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

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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 \(\beta\) subunit of the N-type Ca\(^{2+}\) channel with an α\(_{1B}\) subunit-specific monoclonal antibody showed the association of β\(_{1A}\), β\(_{1B}\), and β\(_{1C}\) subunits.

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, α, δ, and β), with a variable channel-specific subunit (γ or 95 kDa, respectively) (3). The genes encoding the α 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 (S). Recent studies have identified complementary interaction domains on the α\(_{1}\) and β subunits (6, 7).

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The β subunit regulates channel activity by binding to a highly conserved portion of the cytoplasmic linker of the I–III loop of all α\(_{1}\) subunits, known as the α\(_{1}\) subunit interaction domain (AID). 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 autoimmune 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—\(\beta\)-Conotoxin (CTx) GVIA, [\(^{35}\)S]methylene, 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 (Unysyn 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 I–III cytoplasmic loop of the α\(_{1B}\) subunit (29). It reacts selectively with high affinity brain receptors for \(\omega\)-CTx GVIA.

SDS-PAGE and Immunoblot Analysis—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 immunofinity-purified as described previously (14), using the appropriate fusion protein for β\(_{1A}\) (residues 428–597;...
GenBank accession number X61394), \( \beta_3 \) (residues 462–579; GenBank accession number M80545), \( \beta_2 \) (residues 369–484; GenBank accession number M88751), and \( \beta_4 \) (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 \(^{125}\text{I} \)-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 CTx GVIA for 1 h at 22°C. Then, the membranes were sedimented by centrifugation in a Beckman TL 100 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 mM leupeptin, 0.7 mM pepstatin A, 76.8 mM 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 \(^{125}\text{I} \)-counting.

\( \beta_4 \) subunits were extracted from the membrane in solubilization buffer for 1 h at 4°C. The resulting pellet was resuspended in solubilization buffer (1 ml) overnight at 4°C. The beads were washed as described above and quantified by \(^{125}\text{I} \)-counting. The amount of precipitation in each case was determined relative to that of Sheep 46 (Sh46; 100%).

Immunofluorescence Enrichment of Ca\(^{2+} \) Channels Containing \( \alpha_{1B} \)—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 \(^{125}\text{I} \)-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 \(^{125}\text{I} \)-counting and pooled. The enriched channels were then incubated overnight with mAb CC18 coupled to Avidinhydrozide (~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 mM Tris-HCl, pH 8.0 (125 μl). The peak fractions were detected by \(^{125}\text{I} \)-counting and were concentrated in an Amicon ultrafiltration unit using a YM-100 membrane. The subunit composition was analyzed by SDS-PAGE and immunoblotting.

Binding of AIDG-Glutathione S-Transferase Fusion Protein to \(^{35}\text{S} \)-Labeled \( \beta \) Subunits—The affinity of each of the in vitro synthesized \(^{35}\text{S} \)-labeled \( \beta \) subunits for AIDG was determined as described previously (8). Briefly, various \(^{35}\text{S} \)-labeled \( \beta \) subunit probes (\( \beta_{1A}, \beta_{2A}, \beta_{3A}, \beta_{4A} \)) were synthesized by coupled in vitro transcription and translation with the TNT \( ^{\text{TM}} \) kit (Promega). 0.7–1.3 pm \(^{35}\text{S} \)-labeled \( \beta \) subunit was incubated overnight at 4°C in PBS (1 ml) with increasing concentrations (100 pm to 1 nm) of the AIDG-glutathione S-transferase fusion protein (residues 378–434 of the \( \alpha_{1B} \) 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.
the \( \beta_3 \) subunit precipitated the largest amount of toxin binding (56.1 \( \pm \) 8.3\%), although a \( \beta_3 \) subunit antibody was previously shown to sediment a larger fraction of the receptors. In the earlier study, however, the \( \beta_3 \) subunit antibodies (affinity-purified from Sheep 46) may have been cross-reactive with the \( \beta_4 \) subunit, which at that time had not been cloned. In the present study, the \( \beta_3 \) subunit-specific antibody was raised directly against a C-terminal fusion protein (Sheep 49) and was shown to be specifically reactive with the \( \beta_3 \) subunit (30). Interestingly, the \( \beta_4 \) subunit-specific antibody also sedimented a significant proportion of receptors (30.5 \( \pm \) 2.1\%), suggesting that it is a major component of the purified N-type \( \text{Ca}^{2+} \) channels. Moreover, coinubation of saturating amounts of the \( \beta_3 \) and \( \beta_4 \) subunit antibodies with the labeled receptor precipitated 84 \( \pm \) 0.7\%, which is approximately equivalent to the sum of precipitation by both sera incubated separately (56.1 \( \pm \) 8.3\%) (\( \beta_3 \) + 30.5 \( \pm \) 2.1\%) (\( \beta_4 \)). This further confirmed the specificity of these antibodies and demonstrated that both \( \beta \) 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 \( \beta_{1b} \) subunit-specific antibody also precipitated a significant amount of the labeled receptors after subtracting the nonspecific precipitation (10.3 \( \pm \) 1.6\%), suggesting that it also associates with the \( \alpha_3\delta_2 \) subunit of the N-type \( \text{Ca}^{2+} \) channel in brain. In contrast, the \( \beta_{2a} \) subunit antibody did not precipitate significant amounts of \( 1^{251} \)-\( \omega \)-CTX GVIA binding over the nonspecific binding, which was reproducibly \(<\)3\%, suggesting either that the \( \beta_{2a} \) subunit may not be associated with the brain N-type \( \text{Ca}^{2+} \) channel or that its expression level in brain is too low to detect (26). These immunoprecipitation data represent the first evidence that three different \( \beta \) subunits associate with the \( \alpha_{1b} \) subunit in the native N-type \( \text{Ca}^{2+} \) channel.

