Association of Neuronal Calcium Channels with Modular Adaptor Proteins*

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Presynaptic voltage-gated calcium (Ca\(^{2+}\)) channels mediate Ca\(^{2+}\) influx into the presynaptic terminal that triggers synaptic vesicle fusion and neurotransmitter release. The immediate proximity of Ca\(^{2+}\) channels to the synaptic vesicle release apparatus is critical for rapid and efficient synaptic transmission. In a series of biochemical experiments, we demonstrate a specific association of the cytosolic carboxyl terminus of the N-type Ca\(^{2+}\) channel pore-forming \(\alpha_{1B}\) subunit with the modular adaptor proteins Mint1 and CASK. The carboxyl terminus of \(\alpha_{1B}\) binds to the first PDZ domain of Mint1 (Mint1-1). The proline-rich region present in the carboxyl terminus of \(\alpha_{1B}\) binds to the SH3 domain of CASK. Mint1-1 is specific for the E/D-PDZ domain-binding motif is present only in the “long” carboxyl-terminal splice variants of N-type (\(\alpha_{1B}\)) and P/Q-type (\(\alpha_{1A}\)) Ca\(^{2+}\) channels, but not in R-type (\(\alpha_{1R}\)) or L-type (\(\alpha_{1C}\)) Ca\(^{2+}\) channels. Our results directly link presynaptic Ca\(^{2+}\) channels to a macromolecular complex formed by modular adaptor proteins at synaptic junction and advance our understanding of coupling between cell adhesion and synaptic vesicle exocytosis.

Synapses are highly specialized structures. Proper localization of synaptic components is maintained via a complex network of protein-protein interactions, often mediated via modular adaptor proteins (1). In the postsynaptic cell the carboxyl termini of NMDA\(^{1}\) receptors, potassium channels and neurotransmitters bind to three PDZ domains of PSD-95 (2–4). Tripartite complexes composed of modular adaptor proteins CASK, Mint1, and Veli are present in the presynaptic terminal (5). Adaptor domains of these proteins are not involved in formation of the tripartite complex, leaving them free to recruit \(\beta\)-neurexins (via CASK-PDZ domain (6)) and other signaling molecules, which have not yet been identified.

N-type, P/Q-type, and R-type presynaptic Ca\(^{2+}\) channels mediate rapid Ca\(^{2+}\) influx into the presynaptic terminal that triggers synaptic vesicle fusion and neurotransmitter release (7, 8). The immediate proximity of Ca\(^{2+}\) channels to the synaptic vesicle release apparatus is critical for rapid and efficient synaptic transmission (9). We hypothesized that in addition to previously recognized interaction with t-SNARE protein syntaxin (10, 11), proper localization of Ca\(^{2+}\) channels in the presynaptic terminal could also be mediated via association with modular adaptor proteins.

Here we report specific association of N-type Ca\(^{2+}\) channel pore-forming subunit (\(\alpha_{1B}\)) carboxyl termini with the first PDZ domain of Mint1 and SH3 domain of CASK. Our results directly link presynaptic Ca\(^{2+}\) channels to the synaptic junction macromolecular complex and advance our understanding of coupling between cell adhesion and synaptic vesicle exocytosis.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids were constructed in prey pVP16-3 and bait pLexA for use as yeast-two hybrid vectors; in pGEX-KG for expression as GST fusion proteins; in pCMV5, HA-pCMV5, and FLAG-pCMV5 (NH\(_2\)-terminal epitope tags) for expression in COS7 cells. The following human \(\alpha_{1B}\) (12) carboxyl-terminal expression plasmids were generated (listed by encoded residue numbers): NC4 = 2201–2333, NC4-D2334X = 2201–2333, NC3 = 2021–2339, NC3-D2334X = 2021–2333, NC6 = 2021–2321, NC14 = 1809–2038. The Mint1 expression vectors encode the following residues of rat protein (13): Mint1-1 = 647–751, Mint1-2 = 745–839, Mint1-3 = 1–839. CASK-SH3 construct is 947–1242 of human sequence (6), PSD95-SH3 = 386–554 of rat sequence (3). NMDAR2A, NRX1A (pBTM116Nea), Mint1-PDZ1-2, and Mint1-PBT constructs were described previously (4–6). Mutant NC constructs were generated by PCR and sequenced.

