Evidence for a 95 kDa Short Form of the α_{1A} Subunit Associated with the ω-Conotoxin MVIIC Receptor of the P/Q-type Ca^{2+} Channels

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Neuronal voltage-dependent Ca^{2+} channels have been isolated previously and shown to contain a primary α_1 pore-forming subunit as well as auxiliary $\alpha_2\delta$ and β subunits, in addition to an uncharacterized 95 kDa protein. In the present study, using multiple approaches, we have extensively characterized the molecular structure of the 95 kDa protein. Separation of the P/Q- and N-type neuronal Ca^{2+} channels showed that the 95 kDa protein is associated exclusively with the ω-Conotoxin MVIIC receptor of the P/Q-type channels. Analysis of purified synaptic plasma membranes and the isolated P/Q-type channels, using α_{1A}-specific antibodies, suggested a structural relationship between the α_{1A} subunit and the 95 kDa protein. This finding was supported by protein–protein interaction data, which revealed that the β subunit can associate with the 95 kDa protein in addition to the α_{1A} subunit. Changes in electrophoretic mobility after enzymatic treatment with Endo F indicated that the 95 kDa protein is glycosylated. Furthermore, microsequencing of the 95 kDa protein yielded 13 peptide sequences, all of which are present in the first half of the α_{1A} subunit up to amino acid 829 of the cytoplasmic linker between repeats II and III. Taken together, our results strongly suggest that the 95 kDa glycoprotein associated with the P/Q-type Ca^{2+} channels is a short form of the α_{1A} subunit.

Key words: P/Q-type Ca^{2+} channels; 95 kDa subunit; α_{1A} subunit; N-type Ca^{2+} channel; β subunit; ω-Conotoxin MVIIC; ω-Conotoxin GVIA

Voltage-dependent Ca^{2+} channels are essential for controlling cell–cell communication in the CNS (for review, see Wheeler et al., 1994a; Dunlap et al., 1995). In particular, N- and P/Q-type Ca^{2+} channels have been shown to play a central role in regulating neurotransmitter release (Luebke et al., 1993; Takahashi and Momiyama, 1993; Turner et al., 1993; Wheeler et al., 1994b) via direct interactions with proteins of the synaptic vesicle docking/fusion complex at the nerve terminal (Sheng et al., 1994, 1996; Mochida et al., 1996; Rettig et al., 1996). These channels can be distinguished electrophysiologically and pharmacologically by using two naturally occurring neurotoxins, ω-Conotoxin GVIA (ω-Ctx GVIA; Boland et al., 1994; Turner and Dunlap, 1995) and ω-Conotoxin MVIIC (ω-Ctx MVIIC; Hillyard et al., 1992; Turner and Dunlap, 1995; McDonough et al., 1996).

Extensive biochemical studies have shown that neuronal N- and P/Q-type Ca^{2+} channels are heteromultimeric complexes of α_1, $\alpha_2\delta$, and β subunits (Ahlijanian et al., 1990; McEnery et al., 1991; Witcher et al., 1993a,b; Leveque et al., 1994; Martin-Moutot et al., 1995; Liu et al., 1996). Analysis of the subunit composition also has shown the presence of a fourth polypeptide (95–110 kDa) of unknown properties (McEnery et al., 1991; Witcher et al., 1993a,b; Leveque et al., 1994).

Functional differences between N- and P/Q-type Ca^{2+} channels are attributed to several factors, including the expression of distinct α_1 subunit proteins and the selective association of auxiliary subunits. Molecular cloning has revealed different neuronal Ca^{2+} channel α_1 genes (Snutch and Reiner, 1992). cDNA of these α_1 subunits has been isolated, and its functional expression has revealed properties that fall into the P/Q and N categories (α_{1A} and α_{1B}, respectively; Mori et al., 1991; Fujita et al., 1993). Likewise, the molecular properties of the N-type channels have been closely examined recently by using a monoclonal antibody raised specifically against the cytoplasmic II–III loop of the α_{1B} subunit (Scott et al., 1996). The results of this investigation have revealed extensive β subunit heterogeneity of the N-type channels. Parallel biochemical studies on P/Q-type channels, using ^{125}I-ω-Ctx MVIIC and a polyclonal antibody to the α_{1A} subunit, disclosed similar diversity of β subunits associated with these channels (Liu et al., 1996).

Although extensive studies on the α_1, $\alpha_2\delta$, and β subunits of voltage-dependent Ca^{2+} channels have been performed (for review, see Catterall, 1995; De Waard et al., 1996), little is known about the structure of the 95 kDa protein associated with neuronal Ca^{2+} channels. To investigate the molecular properties of this protein, we separated the N- and P/Q-type channels, using site-directed monoclonal antibodies. This not only has allowed us to examine the subunit composition of both of these channel types but also has permitted us to investigate the structure of the 95 kDa protein by a number of biochemical techniques. The identity of this protein ultimately was established by protein microsequenc-

Received Sept. 10, 1997; revised Oct. 28, 1997; accepted Oct. 31, 1997.

R.F. is supported by a Human Frontier Science Program postdoctoral fellowship. K.P.C. is an Investigator of the Howard Hughes Medical Institute (HHMI). The protein sequencing was performed by Dr. C. A. Slaughter and C. Moomaw at the HHMI Biopolymers Facility (University of Texas Southwestern Medical Center, Dallas, TX) and by R. F. Cook and N. Thorngren at the Biopolymers Laboratory, Center for Cancer Research (MIT, Cambridge, MA). Monoclonal antibody CC 18 was raised in collaboration with Dr. V. A. Lennon (Mayo Clinic, Rochester, MN). We thank J. C. Miller for his expert technical assistance. We are also grateful to C. Leveille, D. Venzke, and Drs. G. Biddlecome, K. Bielefeldt, and C. A. Gurnett for critically reading this manuscript.

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 0270-6474/98/180641-07$05.00/0

ing. Hence, using multiple approaches, we show conclusively in this report that the 95 kDa glycoprotein associates with the P/Q-type Ca^{2+} channels and is a short form of the α_{1A} subunit.

