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β Subunit Heterogeneity in N-type Ca^{2+} Channels*

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Victoria E. S. Scott, Michel De Waard, Hongyan Liu‡§, Christina A. Gurnett§, David P. Venzke, Vanda A. Lennon¶‖, and Kevin P. Campbell

From the Howard Hughes Medical Institute and the ‡Program in Neuroscience, Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242 and the ¶Neuroimmunology Laboratory, Departments of Immunology and Neurology, Mayo Clinic, Rochester, Minnesota 55905

The β subunit of the voltage-dependent Ca^{2+} channel is a cytoplasmic protein that interacts directly with an α_1 subunit, thereby modulating the biophysical properties of the channel. Herein, we demonstrate that the α_{1B} subunit of the N-type Ca^{2+} channel associates with several different β subunits. Polyclonal antibodies specific for three different β subunits immunoprecipitated ^{125}I-ω-conotoxin GVIA binding from solubilized rabbit brain membranes. Enrichment of the N-type Ca^{2+} channels with an α_{1B} subunit-specific monoclonal antibody showed the association of β_{1b}, β_3, and β_4 subunits. Protein sequencing of tryptic peptides of the 57-kDa component of the purified N-type Ca^{2+} channel confirmed the presence of the β_3 and β_4 subunits. Each of the β subunits bound to the α_{1B} subunit interaction domain with similar high affinity. Thus, our data demonstrate important heterogeneity in the β subunit composition of the N-type Ca^{2+} channels, which may be responsible for some of the diverse kinetic properties recorded from neurons.

Voltage-dependent Ca^{2+} channels are essential for regulating Ca^{2+} concentrations in many cells. Based upon electrophysiological and pharmacological properties, these channels have been classified into five major groups (L, N, T, R, and P/Q types) (1, 2). L-type Ca^{2+} channels are central to excitation-contraction coupling in skeletal and cardiac muscle, while T-type channels are involved in pacemaker activity. The N-, P/Q-, and R-type Ca^{2+} channels are found predominantly in the central and peripheral nervous systems and have major roles in controlling neurotransmitter release. The skeletal muscle L-type and the brain N-type Ca^{2+} channels have both been purified. Although functionally distinct, these have similar subunit compositions (α_1, $\alpha_2\delta$, and β), with a variable channel-specific subunit (γ or 95 kDa, respectively) (3, 4). The genes encoding the α_1 pore-forming subunits have been separated into six groups (S, A, B, C, D, and E), each containing multiple splice variants, while the β subunits have been classified into four major classes (namely β_1, β_2, β_3, and β_4), also containing several splice variants (5). Recent studies have identified complementary interaction domains on the α_1 and β subunits (6, 7). The β subunit regulates channel activity by binding to a highly conserved portion of the cytoplasmic linker of the I–II loop of all α_1 subunits, known as the α_1 subunit interaction domain (AID).[1] The β subunit's interaction site, known as the β subunit interaction domain, encompasses ~30 amino acids in the amino-terminal portion of the second highly conserved domain. *In vitro* binding studies have shown that an α_1 subunit binds a single β subunit in a 1:1 stoichiometry (8).

N-type Ca^{2+} channels are involved in regulating neurotransmitter release in the central and peripheral nervous systems and in controlling endocrine secretion. These also serve as autoantigens in paraneoplastic neurologic disorders and may be the target of pathogenic autoantibodies responsible for autonomic dysfunction in the Lambert-Eaton myasthenic syndrome (9). Electrophysiological analysis of the N-type Ca^{2+} channels in different neurons has revealed an unusual degree of diversity in the rates of inactivation (10–12). The structural basis of this functional diversity is not understood. Herein, we examine which β subunits are associated with the N-type Ca^{2+} channels from rabbit brain using a monoclonal antibody specific for the α_{1B} subunit and polyclonal antibodies for three different β subunit genes. Several lines of evidence are presented that demonstrate that there is heterogeneity in the β subunit of native N-type channels, and this may account for some of the functional diversity of channel kinetic properties recorded from neurons.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-ω-Conotoxin (CTx) GVIA, [^{35}S]methionine, and the ECL kit were purchased from Amersham Corp. Digitonin was obtained from ICN Biomedicals and purified as detailed elsewhere (3). Other biochemicals used were protein G-Sepharose (Pharmacia Biotech Inc.), horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim), and Avidchrom hydrazide gel (Unisyn Technologies). All other chemicals were of reagent grade. The GraFit Version 3.0 curve fitting program was purchased from Sigma.

Production of a Monoclonal Antibody (mAb) to the α_{1B} Subunit—mAb CC18 was secreted by a hybridoma produced from the splenic B lymphocyte of a rat hyperimmunized with a fusion protein corresponding to the II–III cytoplasmic loop of the α_{1B} subunit (29). It reacts selectively with high affinity brain receptors for ω-CTx GVIA.