Yeast Two-hybrid Methods—L40 yeast strain was transformed with a corresponding pair of bait and prey plasmids; the background of prey interaction was determined from \(\beta\)-galactosidase activity in the yeast cell extracts using o-nitrophenol-\(\beta\)-D-galactoside substrate as previously described (4), normalized per total protein content of cell lysate and presented in relative units.

In Vitro Binding Assays—GST fusion proteins were expressed in BL21 Escherichia coli strain, purified from bacterial lysates on glutathione-agarose beads and used in pull-down experiments. Mint, CASK, and NC proteins were expressed in COS7 cells by DEAE-dextran transfection (14). 72 h after transfection, cells were collected and solubilized for 30 min at 4 \(^\circ\)C in extraction buffer containing 0.5% Triton X-100, 20 mM imidazole, pH 6.8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors. Extracts were clarified by centrifugation and incubated for 1 h at 4 \(^\circ\)C with the corresponding GST fusion protein. Beads were washed three times with the extraction buffer and the beads harbored proteins were separated on SDS-polyacrylamide gel electrophoresis and probed with the anti-Mint1 polyclonal antibody or monoclonal anti-HA.11 (Berkley Antibody Company) and anti-FLAG-M5 (Sigma) antibodies using ECL (Fierce) detection method.

Brain Immunoprecipitations—Anti-Mint1 polyclonal antibody, raised against full-length GST-Mint1, was described previously (13); anti-NN antibody was raised against rat \(\alpha_{1B}\) amino-terminal peptide (MVRFEILGDDGGRYYG). Rat brain synaptosomes (PSD) were prepared as described (15) and solubilized for 1.5 h at 4 \(^\circ\)C in extraction buffer containing 25 mM HEPES/NaOH, pH 7.2, 160 mM sucrose, 2 mM EDTA, 10% glycerol, 1% CHAPS with addition of protease inhibitors. The lysate was clarified by centrifugation at 100,000 g for 40 min, and the supernatant was used in immunoprecipitations. Brain samples of rat lysate were preincubated with 0.5 mM \(\beta\)-GP via crosslinking. Anti-Mint1 monoclonal antibody or anti-FLAG-M5 (Sigma) antibodies using ECL (Fierce) detection method.

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immune sera. Beads were pelleted by centrifugation, washed three times with 5-fold diluted extraction buffer, and 125I radioactivity in the pellet was quantified by γ counting.

RESULTS AND DISCUSSION

Putative SH3 and PDZ Domain Binding Motifs in the Sequence of Neuronal Ca2+ Channels—To define specific regions of channel sequence that may potentially mediate the interactions of synaptic Ca2+ channels with cytosolic proteins, we performed primary sequence alignment of carboxyl-terminal regions of human α1B (N-type), α1A (P/Q-type, abbreviated as Q-type), and α1E (R-type) subunits (12, 16, 17) at Clustalw-EBI site. Three aligned sequences were highly conserved for the period of about 200 amino acids following the last transmembrane region (TMR-IVS6) and then sharply deviated from each other for most of the remaining sequence (Fig. 1A). We identified two conserved patches embedded within this highly variable region. The first conserved region is enriched with proline residues and contains a number of potential SH3-domain binding motifs PXXP (18). This region encompasses the part of the channel that has been recently implicated in modulatory interactions with G-protein βγ subunits (19) and with auxiliary β subunits (20). The second conserved region is the very carboxyl terminus of neuronal Ca2+ channels pore-forming subunits (Fig. 1A), all of which end with DXXC-COOH motif. PDZ domains bind to carboxyl termini of target proteins (1), and we reasoned that the conserved DXXC-COOH motif may correspond to a novel PDZ domain binding consensus sequence.

Mint1-1 PDZ Domain Specifically Binds N-type Ca2+ Channel Carboxyl Termini—To identify a PDZ domain that associates with the conserved DXXC-COOH motif, we examined the interaction of NC4 bait with a prey library of PDZ domains in liquid yeast two-hybrid assays. PDZ domains of PSD93, SAP102, PSD95, NOS, GRIP, Z01, Dlg, spinophilin, CASK, and Mint1 were tested. The strongest interaction of NC4 bait was observed with the first PDZ domain of Mint-1 (Mint1-1).