MATERIALS AND METHODS

Materials. ^{125}I-ω-Ctx GVIA, [^{35}S]methionine, and the enhanced chemiluminescence (ECL) kit were obtained from Amersham Life Science (Arlington Heights, IL). ^{125}I-ω-Ctx MVIIC was from DuPont NEN (Boston, MA). Digitonin, purchased from ICN Biomedicals (Costa Mesa, CA), was purified as previously described (Leung et al., 1987). Other biochemicals used were protein G-Sepharose (Pharmacia Biotech, Piscataway, NJ), horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN), Hydrazide Avidgel (Unisyn Technologies, Hopkinton, MA), and peptide *N*-glycosidase F (Endo F; Oxford GlycoSystems, Bedford, MA). All other chemicals were of reagent grade.

Separation of the N- and P/Q-type Ca^{2+} channels. Rabbit brain membranes (~1 gm of protein) were prepared as detailed elsewhere (Witcher et al., 1993b), and the channels were extracted by solubilization in 10 mM HEPES-NaOH, pH 7.4, 0.5 M NaCl, and five protease inhibitors (0.23 mM phenylmethylsulphonyl fluoride, 0.64 mM benzamidine, 1 mM leupeptin, 0.7 mM pepstatin A, and 76.8 nM aprotinin) containing 0.6% digitonin for 1 hr at 4°C. After centrifugation at 35,000 rpm for 37 min in a 45 Ti rotor, the detergent extract was diluted twofold with ice-cold deionized water and applied to a heparin agarose column (50 ml) preequilibrated with 5 column volumes of buffer A (10 mM HEPES-NaOH, pH 7.4, 0.1 M NaCl, and protease inhibitors) containing 0.1% digitonin at a flow rate of 5 ml/min. The column was washed extensively with buffer A and eluted in the same buffer containing 0.7 M NaCl, collecting 75 ml (heparin pool). Then the enriched channels were incubated overnight with the monoclonal antibody (mAb) CC 18 (α_{1B}-specific antibody; Scott et al., 1996) coupled to Hydrazide Avidgel (~2 ml of settled resin) prepared according to the manufacturer's instructions. The resin was washed extensively with buffer A and eluted with 50 mM glycine-HCl, pH 2.5, containing 0.6 M NaCl and 0.1% (w/v) digitonin (7.5 ml). The channels were neutralized immediately with 2 M Tris-HCl, pH 8.0 (1 ml). Then the void of the mAb CC 18 column (which is devoid of N-type channels) was incubated for 3 hr at 4°C with the mAb $VD2_1$ (β subunit-specific antibody; Witcher et al., 1993a) in a Hydrazide Avidgel column (~2 ml of settled resin), washed, and eluted as mentioned above. The isolated N- and P/Q-type channels were concentrated in a Centricon 30 (Amicon, Beverly, MA) by centrifugation at 5000 rpm for 2 hr at 4°C. The subunit composition was analyzed by SDS-PAGE and immunoblotting.

ω-Ctx binding to neuronal Ca^{2+} channels. N- and P/Q-type channel binding activity was assayed with ^{125}I-ω-Ctx GVIA and ^{125}I-ω-Ctx MVIIC, respectively, at different stages throughout the separation, as detailed elsewhere (Liu et al., 1996). Briefly, aliquots (50 μl) of solubilized membrane extracts were resuspended in a total volume of 300 μl of binding buffer (10 mM HEPES, pH 7.4, 0.2 mg/ml bovine serum albumin, 100 mM NaCl, and protease inhibitors) and incubated with a saturating concentration (1 nM) of ^{125}I-ω-Ctx GVIA for 1 hr at room temperature. The receptor–ligand complexes were collected and washed rapidly with ice-cold binding buffer on Whatman GF/B filters with a cell harvester (Brandel, Gaithersburg, MD). Nonspecific binding was determined by the addition of 100-fold excess nonradioactive ω-Ctx GVIA 10–15 min before the addition of the radiolabeled toxin. Specific binding was calculated by subtracting nonspecific binding from total binding. ω-Ctx MVIIC binding was assayed similarly; however, in this case the binding buffer also contained 0.1 mM EDTA and 0.1 mM EGTA.

SDS-PAGE and immunoblot analysis. Samples were analyzed by SDS-PAGE on 3–12% gradient gels, using the Laemmli buffer system (Laemmli, 1970). After electrophoresis the SDS gels were transferred to nitrocellulose membranes and immunoblotted as described previously (Witcher et al., 1993a; Scott et al., 1996). In short, the membranes were incubated overnight with immunoaffinity-purified polyclonal antibodies to fusion proteins containing specific regions of the α_1 subunit (amino acids 779–969, Sheep 37) or the 95 kDa protein (Sheep 46; Witcher et al., 1993a). The specific protein bands were detected with either the horseradish peroxidase or ECL detection method (according to the manufacturers' instructions). Synaptic plasma membranes were prepared from rabbit brain by using the method of Jones and Matus (1974).

Overlay assay of the [^{35}S]-β_3 subunit on the isolated P/Q-type Ca^{2+} channel. The Ca^{2+} channel β_3 subunit was translated *in vitro* and labeled with [^{35}S]methionine, using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI) as described elsewhere (Pragnell et al., 1994). P/Q-type channels from a single purification were concentrated, and the subunits were resolved on a 3–12% SDS gel. After electrophoresis the proteins were transferred onto nitrocellulose, and the membrane was blocked by incubation for 1 hr in blocking buffer (5% bovine serum albumin and 0.5% fat-free milk prepared in PBS, pH 7.5). Then the membrane was incubated with [^{35}S]-β_3 subunit (1×10^6 cpm) overnight at 4°C in the blocking buffer. To remove unbound [^{35}S]-β_3 subunit, we washed the membrane extensively with blocking buffer for 1 hr at room temperature before completely drying and exposing it to x-ray film.

Deglycosylation of the P/Q-type Ca^{2+} channels. P/Q-type Ca^{2+} channels from one isolation procedure were concentrated to 100 μl, using a centrifugal concentrator (Centricon 30; Amicon) and boiled in the presence of 1% SDS for 3 min to fully unfold and denature the protein. The sample was diluted fivefold to a final concentration of 0.2% SDS with water. Triton X-100 (final concentration 1%) and protease inhibitors were added before deglycosylation with 3 U of Endo F and incubation at 37°C overnight. The reaction was halted by the addition of Laemmli sample buffer, followed by SDS-PAGE analysis and Western blotting, using an anti-95 kDa protein-specific antibody (affinity-purified sheep polyclonal antibodies) directed to the II–III loop of the α_1 (amino acids 779–969; Witcher et al., 1993a).

