

Alternative Splicing of a β_4 Subunit Proline-Rich Motif Regulates Voltage-Dependent Gating and Toxin Block of $\text{Ca}_v2.1$ Ca^{2+} Channels

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Ca^{2+} channel β subunits modify α_1 subunit gating properties through direct interactions with intracellular linker domains. In a previous report (Helton and Horne, 2002), we showed that alternative splicing of the β_4 subunit had α_1 subunit subtype-specific effects on Ca^{2+} channel activation and fast inactivation. We extend these findings in the present report to include effects on slow inactivation and block by the peptide toxin ω -conotoxin (CTX)-MVIC. N-terminal deletion and site-directed mutagenesis experiments revealed that the effects of alternative splicing on toxin block and all aspects of gating could be attributed to a proline-rich motif found within N-terminal β_{4b} amino acids 10–20. Interestingly, this motif is conserved within the third postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 domain of the distantly related membrane-associated guanylate kinase homolog, PSD-95. Sequence identity of ~30% made possible the building of β_{4a} and β_{4b}

three-dimensional structural models using PSD-95 as the target sequence. The models (1) reveal that alternative splicing of the β_4 N terminus results in dramatic differences in surface charge distribution and (2) localize the proline-rich motif of β_{4b} to an extended arm structure that flanks what would be the equivalent of a highly modified PSD-95 carboxylate binding loop. Northern blot analysis revealed a markedly different pattern of distribution for β_{4a} versus β_{4b} in the human CNS. Whereas β_{4a} is distributed throughout evolutionarily older regions of the CNS, β_{4b} is concentrated heavily in the forebrain. These results raise interesting questions about the functional role that alternative splicing of the β_4 subunit has played in the evolution of complex neural networks.

Key words: calcium channel; β_4 subunit; PSD-95; alternative splicing; gating; N terminus; ω -CTX-MVIC

Voltage-gated Ca^{2+} channels participate in an extensive array of cellular activities including excitation–contraction coupling, transcription, and neurotransmitter release. Neuronal Ca_v2 channels are assemblies of up to five subunits, α_1 , α_2/δ , β , and γ . The α_1 subunit consists of four homologous repeats (I–IV) of six helices (S1–S6) that arrange to form the selectivity filter and pore. The 24 transmembrane helices are connected by a series of alternating intracellular and extracellular loops. These loops are targets for a host of modifying proteins, including β subunits, G-proteins, calmodulin, and syntaxin, as well as the peptide toxins of venomous spiders and marine snails (Catterall, 2000). Interaction of these proteins with α_1 subunits typically alters the voltage dependency and kinetics of channel gating, which in turn modifies Ca^{2+} entry into neurons.

Ultimately, gating behavior is determined by the interactions of individual amino acid side chains with the electrostatic forces within their microenvironments. This is especially true for the positively charged S4 helical segments that constitute the voltage sensors in Na^+ , Ca^{2+} , and K^+ channels. Biophysical studies have shown that depolarization disrupts S4 side-chain interactions of

Shaker K^+ channels to the extent that S4 helices rotate 180° along their axes (Cha et al., 1999; Glauner et al., 1999). This motion likely triggers a cascade of side-chain disruptions that ultimately leads to rotation and separation of the intracellular S6 segments that form the K^+ channel gate (Bezannilla, 2000). Such a mechanism is supported by recent studies delineating the conformational changes associated with open and closed states of bacterial two-membrane-spanning K^+ channels (Jiang et al., 2002b) and is generally applicable to Na^+ and Ca^{2+} channel gating.

Attempts have been made to assign specific gating functions to individual Ca^{2+} channel homology domains. Early chimera studies indicated that the IS6 segment was critical for setting the rate of fast inactivation (Zhang et al., 1994); however, substitution of IIS6 and IIS6 of the $\text{Ca}_v2.3$ channel into the slow inactivating $\text{Ca}_v1.2$ channel caused a leftward shift in the voltage dependence of inactivation and increased the rate of $\text{Ca}_v1.2$ channel inactivation to near $\text{Ca}_v2.3$ rates (Stotz et al., 2000). Effects on gating have been reported for amino acid substitutions in IS3 (Zhong et al., 2001), the I-II linker (Berrou et al., 2001), IIS6 (Stotz and Zamponi, 2001), extracellular linkers IIS3-S4 (Lin et al., 1997), and IVS3-S4 (Hans et al., 1999), and IVS6 (Berjukow et al., 2001). Additive effects on $\text{Ca}_v1.2$ channel inactivation were reported recently for individual IS6, IIS6, IIS6, and IVS6 substitutions (Shi and Soldatov, 2002). Together, these data support a structural model of α_1 subunits in which individual transmembrane segments are interdependently entwined (Horn, 2000).

Our results indicate that this model also applies to β subunit interactions with α_1 subunit intracellular linkers. We have shown previously that β_4 subunit alternative splicing had α_1 subtype-

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WI) using the $\beta_4\Delta N$ mutant as the template. The $\beta_4\Delta N$ mutant is missing the first coding 147 bp corresponding to the 49 aa N terminus of β_4 [clone from Helton and Horne (2002)]. The MTN blot was hybridized overnight at 42°C in hybridization buffer [5× SSC, 5% w/v blocking reagent (Roche), 0.1% *N*-lauroylsarcosine, 0.02% w/v SDS, 50% w/v formamide] plus 100 $\mu\text{g/ml}$ herring sperm DNA (Promega). The probe concentration was 1 million counts/ml. The blot was washed with successive stringency washes (four washes, 15 min each at 37°C) ranging from 2× SSC/0.1% SDS to 0.1× SSC/0.1% SDS. The blot was then exposed to radiographic film for 12 hr at –80°C. One microgram of cRNA for both β_{4a} and β_{4b} was run out on a 1% denaturing formaldehyde gel along with a poly(A)-tailed cRNA mass ladder (RNA Molecular Weight Marker 1; Roche). The β_{4a} cRNA is longer than the β_{4b} cRNA because of the additional ~400 nt of 5' untranslated sequence.