Mint1-1 (13) is a modular adapter protein containing a Munc18-interacting domain (MI), a CASK-interacting domain (CI), a PTB domain, and two "orphan" PDZ domains for which ligands have not yet been identified (Fig. 1B). A strong association of NC4 bait with Mint1-1 domain was observed (553 ± 36 arbitrary units, n = 3; Fig. 1B), whereas binding to the Mint1-2 domain was significantly weaker (197 ± 52 arbitrary units, n = 3). As expected for PDZ domain-mediated association (1), the interaction with Mint1-1 is critically dependent on the most carboxyl-terminal region of the channel sequence, as Mint1-1 does not bind to truncated NC4-D2334X protein and mutated NC4-W2338A protein (Trp2338 → Ala) (Fig. 1B). Mint1-1 did not bind to the NMDA receptor and neurexin 1α carboxyl-terminal baits (Fig. 1B).

To independently test for the ability of the carboxyl termini of N-type Ca2+ channels to bind Mint1, we expressed full-length Mint1 in COS cells and performed pull-down experiments with GST-NC4 protein. Anti-Mint1 antibody detected an immunoreactive band with an apparent molecular weight corresponding to Mint1-1 in samples incubated with recombinant GST-NC4, but not with GST alone or with GST-NC4-D2334X (Fig. 1C). Interaction of Mint1-1 with GST-NC4 could be disrupted by addition of 1 mM competitive synthetic peptide HDQDHWC (Fig. 1C), corresponding to the eight carboxyl-terminal amino acids of α1B sequence.

In a complementary series of experiments, we expressed an HA-tagged NC4 protein (HA-NC4) in COS cells and determined that GST-Mint1-PDZ1–2, but not GST alone or GST-Mint1-PTB, precipitated an anti-HA immunoreactive band from the COS cell lysate (Fig. 1D). In agreement with the yeast two-hybrid data (Fig. 1B), GST-Mint1-PDZ1–2 did not precipitate

![Fig. 1](image)

**Fig. 1.** Mint1-1 PDZ domain specifically binds N-type Ca2+ channel carboxyl termini. A, general organization of neuronal voltage-gated Ca2+ channels carboxyl termini. PXXP marks conserved proline-rich region, DXXC-COOH marks the conserved carboxyl-terminal motif. Amino acid numbers correspond to human α1a (N-type) sequence (12). B, N-type Ca2+ channel carboxyl termini bind Mint1-1 in yeast two-hybrid assay. Domain structure of Mint1 (5, 13). The strength of interaction between carboxyl-terminal baits and Mint1-1 PDZ domain was measured in yeast two-hybrid liquid assay. β-Galactosidase activity is indicated in arbitrary units ± S.E. (n = 3). C and D, carboxyl termini of N-type Ca2+ channels bind Mint1 in vitro. C, GST protein, GST-NC4, and GST-NC4-D2334X fusion proteins were used to pull down recombinant Mint1 from COS cell lysate. The first lane contains 0.1% of the amount of COS cell extract used for pull-down experiments in all other lanes. Anti-Mint1 polyclonal antibodies are used for detection. D, GST protein, GST-Mint1-PTB, and GST-Mint1-PDZ1–2 fusion proteins were used to pull down recombinant HA-NC4, HA-NC4-D2334X, and HA-NC4-W2338A proteins from COS cell lysate. Input lanes contain 1/30 of the amount of COS cell extract used in pull-down experiments with the corresponding protein. Anti-HA monoclonal antibodies were used for detection. The experiments presented on C and D were repeated in triplicate with similar results. E, anti-Mint1 polyclonal antibodies specifically immunoprecipitate 125I-ω-GVIA conotoxin binding sites from rat brain. A number of specific 125I-ω-GVIA conotoxin binding sites precipitated by anti-Mint1 antibody are shown as percentage to a number of sites precipitated by anti-NN antibody in parallel experiments. Sequence of competitive NC peptide is RGGRRQPLQPTLPRPSITGGHPDQDHWC. The results are shown as mean ± S.E. (n = 3). Similar results were obtained with two different rat brain synaptosomal preparations.

HA-NC4-D2334X or HA-NC4-W2338A mutant proteins from the COS cell lysate (Fig. 1D).