Microsequencing of the 95 kDa protein. Amino acid sequence information was obtained from the 95 kDa protein, using two different methods. The first involved sequencing the 95 kDa protein from a polyvinylidene difluoride transfer membrane (Immobilon-P; Millipore, Bedford, MA). Briefly, the molecular composition of the P/Q-type channels was resolved by SDS-PAGE and the gel electrotransferred to the Immobilon-P membrane. The protein band corresponding to the 95 kDa protein was excised. Then the immobilized protein was digested with trypsin. Peptides that were released were separated by reverse-phase HPLC on a 2.1 × 100 mm RP-300 column (Perkin-Elmer, Foster City, CA), using a mobile phase of 0.1% trifluoroacetic acid. Elution was accomplished with a 100 min gradient of 0–70% acetonitrile. Peptides were subjected to automated Edman degradation, using an Applied Biosystems model 477A amino acid sequencer (Foster City, CA) with standard manufacturer's programming and chemicals. The second method for acquisition of peptide sequence information involved the generation of peptides by digesting the 95 kDa protein band directly excised from the gel, using endoproteinase lys-C (from *Acromobacter lyticus*; Wako Chemicals, Richmond, VA). The resulting peptides were separated on a Hewlett Packard model 1090 HPLC (Wilmington, DE). Then peptide fractions were analyzed on an Applied Biosystems model 477A amino acid sequencer as described above. In both cases the resultant sequences were screened against the GenBank CDS database, using a BLAST search to determine the homology with any other known proteins.

RESULTS

Separation of the neuronal N- and P/Q-type Ca^{2+} channels

N- and P/Q-type Ca^{2+} channels were separated by immunoaffinity chromatography by using the α_{1B}-specific monoclonal antibody (mAb CC18) in combination with the β subunit-specific monoclonal antibody (mAb $VD2_1$), and the subunit composition of these two neuronal Ca^{2+} channels was examined. The proteins were extracted from rabbit brain membranes and applied to a heparin agarose column. After elution from the heparin column with high salt, the preparation of channels was incubated with the α_{1B} antibody resin overnight; then the void was collected. Conditions in which ^{125}I-ω-Ctx MVIIC binds to the P/Q-type Ca^{2+} channels and not the N-type channels have been determined previously (Liu et al., 1996), and these exact conditions have been applied throughout the present study. We assayed binding of ^{125}I-ω-Ctx GVIA and ^{125}I-ω-Ctx MVIIC to the N- and P/Q-type channels, respectively, in both the heparin eluate and the void from the α_{1B} column (Fig. 1). Because the α_1 subunit that is present in the N-type calcium channels is α_{1B}, virtually all of the ^{125}I-ω-Ctx GVIA specific binding observed originally in the heparin pool (Fig. 1*A, left bar*) bound to the α_{1B} Hydrazide

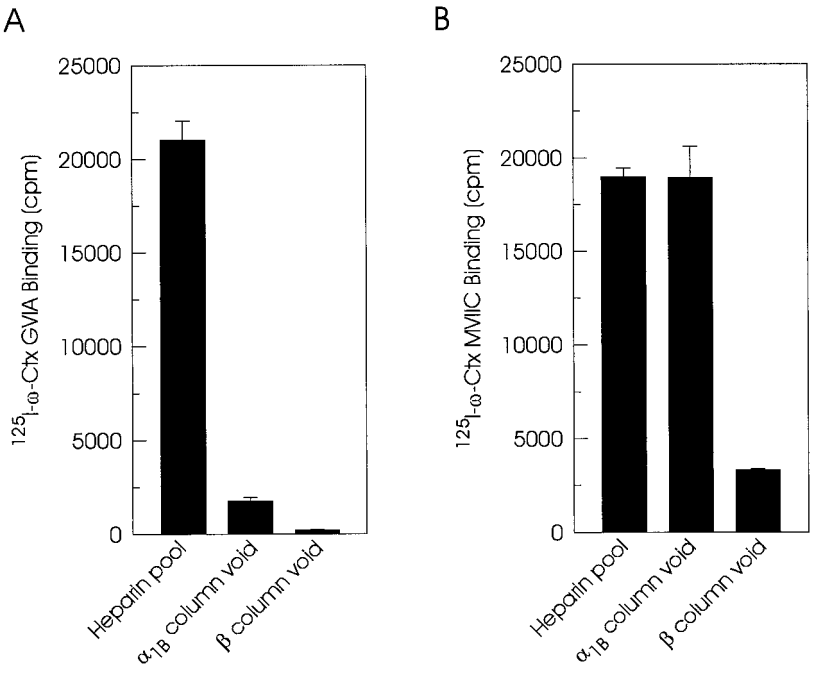

Figure 1. Separation of N- and P/Q-type channels isolated from rabbit brain. N- and P/Q-type channels were isolated from rabbit brain membranes as described under Materials and Methods. The solubilized channels were concentrated on a heparin agarose column, and the N- and P/Q-type channels were resolved by using α_{1B} and β subunit Avidgel columns in series. Aliquots of the heparin pool and the void from both of these antibody columns were assayed for both ^{125}I-ω-Ctx GVIA (*A*) or ^{125}I-ω-Ctx MVIIC (*B*) binding, using a saturating concentration of radiolabeled toxin. The N-type calcium channels (as determined by high-affinity ^{125}I-ω-Ctx GVIA binding) bound to the α_{1B} column, whereas the P/Q-type channels (represented by ^{125}I-ω-Ctx MVIIC specific binding) were present in the void of the α_{1B} column. The β column bound practically all of the P/Q-type calcium channels present in the void of the α_{1B} column.

