indicated antibodies combined in the same assay (means ± S.D., n = 6). C, immunoadsorption assays were performed with 125I-ωMVIIC receptors labeled in the absence of native ω-conotoxin (shaded) or in the presence of 0.3 μM ωMVIIC (open) or ωGVIA (hatched) (means ± S.D., n = 6).
Cerebral synaptosomes were solubilized in CHAPS and applied to either monoclonal anti-syntaxin 1 antibodies (lanes 1 and 3) or non-immune mouse IgG (lanes 2 and 4) covalently coupled to Protein A-Sepharose. After washing, bound proteins were eluted by a step to pH 10.5 and analyzed by Western blotting with anti-α1A peptide antibodies (rbA-2) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the cognate α1A peptide.

Sheng et al. (1994) did not detect binding of fusion proteins containing part of the cytoplasmic loop (residues 723–868) linking homologous domains II-III of the α1A subunit to syntaxin 1A. Our data indicate that there are alternative sites of interaction between SNARE complexes and P/Q-type calcium channel subunits, although they have yet to be identified at the sequence level.

Current models of exocytosis are based on sequential protein-protein interactions leading to the assembly of a multi-molecular complex, located at the interface between a synaptic vesicle and the presynaptic plasma membrane (reviewed by Södhof (1995)). The core interaction concerns a vesicle membrane protein VAMP, which binds to two proteins that are predominantly located at the plasma membrane: syntaxin and SNAP 25. A second vesicle protein synaptotagmin can bind to proteinsoftheexocytoticcomplexcouldcontributetotherapidepolarization.

The calcium sensors that trigger vesicle fusion, and may also regulate the ability of channels to open in response to depolarization.

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