SDS-PAGE and Immunoblot Analyses—Proteins were analyzed by SDS-PAGE on 3–12 or 5–16% gradient gels using the Laemmli buffer system (13). Gels were transferred to nitrocellulose and immunoblotted as described previously (14). The specific protein bands were detected using either the horseradish peroxidase or ECL detection methods (according to the manufacturers' instructions). Antibodies to fusion proteins containing the diverse C-terminal portion of each of four β subunits (30) were immunoaffinity-purified as described previously (14), using the appropriate fusion protein for β_{1b} (residues 428–597;

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§ American Heart Association Iowa Affiliate Predoctoral Fellow.

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** Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Inst., University of Iowa College of Medicine, 400 EMRB, Iowa City, IA 52242. Tel.: 319-335-7867; Fax: 319-335-6957; E-mail: kevin-campbell@uiowa.edu.

[1] The abbreviations used are: AID, α_1 subunit interaction domain; AID_B, α_{1B} subunit interaction domain; CTx, conotoxin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HPLC, high pressure liquid chromatography.

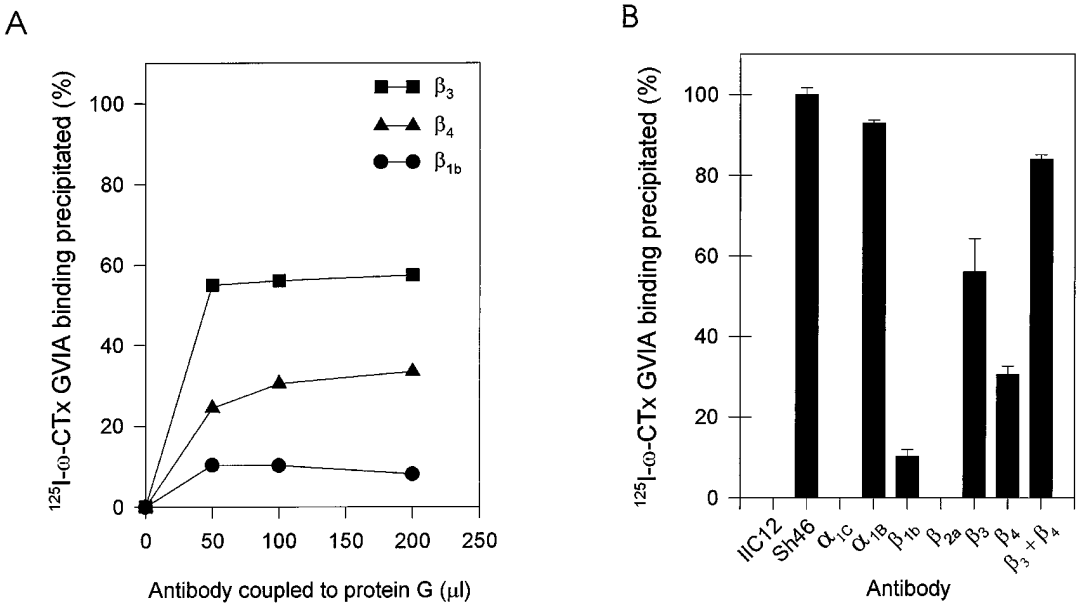

FIG. 1. **Immunoprecipitation of ^{125}I-ω-CTx GVIA binding with subunit-specific antibodies.** Aliquots (~20 mg) of rabbit brain membranes were labeled with ^{125}I-ω-CTx GVIA in the presence and absence of 1000-fold unlabeled toxin and solubilized as described under "Experimental Procedures." *A*, to determine the maximum amount of antibody required to sediment a constant amount of solubilized ^{125}I-ω-CTx GVIA-labeled receptors, increasing quantities of antibody coupled to protein G Sepharose were incubated with an aliquot of the labeled channels (1 ml) overnight at 4 °C. The beads were washed three times with 10 mM HEPES/NaOH, pH 7.5, 0.1 M NaCl, and 0.1% (w/v) digitonin containing five protease inhibitors, and immunoprecipitation was quantified by γ-counting. *B*, saturating concentrations of each antibody coupled to protein G-Sepharose were incubated with solubilized ^{125}I-ω-CTx GVIA receptor complexes (~1 ml) overnight at 4 °C. The beads were washed as described above and quantified by γ-counting. The amount of precipitation in each case was determined relative to that of Sheep 46 (*Sh46*; 100%). IIC12 is a mAb raised against the skeletal muscle dihydropyridine receptor.

GenBank accession number X61394), β_{2a} (residues 462–578; GenBank accession number M80545), β_3 (residues 369–484; GenBank accession number M88751), and β_4 (residues 419–519; GenBank accession number L02315).