Avidgel column. In consequence, only a minimal fraction of the ^{125}I-ω-Ctx GVIA binding was found in the void of that column (Fig. 1*A, middle bar*). Analogously, when the isolated N-type channels were applied to a β subunit column antibody, the void of that column showed no ^{125}I-ω-Ctx GVIA specific binding, indicating that all of the channels in the preparation bound the column (Fig. 1*A, right bar*). In contrast, equal amounts of the P/Q-type channels, as determined by high-affinity ^{125}I-ω-Ctx MVIIC binding, were present in both the heparin pool (Fig. 1*B, left bar*) and the α_{1B} column void (Fig. 1*B, middle bar*). These results clearly indicate that the P/Q-type channels (α_{1A} subunit-containing channels) did not bind to the α_{1B} Hydrazide Avidgel column. However, when the isolated P/Q-type channels were applied to the β subunit-specific ($VD2_1$) antibody column, nearly all of the ω-Ctx MVIIC binding activity bound (Fig. 1*B, right bar*), and only a small fraction of the binding activity was found in the void as expected. In summary, all of the ω-Ctx GVIA binding, which represents N-type Ca^{2+} channels, binds to the α_{1B} column, whereas the ω-Ctx MVIIC binding, which represents the P/Q channels, is present in the void of the α_{1B} column. The $VD2_1$ column, on the other hand, binds the remainder of the ω-Ctx MVIIC binding.

After elution from each respective antibody column with glycine containing buffer solution, the subunit composition of the isolated N- and P/Q-type channels was examined by SDS-PAGE, followed by Western blotting. Proteins in both isolated channel preparations were examined by using polyclonal antibodies to the II–III loop of the α_1 subunit (Sheep 37; Witcher et al., 1993a). In the case of the N-type channel, these antibodies recognized primarily a protein with a molecular size of 210 kDa (α_{1B}; Fig. 2*A, left*). In contrast, the isolated P/Q-type channels showed a more complex pattern of immunoblotting. In this case, the same antibodies reacted with distinct proteins ranging in size from 95 to 220 kDa (Fig. 2*A, right*), suggesting the presence of different isoforms of the α_{1A} subunit. Earlier immunoblotting studies with partially purified channels identified an $\alpha_2\delta$ subunit and four different β subunits (β_{1b}, β_2, β_3, and β_4; Liu et al., 1996). Herein, a 95 kDa protein was shown to be associated with the P/Q-type

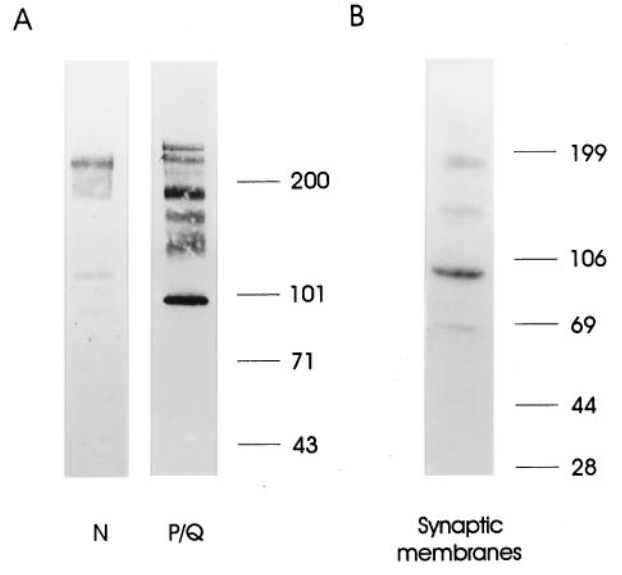

Figure 2. Analysis of the isolated N- and P/Q-type Ca^{2+} channels by SDS-PAGE and Western blotting. *A,* The purified N- and P/Q-type Ca^{2+} channels were analyzed by SDS-PAGE on a 3–12% SDS gradient gel, followed by immunoblotting with affinity-purified α_1 antibodies, and the bands were visualized with a horseradish peroxidase-conjugated secondary antibody. *B,* An aliquot (200 μg) of synaptic plasma membranes was subjected to SDS-PAGE and analyzed by immunoblotting as described above. Molecular weight markers ($\times 10^{-3}$) are indicated in each case (*right*).

and not the N-type channels, as was previously reported (McEnery et al., 1991; Witcher et al., 1993a,b; Leveque et al., 1994).

A potential alternative explanation for the presence of these antigenically similar proteins in the purified P/Q-type channel preparations could be proteolysis that occurred during the isolation procedure; however, several lines of evidence contest this possibility. The significant sequence homology between the α_{1A} and the α_{1B} subunits would suggest that proteolysis probably

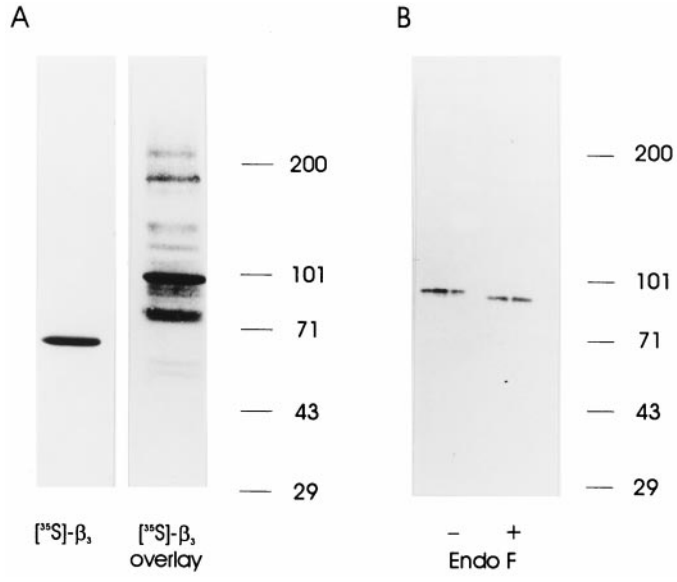

Figure 3. Biochemical properties of the 95 kDa protein. *A*, Analysis of the β subunit interaction assay with P/Q-type channels. An aliquot of *in vitro* translated [^{35}S]-labeled β_3 subunit (5×10^5 cpm) was subjected to SDS-PAGE on a 3–12% gradient gel and visualized by autoradiography (*left*). The β-interaction assay was performed on the isolated P/Q-type channels after the subunits were resolved by SDS-PAGE and the protein was transferred onto nitrocellulose membrane. The [^{35}S]-labeled β_3 subunit was incubated with the membrane overnight and washed extensively to remove unbound [^{35}S]-labeled β_3 subunit; the specifically interacting proteins were determined by autoradiography (*right*). *B*, Deglycosylation of the isolated P/Q-type channels. The P/Q-type channels from a single purification procedure were deglycosylated with Endo F. The sample was subjected to SDS-PAGE analysis on a 3–12% gradient gel before immunoblotting with antibodies specific for the 95 kDa protein. − and + Endo F indicate untreated and deglycosylated proteins, respectively. Molecular mass standards ($\times 10^{-3}$) are indicated on the *right*.

would occur similarly on both α_1 subunits. Moreover, freshly purified synaptic plasma membranes when probed with the same antibody contained similarly reactive proteins (Fig. 2*B*). Furthermore, the 95 kDa protein is a predominant species in both the membrane and purified preparations. More importantly, inclusion or omission of protease inhibitors (data not shown) did not affect the relative quantities of these antigenic variants. These antibody data suggest that the 95 kDa protein associated with P/Q-type neuronal channels is structurally related to the α_{1A} subunit.