To determine an ability of N-type Ca2+ channels to associate with Mint1 in vitro, we performed immunoprecipitation experiments with rat brain synaptosomes. We found that anti-Mint1 polyclonal antibodies specifically precipitated a significant number of 125I-ω-GVIA binding sites (Fig. 1E). An ability of anti-Mint1 antibodies to precipitate 125I-ω-GVIA binding sites was reduced by 70% in the presence of 1 mM NC peptide (Fig. 1E), composed of putative SH3 and PDZ binding motifs in
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Fig. 2. Ligand specificity of Mint1-1 PDZ domain. A, Mint1-1 is specific for E/D-X-W-C/S-COOH consensus sequence. A series of mutant NC4 baits was generated and tested for an ability to interact with Mint1-1 in liquid yeast two-hybrid assay. \( \beta \)-Galactosidase activity is indicated in arbitrary units \( \pm \) S.E. (n \( \geq \) 3). B, Mint1-1 is specific for long splice variants of N-type and Q-type Ca\textsuperscript{2+} channels. The strength of NC4-based chimeric bait interactions with Mint1-1 was analyzed in liquid yeast two-hybrid assay. \( \beta \)-Galactosidase activity is indicated in arbitrary units \( \pm \) S.E. (n \( \geq \) 3). Carboxyl-terminal sequences of splice variants of various pore-forming Ca\textsuperscript{2+} channel subunits, shown roughly to scale, were retrieved from the GenBank\textsuperscript{TM} data base. Species and accession numbers are as follows: human N-type, M94172 and M94173 (12); human Q-type, U79668, U79664, and U79666 (16); rabbit R-type, 2011160A and 2011160B (22); cardiac rabbit \( \gamma \)-type, 1512308A (23). Sites of leucine (tg\textsuperscript{2}) mutation in mouse \( \alpha_{1A} \) gene (26) and SCA6-causing polyglutamine expansion \((Q_{6})\) in human \( \alpha_{1A} \) subunit (16) are indicated. C, the E/D-X-W-C/S-COOH motif is evolutionary conserved in the pore forming subunit of N-type and P/Q-type voltage-gated Ca\textsuperscript{2+} channels. Species and accession numbers are as follows: \( \alpha_{1A} \) (N-type), M94172 (human); Q02294 (rat); D14157 (rabbit); \( \alpha_{1B} \) (P/Q-type), U79666 (human); AF051526 (rat); rabbit splice variant is predicted by analogy with the human channel from X57869 nucleotide data base entry; doe-4, 477714 (\( D. \) ommata (27)); SQCC1, D66600 (\( D. \) bleckerei (28)); Dmca1A, PF1645 (\( D. \) melanogaster (29)).

N-type Ca\textsuperscript{2+} channel carboxyl termini. We concluded that at least 25% of rat brain N-type Ca\textsuperscript{2+} channels is complexed with Mint1 in vivo, presumably via carboxyl-terminal association with Mint1-1 PDZ domain.

Ligand Specificity of Mint1-1 Defines Class III PDZ Domain Family—The carboxyl termini of N-type channels specifically bind Mint1-1 PDZ domain (Fig. 1). To delineate the structural determinants responsible for this interaction, we mutated the last six carboxyl-terminal amino acids in the NC4 bait and measured the strength of interaction of resulting mutants with the Mint1-1 PDZ domain in a yeast two-hybrid assay. A series of truncation, alanine substitution, and charge reversal mutants was analyzed (Fig. 2A). The obtained data set leads us to define the E/D-X-W-C/S-COOH consensus motif as the Mint1-1 recognition sequence. This sequence is different from the previously characterized class I (S/T-X-Y-COOH) or class II (E/D-Y-COOH) PDZ domains ligands (21) (\( \Phi \) stands for a hydrophobic residue). We propose that Mint1-1/Ca\textsuperscript{2+} channels interactions define a novel class of PDZ domains (class III).

Do all Ca\textsuperscript{2+} channels bind Mint1-1? To answer this question, we tested the ability of all known human N-type (12), human Q-type (16), rabbit R-type (22) Ca\textsuperscript{2+} channel splice variants and rabbit cardiac \( \gamma \)-type (23) Ca\textsuperscript{2+} channel to bind Mint1-1 in yeast two-hybrid liquid assays by replacing the last six amino acids of the NC4 bait with the last six amino acids of corresponding channel subtype or splice isoform. From all the baits tested, only baits corresponding to the “long” splice variants of human N-type (NC4-DQDHWC) and Q-type (NC4-SDDDWCC) interacted with Mint1-1 (Fig. 2B). This exquisite specificity of Mint1-1 points to a potential biological relevance of Mint1-1/Ca\textsuperscript{2+} channels interaction.