Immunoprecipitation of N-type Ca^{2+} Channels—Antibodies were incubated overnight with protein G-Sepharose beads in PBS at 4 °C. The beads were then washed three times with PBS prior to resuspension in an equal volume of PBS. An aliquot of rabbit brain membranes (~20 mg) was incubated with ^{125}I-ω-CTx GVIA (0.5 nM) in 10 mM HEPES/NaOH, pH 7.5, containing 0.1 M NaCl and 0.2 mg/ml bovine serum albumin in the presence and absence of 1000-fold unlabeled ω-CTx GVIA for 1 h at 22 °C. Then, the membranes were sedimented by centrifugation in a Beckman TL100 centrifuge at 50,000 rpm for 10 min at 4 °C. The resulting pellet was resuspended in solubilization buffer (10 mM HEPES/NaOH, pH 7.5, containing 1 M NaCl, 0.23 mM phenylmethylsulfonyl fluoride, 0.64 mM benzamidine, 1 μM leupeptin, 0.7 μM pepstatin A, 76.8 nM aprotinin, and 1% (w/v) digitonin) to a final volume of 7 ml and incubated at 4 °C for 1 h. Particulate material was removed from solution by sedimentation at 100,000 rpm for 30 min, and the supernatant was diluted 3–5-fold with ice-cold distilled, deionized water. Aliquots (1 ml) of the labeled solubilized extract were then incubated with saturating concentrations of each antibody-protein G-Sepharose bead complex at 4 °C overnight with agitation. Subsequently, the beads were sedimented by centrifugation and washed twice with ice-cold buffer A (10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl plus protease inhibitors as listed above) containing 0.1% (w/v) digitonin prior to quantification by γ-counting.

N-terminal Sequence Analysis of the 57-kDa Subunit of the N-type Ca^{2+} Channel—The N-type Ca^{2+} channel was purified as described previously (4), and the subunits were resolved by SDS-PAGE and transferred onto an Immobilon PSQ membrane in 10 mM CAPS/NaOH, pH 11, containing 10% (w/v) methanol for 3 h. The 57-kDa subunit was visualized by Coomassie Brilliant Blue staining, excised, and digested with trypsin for 18 h at 37 °C. The resulting peptides were separated by reverse-phase HPLC and then subjected to Edman degradation.

Transient Transfection of β Subunits into COS-7 Cells—COS-7 cells were obtained from the American Type Culture Collection and grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. cDNAs encoding the β_3 and β_4 subunits were subcloned into the vector pcDNA3 (Invitrogen). Plasmid DNA (30 μg) was introduced into 1×10^7 cells by electroporation at 950 microfarads and 320 V in a Bio-Rad Gene Pulser. To increase cell viability, cells were electroporated in Cytomix buffer (15). After 72 h, cells were washed and harvested in PBS. Crude cell lysate was prepared by homogenization in PBS with 0.23 mM phenylmethylsulfonyl fluoride and 0.64 mM benzamidine. Cell pellets were collected by centrifugation at 14,000 rpm for 10 min and resuspended in Laemmli sample buffer (13).

Immunoaffinity Enrichment of Ca^{2+} Channels Containing α_{1B}—Protease inhibitors were included in all buffers at the concentrations indicated above to minimize proteolysis of the receptors throughout the purification. Rabbit brain membranes (~2 mg), prepared as detailed elsewhere (14), were prelabeled with ^{125}I-ω-CTx GVIA as described above and used as a tracer to detect the channels throughout the purification. Labeled receptors, together with unlabeled protein (~1 g), were extracted from the membrane in solubilization buffer for 1 h at 4 °C. After centrifugation at 35,000 rpm for 37 min in a 45 Ti rotor, the detergent extract was diluted 3-fold with ice-cold distilled, deionized water and applied to a heparin-agarose column pre-equilibrated with buffer A 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 5-ml fractions. Peak fractions were detected by γ-counting and pooled. The enriched channels were then incubated overnight with mAb CC18 coupled to Avidchrom hydrazide (~2 ml of settled resin) prepared according to the manufacturer's instructions. The resin was washed extensively with buffer A containing 0.7 M NaCl and then eluted with 50 mM glycine HCl, pH 2.5, containing 0.6 M NaCl and 0.1% (w/v) digitonin. Fractions (1 ml) were immediately neutralized with 2 M Tris-HCl, pH 8.0 (125 μl). The peak fractions were detected by γ-counting and were concentrated in an Amicon ultracentrifugation unit using a YM-100 membrane. The subunit composition was analyzed by SDS-PAGE and immunoblotting.