Ca^{2+} channel β subunit interacts with the 95 kDa protein

To investigate the relationship between the α_1 subunit and the 95 kDa protein further, we performed a β subunit interaction assay. This assay was developed in a previous study (Pragnell et al., 1994), which identified a region in the I–II loop in all known α_1 subunits that can bind Ca^{2+} channel β subunits. The β_3 subunit in a pcDNA3 expression vector containing a T7 RNA polymerase site was translated *in vitro* in the presence of [^{35}S]methionine, and the translation product was analyzed by SDS-PAGE and autoradiography. The left panel in Figure 3*A* shows the migration of the polypeptide identified as the translated product, which exhibited a molecular mass similar to that expected from its amino acid sequence (~58 kDa). Then the [^{35}S]-β_3 probe was incubated with the P/Q-type channels, after separation of the subunits by electrophoresis on a 3–12% gradient gel and transfer onto nitrocellulose membrane (Fig. 3*A, right*). The resulting overlay identified a number of proteins of similar molecular size to those identified in immunoblotting studies above (Fig. 2*A, right*), suggesting that each of these proteins contained the site of interaction between the α_1 and β subunits of voltage-dependent Ca^{2+} channels. The overlay assay also revealed a protein of slightly smaller molecular weight (~75 kDa) that appeared not to be recognized by the α_1 antibodies (Fig. 2) and might represent the C-terminal part of α_{1A} subunit. Accordingly, it has been shown that a second β subunit interaction site exists within the C terminus of the neuronal α_{1E} subunit distinct from the previously known interaction domain located between repeat I and II of α_1 subunits (Tareilus et al., 1997). More interestingly, the presence of a second β interaction site also has been observed recently in the C terminus of the α_{1A} subunit (Walker et al., 1997).

95 kDa polypeptide is a glycoprotein

Another biochemical property of the 95 kDa protein was investigated by deglycosylation studies with the isolated P/Q-type channels and Western blotting of the resultant sample. When a 95 kDa-specific antibody was used, the blot showed a distinct shift in the mobility of the 95 kDa protein to 90 kDa on a 3–12% gradient gel after Endo F treatment (Fig. 3*B*). Interestingly, there are two potential glycosylation sites on the α_{1A} subunit, on Asn 283 and Asn 1665. The first of these sites is present within the sequence of the 95 kDa subunit, which explains the shift in molecular size observed in the blot. These data provided the first indication that an α_1 subunit of voltage-dependent Ca^{2+} channels is glycosylated.

Molecular identity of the 95 kDa protein

To establish the identity of the 95 kDa polypeptide precisely, we performed protein microsequencing on this subunit after large-scale isolation of the P/Q-type channels. After SDS-PAGE analysis to resolve the subunits, two alternative methods were used to obtain protein sequence information from the 95 kDa protein. The first method involved transferring the 95 kDa protein from a SDS gel onto an Immobilon-P membrane and excising the 95 kDa band previously stained with Coomassie blue. Peptides were released with trypsin and resolved by reverse-phase HPLC. Sequence analysis was performed with an automated amino acid sequencer. This yielded a number of signals (peptides 1, 4, 6, 8, 10–12; Fig. 4), which, when screened in a BLAST search, showed strong identity with the amino acid sequence of the α_{1A} subunit cloned from rabbit brain (Table 1).

To investigate this further, we implemented a second method to obtain additional peptide sequence information. Similarly, the sample was subjected to SDS-PAGE, the gel piece containing the 95 kDa protein was excised, and peptides were generated by in-gel digestion, using endoproteinase lys-C. The resulting fragments were resolved by reverse-phase HPLC before protein microsequencing, which yielded six different peptide sequences (peptides 2, 3, 5, 7, 9, 13; Fig. 4).

Consistent with the results obtained in Western blotting, analysis of these signals showed that 10 of the 13 peptides presented 100% amino acid identity with the sequence of the P/Q-type calcium channel α_{1A} subunit (Table 1). Only in three cases were there discrepancies with the protein sequence of the α_{1A} subunit, and in all cases the divergence can be attributed to ambiguous amino acid identification during the microsequencing process. Notably, all 13 sequences were confined to the first half of the α_{1A} subunit up to the middle of the cytoplasmic II–III loop (Fig. 5).