To investigate the \( \beta \) subunit heterogeneity of N-type channels further, immunoaffinity-purified \( \text{Ca}^{2+} \) 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 \( \beta_3 \) subunit, which confirmed the previous observation that this subunit is present in the purified N-type \( \text{Ca}^{2+} \) channel (4). Peptides 4 and 7 showed \(<\)85\% amino acid identity to the more recently cloned \( \beta_4 \) subunit (17) and were absent from the \( \beta_3 \) subunit sequence. The remaining two peptide sequences are present in the second conserved domain of each of the \( \beta \) subunits and could therefore not be specifically assigned to any of the \( \beta \) subunits. These data confirmed that both the \( \beta_3 \) and \( \beta_4 \) subunits are associated with the N-type \( \text{Ca}^{2+} \) channels.

**Table I**

| Peptide | Sequence | \( \beta \) subtype | Residues |
|---------|----------|----------------------|----------|
| 1       | SGNPSSL-DI-N | \( \beta_3 \) | 138-150 |
| 2       | LLAQDSE-D | \( \beta_3 \) | 459-467 |
| 3       | LTQVMMAY | \( \beta_3 \) | 315-323 |
| 4       | RERQAAIQLER-SKPVAFVK | \( \beta_4 \) | 76-97 |
| 5       | RSSLAEVOSE-ERIFE | \( \beta_4 \) | 285-300* |
| 6       | KTSALPIIV-V | \( \beta_4 \) | 323-334* |
| 7       | NLGSTALSYPTAIS-LQ | \( \beta_4 \) | 416-433 |

*a* Residues in the \( \beta_4 \) sequence.
Since the $\beta_3$ subunit has a molecular mass of $\sim 72$ kDa, it was resolved from the $\beta_2$ and $\beta_4$ 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 $\beta_3$ (Sheep 49) and $\beta_4$ (Rabbit 145) subunits was tested in immunoblot experiments. COS-7 cells, which do not contain detectable levels of endogenous $\text{Ca}^{2+}$ channel subunits, were transiently transfected with constructs encoding the $\beta_3$ and $\beta_4$ 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 $\beta_3$ and $\beta_4$ subunit-specific antibodies. Neither antibody recognized any proteins in the untransfected cells. The resulting immunoblots demonstrate the specificity of the $\beta_3$ and $\beta_4$ subunit-specific antibodies since the $\beta_3$ antibodies recognized only the protein in the

**FIG. 3.** Analysis of N-type $\text{Ca}^{2+}$ channels immunoaffinity-enriched using mAb CC18 against the $\alpha_{1B}$ subunit. A, shown is the elution profile of 125I-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 $\beta$ subunit antibodies. Molecular mass markers (in kilodaltons) are shown to the left.
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 (8). These data demonstrate that the AIDB fusion protein interacts with each of the \(\beta_4\) subunits with similar high affinities, unlike the binding to the AID A site, which was highly unlikely.

Recent identification of the interaction domains between the \(\alpha_3\) and \(\beta\) 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 \(\beta\) subunits from each of the four genes was measured. Interestingly, the AID\(_B\) fusion protein interacted with the \(\beta_{1B}\) (\(K_D = 4.7\) nM; 90% of total binding capacity), \(\beta_{2a}\) (\(K_D = 4.8\) nM; 98% of total binding capacity), and \(\beta_3\) (\(K_D = 7.26\) nM; 91% of total binding capacity) subunits with similar high affinities (Fig. 4). Unlike \(\beta_{1B}\), \(\beta_{2a}\), and \(\beta_3\), the \(\beta_4\) 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 \(\beta_4\) 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 \(\beta\) subunits with similar high affinity, unlike the binding to the AID\(_A\) site, which
showed a 20-fold lower affinity for $\beta_3$ than for $\beta_4$ (8).

Although several in vitro expression studies using recombinant protein have shown that $\alpha_1$ subunits form functional Ca$^{2+}$ channels with variable channel kinetics depending upon which $\beta$ subunit is coexpressed (19–24), this is the first report to demonstrate directly that different $\beta$ subunits are associated with the $\alpha_{1B}$ subunit in the native N-type Ca$^{2+}$ channel. The initial suggestion that only the $\beta_2$ subunit was present in the N-type Ca$^{2+}$ channel was made prior to the discovery and cloning of the $\beta_4$ 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 $\alpha_{1S}$ subunit of the skeletal muscle dihydropyridine receptor appears to associate only with the $\beta_{1S}$ subunit (data not shown) since this is the only $\beta$ subunit known to be expressed in skeletal muscle tissue (25). In neurons, the levels of expression of each $\beta$ subunit may, in part, determine the apparent specificity of association between the $\alpha_{1B}$ subunit and various $\beta$ subunits. Our data support this hypothesis since immunoprecipitation of four different $\beta$ subunits with specific antibodies correlates well with the relative amounts of each $\beta$ gene expressed in brain, the most abundant being $\beta_3$ and $\beta_4$, with smaller quantities of $\beta_1$ and negligible proportions of the $\beta_2$ subunit (26). It is thus possible that in different tissue sources, the levels of expression of certain $\beta$ subunits determine the levels of association with the $\alpha_1$ 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 $\alpha$-conotoxin-sensitive K$^+$ channels form heterooligomers with various combinations of four $\alpha$ subunits with and without four ancillary $\beta$ subunits (27). In the ligand-gated $\gamma$-aminobutyric acid type A receptor, particular $\beta$ subunits have also been shown to associate with several different $\alpha$ 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 $\beta$ subunit composition may account for some of the functional diversity of these channels in the nervous system.

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