Binding of AID_B-Glutathione S-Transferase Fusion Protein to ^{35}S-Labeled β Subunits—The affinity of each of the *in vitro* synthesized ^{35}S-labeled β subunits for AID_B was performed as described previously (8). Briefly, various ^{35}S-labeled β subunit probes (β_{1b}, β_{2a}, β_3, and β_4) were synthesized by coupled *in vitro* transcription and translation with the TNT® kit (Promega). 0.7–1.3 pM ^{35}S-labeled β subunit was incubated overnight at 4 °C in PBS (1 ml) with increasing concentrations (100 pM to 1 mM) of the AID_B-glutathione *S*-transferase fusion protein (residues 378–434 of the α_{1B} subunit (16)) noncovalently coupled to glutathione-Sepharose beads. The beads were then washed four times with PBS and subjected to scintillation counting, and the data were analyzed using the GraFit program.

TABLE I

Peptide sequences of the N-type Ca^{2+} channel β subunit

Subunits of the purified N-type Ca^{2+} channel were resolved by SDS-PAGE, electrotransferred onto Immobilon PSQ membrane, and visualized with Coomassie Blue. The proteins with mobilities of 57 kDa were excised and digested with trypsin for 18 h at 37 °C. The resulting peptides were separated by reverse-phase HPLC, followed by direct sequencing. Seven different peptides were sequenced. Comparison with those in the GenBank Data Bank identified three β_3 subunit peptides and four β_4 sequences. Dashes indicate residues that were unidentified by sequencing.

Peptide	Sequence	β subtype	Residues
1	SGNPSSL-DI-N	β_3	138–150
2	LLAQDSE-D	β_3	459–467
3	LTVQMMAY	β_3	315–323
4	REQQAAIQLER- -SKPVAFAVK	β_4	76–97
5	RSSLAEVQSE-ERIFE	$\beta_4 = \beta_2 = \beta_1 > \beta_3$	285–300[a]
6	KTSLAPIIV-V-	$\beta_4 = \beta_3 = \beta_2 > \beta_1$	323–334[a]
7	NLGSTALSPYPTAIS-LQ	β_4	416–433

[a] Residues in the β_4 sequence.

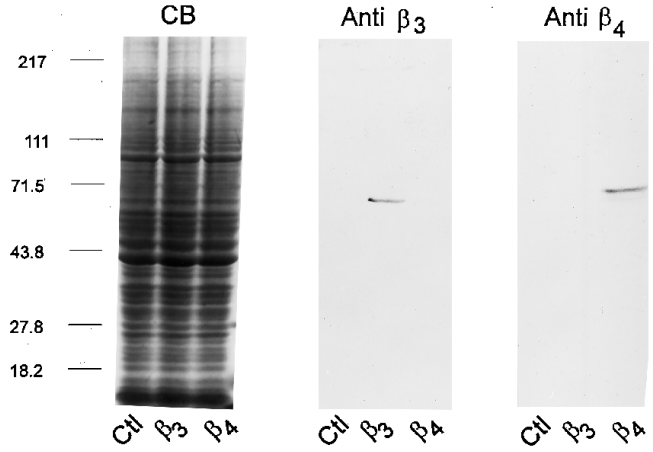

FIG. 2. **Determination of the specificity of the β_3 and β_4 antibodies.** The specificity of the affinity-purified β_3 and β_4 subunit antibodies was determined by transiently transfecting COS-7 cells as described under "Experimental Procedures" with constructs encoding the respective β subunit. After harvesting the cells, aliquots (~150 μg) were subjected to SDS-PAGE on a 5–16% gel, followed by either Coomassie Blue staining (*CB*) or immunostaining with affinity-purified β_3 and β_4 subunit antibodies. *Ctl*, control untransfected cells.

RESULTS AND DISCUSSION

The N-type Ca^{2+} channel subunit composition was investigated using a number of different antibodies. Saturating amounts of four β subunit-specific antibodies were determined by incubating increasing quantities of these antibodies with a constant amount of ^{125}I-ω-CTx GVIA-labeled receptors to establish the maximum immunoprecipitation of solubilized rabbit brain N-type channels (Fig. 1*A*). The maximum immunoprecipitation by a variety of antibodies raised against several components of voltage-dependent Ca^{2+} channels was subsequently compared. Polyclonal antisera against the purified N-type Ca^{2+} channel (Sheep 46) precipitated the ^{125}I-ω-CTx GVIA receptors and provided the 100% control value (Fig. 1*B*). A second polyclonal antibody (Y006) raised against the II–III loop of the α_{1B} subunit precipitated 93 ± 1.8% of the ^{125}I-ω-CTx GVIA receptors with respect to the Sheep 46 antibodies. This confirmed the presence of the α_{1B} subunit in all N-type Ca^{2+} channel oligomers. In contrast, a monoclonal antibody (IIC12) raised against the purified skeletal muscle dihydropyridine receptor (3) was included as a negative control and was shown to precipitate negligible amounts (<3%) of ^{125}I-ω-CTx GVIA receptors. In addition, a polyclonal antibody specific for the α_{1C} subunit (Rabbit 140) did not sediment significant amounts of the N-type channels and served as a second negative control.