Searches of sequence data bases reveal that the E/D-X-W-C/S-COOH motif is present exclusively in N-type and Q-type Ca\textsuperscript{2+} channel pore-forming subunits. With the exception of rabbit N-type Ca\textsuperscript{2+} channel, the E/D-X-W-C/S-COOH motif is evolutionarily conserved in the sequence of Ca\textsuperscript{2+} channels from Drosophila melanogaster to humans (Fig. 2C), indicating its importance for their biological function. Because of Trp \( \rightarrow \) Lys substitution in the –1 position, the R-type Ca\textsuperscript{2+} channel carboxyl terminus does not bind to Mint1-1 (Fig. 2B). The E/D-X-W-C/S-COOH motif is conserved in R-type Ca\textsuperscript{2+} channel sequence from Discopyge onnata to humans (data not shown). It is plausible that another member of class III PDZ domain family may specifically associate with this sequence.

CASK SH3 Domain Binds Proline-rich Region in N-type Ca\textsuperscript{2+} Channel Carboxyl Terminus—In brain Mint1 is strongly associated with CASK (5). CASK (6) is another modular adaptor protein, which consists of Mint1-interacting CaM kinase domain, Veli-interacting domain (VI), \( \beta \)-neurexin binding PDZ domain (6), SH3 domain, 4.1-band protein binding domain (4.1), and guanilate kinase (GK) domain (Fig. 3A). A ligand of the CASK SH3 domain has not yet been identified. We reasoned that the proline-rich region in neuronal Ca\textsuperscript{2+} channel carboxyl termini (Fig. 1A) may associate with the CASK-SH3...
domain. To test this hypothesis, we performed GST-CASK-SH3 pull-down experiments with HA-tagged NC3-D2334X, NC4, NC6, and NC14 (Fig. 3B) proteins expressed in COS cells. We found that GST-CASK-SH3 interacted strongly with NC3-D2334X and NC6, but not with NC4 and NC14 fragments (Fig. 3A), in agreement with the location of proline-rich region in the N-type channel carboxyl terminus. Similar results were obtained in pull-down experiments with full-length GST-CASK (data not shown). The NC3-D2334X fragment associated strongly with GST-CASK-SH3, but only weakly with GST-PSD95-SH3 (Fig. 3B). A signal with a shorter NC6 fragment was observed only with GST-CASK-SH3 (Fig. 3B) and not with GST-PSD95-SH3. Thus, we concluded that the proline-rich region of the N-type Ca\(^{2+}\) channel carboxyl terminus interacts strongly and specifically with the SH3 domain of CASK. Six putative SH3 domain binding motifs (PXXP) are present within this proline-rich region in N-type Ca\(^{2+}\) channel sequence; however, we failed to assign a role of major CASK-SH3 domain binding site to any one of these motifs in further deletion mutagenesis experiments (data not shown). In addition, binding experiments we also established an ability of N-type Ca\(^{2+}\) channel carboxyl terminus to support simultaneous in vitro interactions with Mint1 and CASK (data not shown).

### Macromolecular Signaling Complex in the Synapse—Neuronal Ca\(^{2+}\) channel carboxyl-terminal regions bind to the first PDZ domain of Mint1 (Figs. 1 and 2) and to the SH3 domain of CASK (Fig. 3). In turn, Mint1 and CASK are tightly bound to each other within the tripartite presynaptic protein complex (5). These interactions create a potential for formation of Ca\(^{2+}\) channel-Mint1-CASK ternary complex and recruitment of long N-type and Q-type Ca\(^{2+}\) channel splice variants to a macromolecular signaling complex assembled at synaptic junction (Fig. 4). By means of extracellular neuroxin-neurologin interaction (24, 25), the proposed presynaptic signaling complex is kept in register with the postsynaptic complex built around PSD-95 (4), which includes NMDA receptors and K\(^{+}\) channels (2, 3). By keeping presynaptic Ca\(^{2+}\) channels, fusion-competent synaptic vesicles, and postsynaptic receptors in alignment, the proposed complex (Fig. 4) may account for the extremely high speed and efficiency of synaptic transmission in central synapses.

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