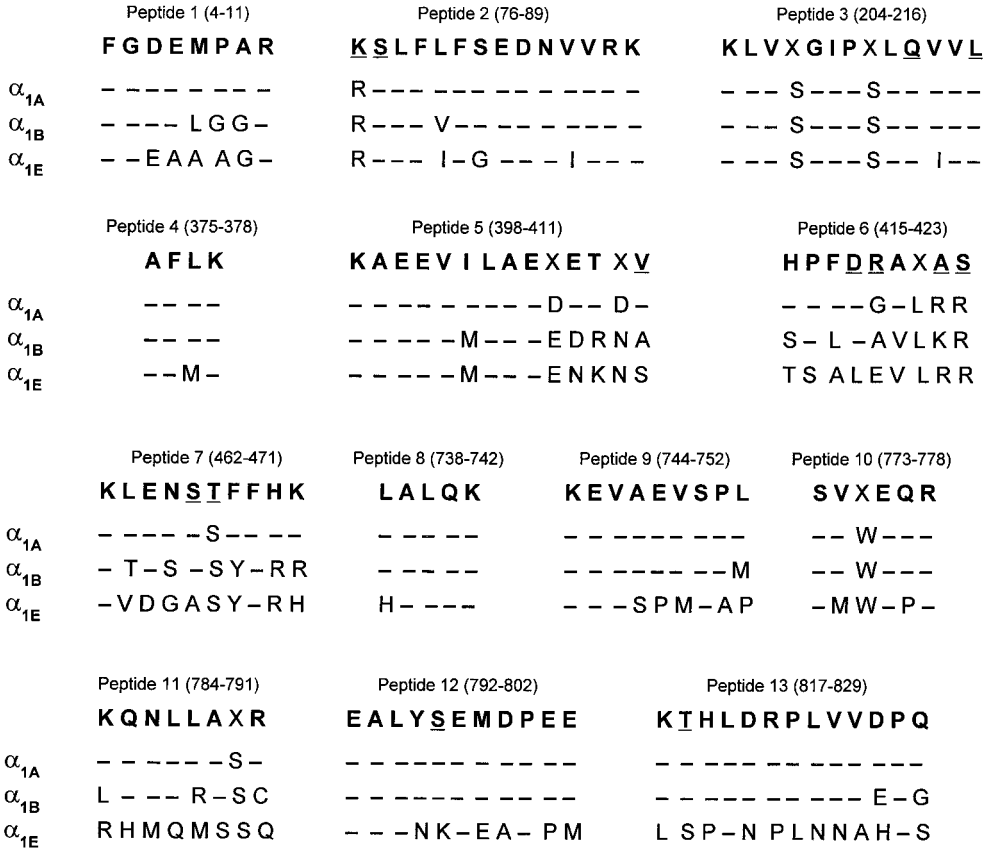

Figure 4. Amino acid sequences of the 13 peptides obtained from the 95 kDa protein and alignment with the sequence of three neuronal Ca^{2+} channel α_1 pore-forming subunits (α_{1A}, α_{1B}, and α_{1E}; GenBank accession numbers I46478, D14157, and X67855, respectively). *Hyphens* indicate identity to the sequence of peptides at the top. *X* indicates uncharacterized residues, and *underlining* designates low confidence determinations. The *numbers* denote the positions in the α_{1A} sequence. Alignment was made manually. The percentage of identity and similarity of the purified peptides and the α_{1A} subunit sequence is notably high (Table 1). All of the microsequenced peptides were confined to the first half of the α_{1A} subunit sequence (see Fig. 5).

Table 1. Percentage of protein sequence similarities of neuronal Ca^{2+} channel α_1 pore-forming subunits and peptides obtained from the 95 kDa protein

Peptide	α_{1A}	α_{1B}	α_{1E}
1	100	63	38
2	93*	86	71
3	100	100	91
4	100	100	75
5	100	64	64
6	63*	25	0
7	90*	40	20
8	100	100	80
9	100	89	44
10	100	100	60
11	100	57	0
12	100	100	45
13	100	85	15

* Discrepancies with α_{1A} subunit sequence are attributable to ambiguous amino acid identification (see Fig. 4).

These data provide convincing evidence that the 95 kDa protein associated with the P/Q-type Ca^{2+} channels is a member of the α_{1A} subunit family. Interestingly, the determined molecular size of this short form of the α_{1A} subunit from the start of the N-terminal sequence (peptide 1) to the end of peptide 13 was 93,784 Da, which is close to that estimated for this protein from SDS-PAGE analysis.

DISCUSSION

Neuronal Ca^{2+} channels were purified first from rat (McEnery et al., 1991; Leveque et al., 1994) and rabbit (Witcher et al., 1993a) brain and shown to contain α_{1B}, $\alpha_2\delta$, and β subunits, together with a specific protein of 110 or 95 kDa, respectively. Although these purification protocols have greatly increased our understanding of neuronal Ca^{2+} channels, in view of the recently realized structural similarities between the N- and P/Q-type Ca^{2+} channels (Liu et al., 1996; Rettig et al., 1996; Scott et al., 1996; Sheng et al., 1996), it is entirely possible that these purified materials may contain a mixture of both channel types. Unfortunately, because of the lack of specific radiolabeled high-affinity ligands that bind to the P/Q-type channels, this was not realized previously (McEnery et al., 1991; Witcher et al., 1993a; Leveque et al., 1994). Recently, however, the conditions for measuring the binding of ^{125}I-ω-Ctx MVIIC specifically to P/Q-type channels were established (Liu et al., 1996), thereby allowing the detection of these channels throughout the present study (see Fig. 1). Moreover, a monoclonal antibody that exclusively reacts with the α_{1B} subunit was developed (Scott et al., 1996). This also has proven very useful for specifically separating the N-type from the P/Q-type Ca^{2+} channels, consequently permitting the subunit composition of each of these channel types to be assessed. Surprisingly, the 95 kDa protein that previously was proposed to be associated with the N-type channel was shown in this study to associate exclusively with the P/Q-type channels after immunoaffinity enrichment (see Fig. 2).

The properties of this protein were examined further, using a number of different biochemical approaches from immunoblotting to protein–protein interaction assays and, finally, to protein microsequencing (Figs. 3, 4). The data that arose from these studies established a close structural relationship between the 95 kDa protein and the α_{1A} subunit of P/Q-type channels. Interestingly, when the α_{1A} subunit was identified originally, Northern