Saturating amounts of each of the β subunit antibodies were used to precipitate the relative amounts of each β subunit associated with the ^{125}I-ω-CTx GVIA receptor. Consistent with previous observations (14), a polyclonal antibody specific for the β_3 subunit precipitated the largest amount of toxin binding (56.1 ± 8.3%), although a β_3 subunit antibody was previously shown to sediment a larger fraction of the receptors. In the earlier study, however, the β_3 subunit antibodies (affinity-purified from Sheep 46) may have been cross-reactive with the β_4 subunit, which at that time had not been cloned. In the present study, the β_3 subunit-specific antibody was raised directly against a C-terminal fusion protein (Sheep 49) and was shown to be specifically reactive with the β_3 subunit (30). Interestingly, the β_4 subunit-specific antibody also sedimented a significant proportion of receptors (30.5 ± 2.1%), suggesting that it is a major component of the purified N-type Ca^{2+} channels. Moreover, coincubation of saturating amounts of the β_3 and β_4 subunit antibodies with the labeled receptor precipitated 84 ± 0.7%, which is approximately equivalent to the sum of precipitation by both sera incubated separately (56.1 ± 8.3% (β_3) + 30.5 ± 2.1% (β_4)). This further confirmed the specificity of these antibodies and demonstrated that both β subunits were not present in the same oligomer since saturating concentrations of both antibodies incubated together did not immunoprecipitate less than the sum of each antibody incubated separately.

Notably, the β_{1b} subunit-specific antibody also precipitated a significant amount of the labeled receptors after subtracting the nonspecific precipitation (10.3 ± 1.6%), suggesting that it also associates with the α_{1B} subunit of the N-type Ca^{2+} channel in brain. In contrast, the β_{2a} subunit antibody did not precipitate significant amounts of ^{125}I-ω-CTx GVIA binding over the nonspecific binding, which was reproducibly <3%, suggesting either that the β_{2a} subunit may not be associated with the brain N-type Ca^{2+} channel or that its expression level in brain is too low to detect (26). These immunoprecipitation data represent the first evidence that three different β subunits associate with the α_{1B} subunit in the native N-type Ca^{2+} channel.

To investigate the β subunit heterogeneity of N-type channels further, immunoaffinity-purified Ca^{2+} channel subunits (4) were separated by SDS-PAGE, electrophoretically transferred onto Immobilon PSQ membrane, and visualized by Coomassie Blue staining. The 57-kDa band was excised and digested with trypsin. This generated seven peptides, which were resolved by reverse-phase HPLC, followed by Edman degradation (Table I). Comparison of the sequences with those in the database showed that peptides 1–3 had >80% amino acid identity to the β_3 subunit, which confirmed the previous observation that this subunit is present in the purified N-type Ca^{2+} channel (4). Peptides 4 and 7 showed >85% amino acid identity to the more recently cloned β_4 subunit (17) and were absent from the β_3 subunit sequence. The remaining two peptide sequences are present in the second conserved domain of each of the β subunits and could therefore not be specifically assigned to any of the β subunits. These data confirmed that both the β_3 and β_4 subunits are associated with the N-type Ca^{2+} channels.

TABLE II
Specificity of mAb CC18

The mAb specific for the α_{1B} subunit (mAb CC18) was prepared by hyperimmunizing a rat with a fusion protein encoding the II–III loop of the pore-forming subunit of the N-type channel. To assess the specificity of this antibody, the N- and P/Q-type channels were labeled with either ^{125}I-ω-CTx GVIA or ^{125}I-ω-CTx MVIIC, and immunoprecipitation was performed using mAb CC18. The reactivity was also tested by enzyme-linked immunosorbent assay using as the antigen the fusion protein for the II–III loops of the α_{1A} and α_{1B} subunits, and serial dilutions of mAbCC18.

Antibody	IgG subtype	Immunoprecipitation of brain receptors (nmol/liter)		End point dilution by ELISA[a] fusion proteins	
		^{125}I-ω-CTx GVIA	^{125}I-ω-CTx MVIIC	α_{1A}	α_{1B}
mAbCC18	2a	4550	0.08	<40	10^6

[a] ELISA, enzyme-linked immunosorbent assay.

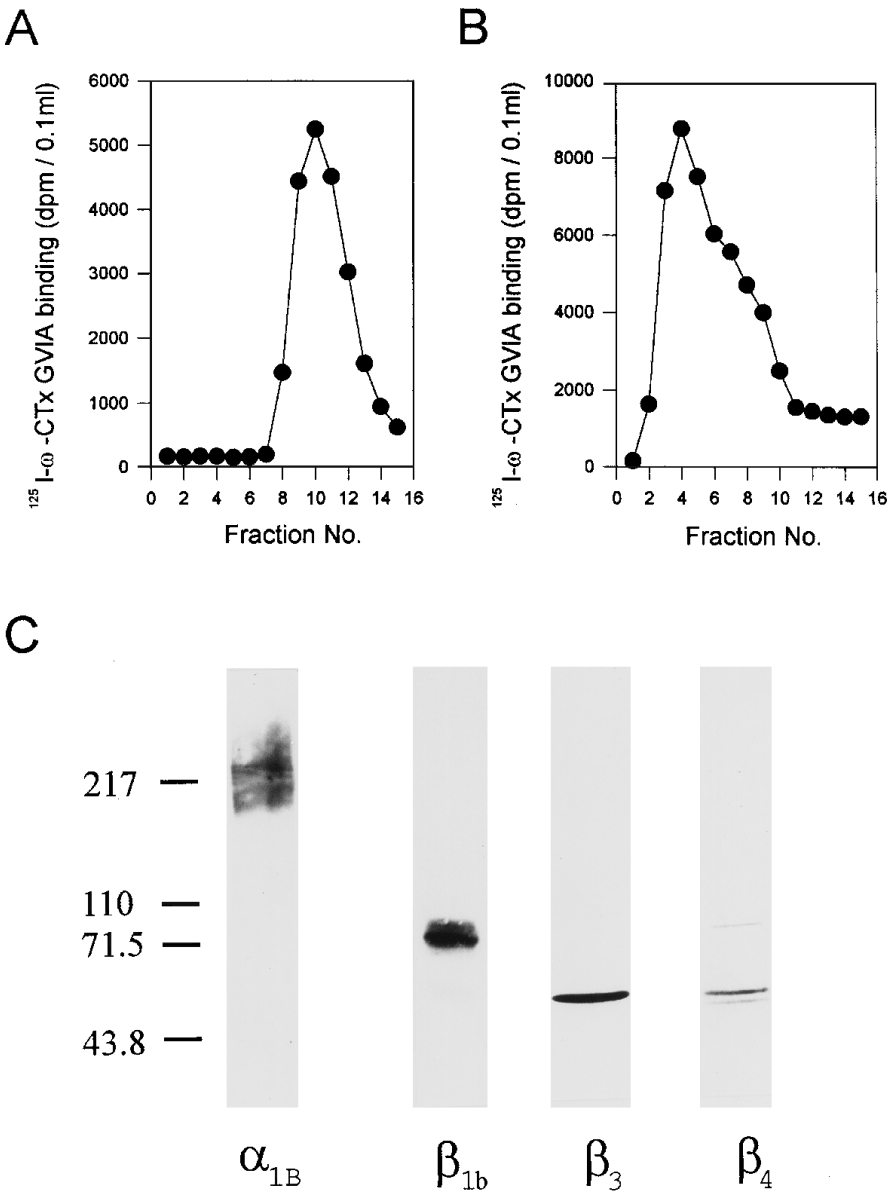

FIG. 3. **Analysis of N-type Ca^{2+} channels immunoaffinity-enriched using mAb CC18 against the α_{1B} subunit.** *A*, shown is the elution profile of ^{125}I-ω-CTx GVIA receptors from the heparin-agarose column. The column was developed with 0.7 M NaCl in buffer A, collecting 5-ml fractions. *B*, shown is the elution profile of the mAb CC18 immunoaffinity column. The eluate from the heparin-agarose column was loaded onto this antibody column, and enriched channels were eluted with 50 mM glycine buffer, pH 2.5. *C*, the mAb CC18 immunoaffinity column eluate (~1–2 μg) was resolved on a 3–12% SDS gel and electrophoretically transferred to nitrocellulose. The subunit composition of the enriched channels was then examined by immunoblotting with mAb CC18 and each of the indicated affinity-purified β subunit antibodies. Molecular mass markers (in kilodaltons) are shown to the left.

Since the β_1 subunit has a molecular mass of ~72 kDa, it was resolved from the β_3 and β_4 subunits (molecular masses of 57 kDa) by SDS-PAGE prior to sequencing, which explains why no specific sequences for this subunit were detected.