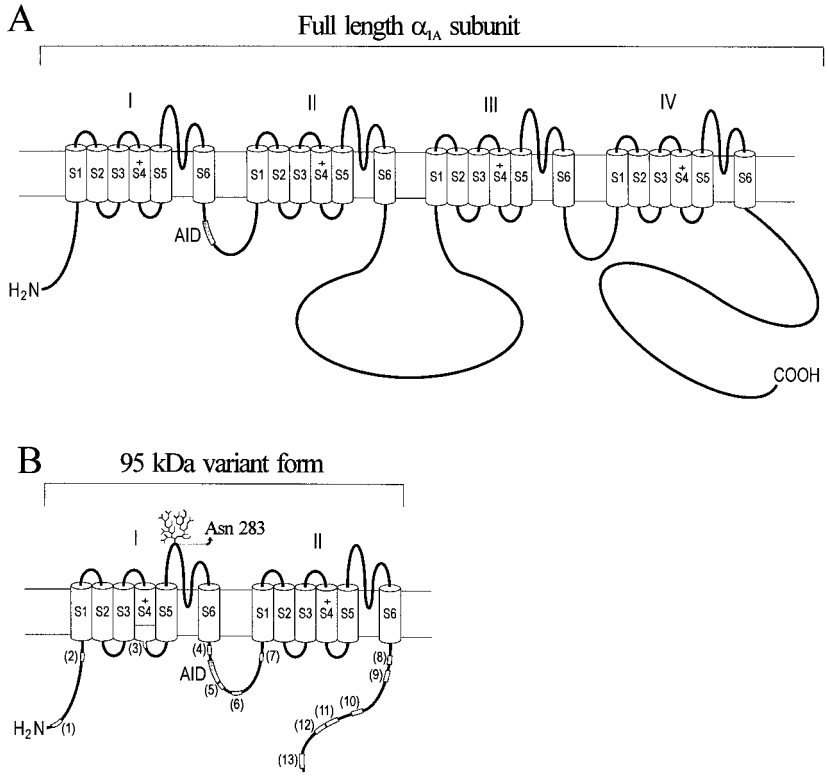

Figure 5. Proposed model for the structure of the 95 kDa subunit of P/Q-type channels. *A*, The cloned Ca^{2+} channel α_{1A} subunit possesses four internal repeated domains (*I–IV*) that are modeled to contain six α-helical transmembrane regions (*S1–S6*), including one (*S4*) that is positively charged and is thought to form part of the voltage sensor. The region separating segments *S5* and *S6* of each domain contains two additional transmembrane segments that together form the pore of the channel. The interaction site with the β subunit has been localized to the cytoplasmic connecting link between domains *I* and *II* (*AID*). *B*, To compare with the α_{1A} subunit structure, the 95 kDa protein was examined by several different biochemical techniques. Microsequencing yielded 13 peptide sequences, each of which is found in the first half of the α_{1A} subunit, as indicated in *parentheses*. Additional studies revealed that the *AID*, the N-linked glycosylation site on *Asn 283*, and part of the *II–III* loop of the α_{1A} subunit are also present in the 95 kDa protein, providing convincing evidence that this protein contains the first half of the α_{1A} subunit and probably corresponds to a short form of this subunit.

blot analysis revealed the presence of two transcripts, one with a size of ~9 kb and a second with a size of 4.4 kb (Snutch et al., 1990). It would appear from these data that the 95 kDa protein described in the present study could, in fact, be a splice variant of the α_{1A} subunit because it contains approximately the first half of the full-length α_{1A} subunit. However, it cannot be excluded that this 95 kDa subunit could have arisen because of post-translational proteolytic processing. This is supported by a recent study that revealed that the NMDA receptor-activated proteolytic processing of the brain α_{1C} subunit yielded neuronal L-type Ca^{2+} channels that have modified kinetic properties (Hell et al., 1996). Given these data, it is tempting to speculate that the association of two 95 kDa proteins could form a functional Ca^{2+} channel equivalent to that generated by the full-length α_{1A} subunit. This possibility is particularly interesting because both of the 95 kDa proteins can bind a β subunit (Fig. 3*A*), potentially yielding a Ca^{2+} channel with novel kinetic properties.

On the other hand, although the expression of the 95 kDa protein may not be sufficient to support channel function, the possibility exists that it may regulate the functional expression of the full-length α_{1A} subunit. Because the 95 kDa protein binds the β subunit, in this scenario the truncated 95 kDa protein would compete with the full-length α_{1A} subunit for this auxiliary subunit. Because the β subunit has been implicated in determining the membrane localization in addition to modulating properties of channel conduction of the α_1 subunit (for review, see Catterall, 1995), this alternative hypothesis predicts that the coexpression of the 95 kDa protein would affect the functional expression of the full-length α_{1A} subunit. Likewise, the 95 kDa protein also might contribute to determining Ca^{2+} current diversity in neurons by interacting with the channel-forming subunits and modifying their biophysical properties and/or altering the α_1 subunit modulation by second messengers.

Genetic studies very recently have linked the α_{1A} subunit of the P/Q-type Ca^{2+} channels to several animal seizure models and human neurological disorders (Fletcher et al., 1996; Ophoff et al., 1996; Doyle et al., 1997; Zhuchenko et al., 1997). Interestingly, mutations within the sequence of the α_{1A} subunit have been found in patients suffering from familial hemiplegic migraine and episodic ataxia type 2 (Ophoff et al., 1996). In line with this finding, it is interesting to note that one of the mutations linked to episodic ataxia type 2 resulted in a frame shift and a premature stop at exon 22, in the IIIS1 region, generating a truncated α_{1A} subunit approximate in size to the 95 kDa protein. This is the first report on the occurrence of a short form of a Ca^{2+} channel α_1 subunit that is linked to a human disorder. Future studies on the functional properties of the 95 kDa protein associated with P/Q-type channels could provide insights into possible mechanisms by which this protein may be linked to migraine.

In conclusion, this study has extensively characterized the molecular structure of the 95 kDa protein of native P/Q-type Ca^{2+} channels by several approaches. Collectively, all of the information obtained in this study on the structure of the 95 kDa protein allows a model to be proposed containing the α_1 interaction domain (AID), the glycosylation site on Asn 283, part of the II–III loop (amino acids 779–969), and the 13 peptide sequences (Fig. 5). These data will be essential for the discovery and development of therapeutic agents for the pharmacological treatment of disorders linked to the voltage-dependent Ca^{2+} channel α_{1A} subunit of the neuronal P/Q-type Ca^{2+} channels.

REFERENCES

Ahlijanian MK, Westenbroek RE, Catterall WA (1990) Subunit structure and localization of dihydropyridine-sensitive calcium channels in mammalian brain, spinal cord, and retina. Neuron 4:819–832.

Boland LM, Morrill JA, Bean BP (1994) ω-Conotoxin block of N-type calcium channels in frog and rat sympathetic neurons. J Neurosci 14:5011–5027.

Catterall WA (1995) Structure and function of voltage-gated ion channels. Annu Rev Biochem 64:493–531.

De Waard M, Gurnett CA, Campbell KP (1996) Structural and functional diversity of voltage-activated calcium channels. In: Ion channels, Vol IV (Narahashi T, ed), pp 41–87. New York: Plenum.

Doyle J, Ren X, Lennon G, Stubbs L (1997) Mutations in the *Cacnl1a4* calcium channel gene are associated with seizures, cerebellar degeneration, and ataxia in *tottering* and *leaner* mutant mice. Mamm Genome 8:113–120.

Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 18:89–98.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy JD, Hawkes R, Frankel WN, Coppeland NG, Jenkins NA (1996) Absence epilepsy in *tottering* mutant mice is associated with calcium channel defects. Cell 87:607–617.

Fujita Y, Mynlieff M, Dirksen RT, Kim MS, Niidome T, Nakai J, Friedrich T, Iwabe N, Miyata T, Furuichi T, Furutama D, Mikoshiba K, Mori Y, Beam KG (1993) Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rabbit brain. Neuron 10:585–598.

Hell JW, Westenbroek RE, Breeze LJ, Wang KK, Chavkin C, Catterall WA (1996) *N*-methyl-D-aspartate receptor-induced proteolytic conversion of postsynaptic class C L-type calcium channels in hippocampal neurons. Proc Natl Acad Sci USA 93:3362–3367.

Hillyard DR, Monje VD, Mintz IM, Bean BP, Nadasdi L, Ramachandran J, Miljanich G, Azimi-Zoonooz A, McIntosh JM, Cruz LJ, Imperial JS, Olivera BM (1992) A new Conus peptide ligand for mammalian presynaptic Ca^{2+} channels. Neuron 9:69–77.

Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Leung AT, Imagawa T, Campbell KP (1987) Structural characterization of the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle. Evidence for two distinct high molecular weight subunits. J Biol Chem 262:7943–7946.

Leveque C, el Far O, Martin-Moutot N, Sato K, Kato R, Takahashi M, Seagar MJ (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.

Liu H, De Waard M, Scott VES, Gurnett CA, Lennon VA, Campbell KP (1996) Identification of three subunits of the high affinity ω-conotoxin MVIIC-sensitive Ca^{2+} channel. J Biol Chem 271:13804–13810.

Luebke JI, Dunlap K, Turner TJ (1993) Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. Neuron 11:895–902.

Martin-Moutot N, Leveque C, Sato K, Kato R, Takahashi M, Seagar M (1995) Properties of ω-conotoxin MVIIC receptors associated with α_{1A} calcium channel subunits in rat brain. FEBS Lett 366:21–25.

McDonough SI, Swartz KJ, Mintz IM, Boland LM, Bean BP (1996) Inhibition of calcium channels in rat central and peripheral neurons by ω-conotoxin MVIIC. J Neurosci 16:2612–2623.

McEnery MW, Snowman AM, Sharp AH, Adams ME, Snyder SH (1991) Purified ω-conotoxin GVIA receptor of rat brain resembles a dihydropyridine-sensitive L-type calcium channel. Proc Natl Acad Sci USA 88:11095–11099.

Mochida S, Sheng ZH, Baker C, Kobayashi H, Catterall WA (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. Neuron 17:781–788.

Mori Y, Friedrich T, Kim MS, Mikami A, Nakai J, Ruth P, Bosse E, Hofmann F, Flockerzi V, Furuichi T, Mikoshiba K, Imoto K, Tanabe T, Numa S (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. Nature 350:398–402.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca^{2+} channel gene CACNL1A4. Cell 87:543–552.

Pragnell M, De Waard M, Mori Y, Tanabe T, Snutch TP, Campbell KP (1994) Calcium channel β subunit binds to a conserved motif in the I-II cytoplasmic linker of the α_1 subunit. Nature 368:67–70.

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA (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.

Scott VE, De Waard M, Liu H, Gurnett CA, Venzke DP, Lennon VA, Campbell KP (1996) β-Subunit heterogeneity in N-type Ca^{2+} channels. J Biol Chem 271:3207–3212.

Sheng ZH, Rettig J, Takahashi M, Catterall WA (1994) Identification of a syntaxin-binding site on N-type calcium channels. Neuron 13:1303–1313.

Sheng ZH, Rettig J, Cook T, Catterall WA (1996) Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 379:451–454.

Snutch TP, Reiner PB (1992) Ca^{2+} channels: diversity of form and function. Curr Opin Neurobiol 2:247–253.

Snutch TP, Leonard JP, Gilbert MM, Lester HA, Davidson N (1990) Rat brain expresses a heterogeneous family of calcium channels. Proc Natl Acad Sci USA 87:3391–3395.

Takahashi T, Momiyama A (1993) Different types of calcium channels mediate central synaptic transmission. Nature 366:156–158.

Tareilus E, Roux M, Qin N, Olcese R, Zhou J, Stefani E, Birbaumer L (1997) A *Xenopus* oocyte β subunit: evidence for a role in the assembly/expression of voltage-gated calcium channels that is separate from its role as a regulatory subunit. Proc Natl Acad Sci USA 94:1703–1708.

Turner TJ, Dunlap K (1995) Pharmacological characterization of presynaptic calcium channels using subsecond biochemical measurements of synaptosomal neurosecretion. Neuropharmacology 34:1469–1478.

Turner TJ, Adams ME, Dunlap K (1993) Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release. Proc Natl Acad Sci USA 90:9518–9522.

Walker D, Bichet D, Campbell KP, De Waard M (1997) A β_4 isoform-specific interaction site in the carboxy terminal region of the voltage-dependent Ca^{2+} channel α_{1A} subunit. J Biol Chem, in press.

Wheeler DB, Sather WA, Randall A, Tsien RW (1994a) Distinctive properties of a neuronal calcium channel and its contribution to excitatory synaptic transmission in the central nervous system. Adv Second Messenger Phosphoprotein Res 29:155–171.

Wheeler DB, Randall A, Tsien RW (1994b) Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. Science 264:107–111.

Witcher DR, De Waard M, Sakamoto J, Franzini-Armstrong C, Pragnell M, Kahl SD, Campbell KP (1993a) Subunit identification and reconstitution of the N-type Ca^{2+} channel complex purified from brain. Science 261:486–489.

Witcher DR, De Waard M, Campbell KP (1993b) Characterization of the purified N-type Ca^{2+} channel and the cation sensitivity of ω-conotoxin GVIA binding. Neuropharmacology 32:1127–1139.

Zhuchenko O, Bailey J, Bonnen P, Ashizawa T, Stockton DW, Amos C, Dobyns WB, Subramony SH, Zoghbi HY, Lee CC (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the α_{1A}-voltage-dependent calcium channel. Nat Genet 15:62–68.