The specificity of the polyclonal antisera that were raised against the C-terminal fusion proteins of the β_3 (Sheep 49) and β_4 (Rabbit 145) subunits was tested in immunoblot experiments. COS-7 cells, which do not contain detectable levels of endogenous Ca^{2+} channel subunits, were transiently transfected with constructs encoding the β_3 and β_4 subunits separately. The cells were harvested, and equal amounts of the protein were subjected to SDS-PAGE on a 5–16% gel. The proteins were electrophoretically transferred onto nitrocellulose and probed with affinity-purified β_3 and β_4 subunit-specific antibodies. Neither antibody recognized any proteins in the untransfected cells. The resulting immunoblots demonstrate the specificity of the β_3 and β_4 subunit-specific antibodies since the β_3 antibodies recognized only the protein in the

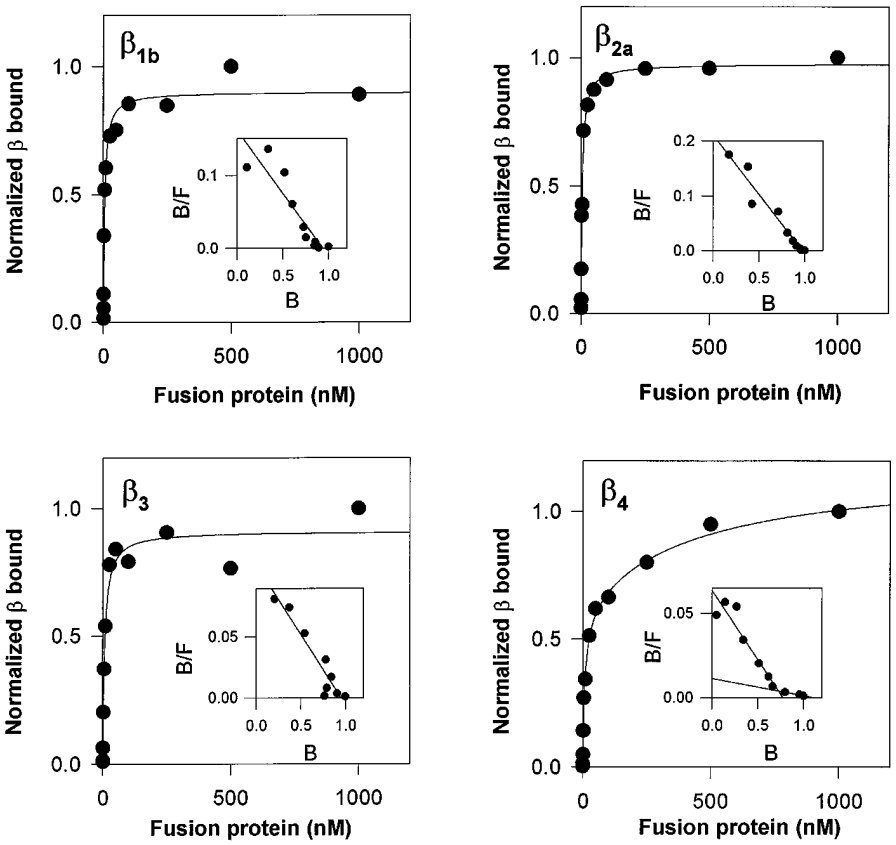

FIG. 4. **Analyses of AID_B-glutathione *S*-transferase fusion protein binding to several β subunits.** Increasing concentrations of AID_B-glutathione *S*-transferase fusion protein (0.1 nM to 1 mM) were coupled to GSH-Sepharose for 1 h and then incubated with 0.7–1.3 pM ^{35}S-labeled β subunits for 15 h at 4 °C. The data were fitted using the GraFit fitting program, yielding apparent K_D values of 4.7 nM (β_{1b}), 4.8 nM (β_{2a}), 7.6 nM (β_3), and 8.4 and 444 nM (β_4). Scatchard plots were fitted by linear regression (*insets*). *B/F*, bound/free.

β_3-transfected cells and none in those transfected with β_4. The β_4 antibodies were likewise shown to be specific for the β_4 subunits expressed in COS-7 cells (Fig. 2).

The subunit composition of the N-type Ca^{2+} channel was further established by the development of a purification scheme using a heparin-agarose column, as was previously published (4), followed by immunoaffinity chromatography using the α_{1B} subunit-specific mAb CC18 coupled to Avidchrom hydrazide. This monoclonal antibody was raised against a fusion protein containing the II–III loop of the α_{1B} subunit. This bound specifically and with high affinity to ^{125}I-ω-CTx GVIA receptors, but did not immunoprecipitate ^{125}I-ω-CTx MVIIC receptors (9) or react with the fusion protein containing the II–III loop of the α_{1A} subunit (Table II) in enzyme-linked immunosorbent assay and immunoblotting experiments, even though there is a low sequence identity in this region between both α_1 subunits (18, 19). Furthermore, this mAb did not immunoprecipitate any significant proportions of [^{3}H]PN 200-110 binding to skeletal muscle triads (data not shown), which was not surprising since there is very little homology between the sequence of the II–III loop of the α_{1B} subunit and the other α_1 subunit genes (α_{1S}, α_{1C}, α_{1E}, and α_{1D} subunits), so the possibility of cross-reactivity of mAb CC18 in either immunoblotting or immunoprecipitation experiments with any other α_1 subunit was highly unlikely.

Rabbit brain membranes were first labeled with ^{125}I-ω-CTx GVIA, solubilized in high ionic strength buffer containing 1% (w/v) digitonin and a mixture of five protease inhibitors, and applied to a heparin-agarose column. Elution of the column with 0.7 M NaCl resulted in ~10-fold enrichment of the channels (Fig. 3*A*). The peak of channel activity was pooled and subsequently applied to the mAb CC18-Avidchrom column. Development with glycine buffer, pH 2.5, yielded a large peak of radioactivity (Fig. 3*B*) that was pooled and analyzed by SDS-PAGE followed by Western blot analysis with antibodies to the α_{1B} subunit and each of the four β subunits. The resulting immunoblots showed the presence of the broad diffusely stained α_{1B} subunit, with an apparent molecular mass ranging from 190 to 230 kDa. This broad band may contain more than one α_1 species since multiple splice variants of the α_{1B} subunit have been shown to exist (19). However, mAb CC18 is raised against the II–III loop and therefore would be unable to distinguish between the different C-terminal splice variants. Interestingly, immunoblotting with affinity-purified β_{1b}, β_3, and β_4 subunit-specific antibodies (Fig. 3*C*) demonstrated the presence of each of these β subunits in the preparation. As predicted from the immunoprecipitation data, the β_{2a} subunit was not detected in the enriched preparation using its specific antibody and the highly sensitive ECL detection method. Although it cannot be excluded that the β_{2a} subunit may interact with the α_{1B} subunit, the protein levels of this subunit or its level of association with α_{1B} is too low to detect in brain.

Recent identification of the interaction domains between the α_1 and β subunits has allowed the development of an assay for studying the specific association of these subunits *in vitro*. The binding affinity of the AID_B fusion protein to *in vitro* translated ^{35}S-labeled β subunits from each of the four genes was measured. Interestingly, the AID_B fusion protein interacted with the β_{1b} (K_D = 4.7 nM; 90% of total binding capacity), β_{2a} (K_D = 4.8 nM; 98% of total binding capacity), and β_3 (K_D = 7.26 nM; 91% of total binding capacity) subunits with similar high affinities (Fig. 4). Unlike β_{1b}, β_{2a}, and β_3, the β_4 subunit appeared to bind to two sites, one with high affinity (8.4 nM; 63% of total binding capacity) and the other with low affinity (444 nM; 55% of total binding capacity). The lower binding affinity may have been due to the binding of some proteolyzed forms of the β_4 subunit that were generated during the synthesis of the probe, as was previously shown (8). These data demonstrate that the AID_B fusion protein can interact with each of the β subunits with similar high affinity, unlike the binding to the AID_A site, which

showed a 20-fold lower affinity for β_3 than for β_4 (8).

Although several *in vitro* expression studies using recombinant protein have shown that α_1 subunits form functional Ca^{2+} channels with variable channel kinetics depending upon which β subunit is coexpressed (19–24), this is the first report to demonstrate directly that different β subunits are associated with the α_{1B} subunit in the native N-type Ca^{2+} channel. The initial suggestion that only the β_3 subunit was present in the N-type Ca^{2+} channel was made prior to the discovery and cloning of the β_4 subtype, which we have demonstrated here to be a significant component of the N-type Ca^{2+} channel by immunoprecipitation, internal sequence information, and Western blotting analysis. Data presented herein revealed that there is more diversity in the N-type Ca^{2+} channel subunit composition than was originally indicated. In contrast, the α_{1S} subunit of the skeletal muscle dihydropyridine receptor appears to associate only with the β_{1a} subunit (data not shown) since this is the only β subunit known to be expressed in skeletal muscle tissue (25). In neurons, the levels of expression of each β subunit may, in part, determine the apparent specificity of association between the α_{1B} subunit and various β subunits. Our data support this hypothesis since immunoprecipitation of four different β subunits with specific antibodies correlates well with the relative amounts of each β gene expressed in brain, the most abundant being β_3 and β_4, with smaller quantities of β_{1b} and negligible proportions of the β_2 subunit (26). It is thus possible that in different tissue sources, the levels of expression of certain β subunits determine the levels of association with the α_1 subunit.

The association of different subunit combinations as a method of generating subtle differences in channel properties has been reported for other ion channels. The neuronal voltage-dependent α-dendrotoxin-sensitive K^+ channels form hetero-oligomers with various combinations of four α subunits with and without four ancillary β subunits (27). In the ligand-gated γ-aminobutyric acid type A receptor, particular β subunits have also been shown to associate with several different α subunits *in vivo* (28), thereby creating more channel heterogeneity. In conclusion, the generation of different N-type Ca^{2+} channel oligomers that differ in their β subunit composition may account for some of the functional diversity of these channels in the nervous system.

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