Molecular modeling. The sequences for rat postsynaptic density-95 (PSD-95) (DLG4_{rat}) and human β_4 (CACNB4) were obtained (accession numbers P31016 and U95020) from the Swiss-prot database. Amino acids 10–96 of β_{4b} were aligned to residues 303–390 of PSD-95 based on secondary structure prediction (nnPredict) and visual inspection. For β_{4a} , amino acids 50–96 of β_{4b} were aligned to residues 345–390 of PSD-95. Using default parameters, the program MODELLER 6 (Sali and Blundell, 1993) was used to produce 50 models each of β_{4b} and β_{4a} structure based on the solved structure of the third PSD-95/Discs large/zona occludens-1 (PDZ) domain of PSD-95 (1BEF). Five models each were chosen for additional analysis based on the molecular probability density function (PDF) output from MODELLER and stereochemical analysis obtained through Ramachandran output from PROCHECK-NMR (Laskowski et al., 1993). The interactions between different atom types within these models and C α root mean square deviation (RMSD) comparisons between the models and 1BEF were characterized with ERRAT (Colovos and Yeates, 1993). The β_{4a} and β_{4b} models chosen for comparison had the fewest disallowed residues (Ramachandran), lowest molecular PDF and RMSD values, and highest percentage of residues in acceptable conformations based on ERRAT and PROCHECK-NMR analysis. Models were visualized with the program MOLMOL (Koradi et al., 1996).

RESULTS

Alternative splicing of the β_4 subunit affects slow inactivation of Ca_v2.1 Ca²⁺ channels

In a previous study (Helton and Horne, 2002), we showed that Ca_v2.1 complexes containing the longer form of an alternatively spliced β_4 subunit N terminus, β_{4b} (49 aa), inactivated at more negative potentials in response to 20 sec conditioning prepulses than complexes containing a shorter form, β_{4a} (15 aa). To determine whether this response extended to slower types of inactivation, we examined in the present study the effects of β_{4a} and β_{4b} on Ca_v2.1 cumulative inactivation elicited by 5 min conditioning prepulses combined with stimulation at 0.25 Hz. Oocytes were stabilized at –80 mV before a 300 msec reference pulse (I_R) to potentials that were predetermined to give peak inward currents (β_{4b} , 0 mV; β_{4a} , +10 mV). The membrane potential was then stepped to and held at the conditioning prepulse potential (ranging from –100 to –20 mV) for 5 min. A 300 msec test pulse (I_T) was elicited from the conditioning prepulse potential every 15 sec (I_5 equals test pulse at 5 min). The kinetics of entry to slow inactivation for Ca_v2.1 complexes containing either β_{4a} or β_{4b} at –40 mV is shown in Figure 1A. For comparison purposes, we fit the data points for both β_{4a} and β_{4b} to two exponentials (smooth curves in the figure). The time constants for the fast component of entry (τ_1) for β_{4a} and β_{4b} were 28.6 ± 2.6 and 18.9 ± 1.2 sec, respectively, and the time constants for the slow component of entry (τ_2) were 769 ± 23.6 and 384 ± 14.8 sec, respectively. Overall, the I_T/I_R ratio for Ca_v2.1 complexes containing β_{4b} decreased to 0.5 in ~70 sec, whereas those containing β_{4a} required ~380 sec (data not shown). This indicated that β_{4b} caused a more than fivefold acceleration of the kinetics of slow inactivation. Representative current traces for reference and 5 min test

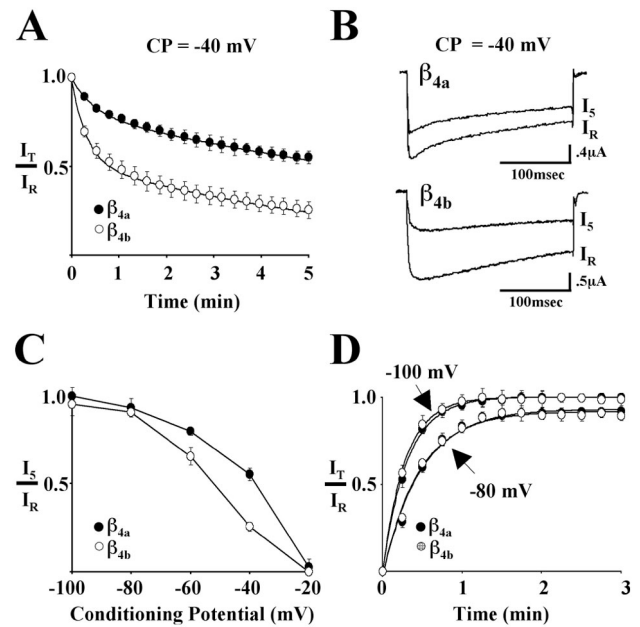


Figure 1. Effects of β_{4a} and β_{4b} on slow inactivation and recovery from slow inactivation of Ca_v2.1 Ca²⁺ channels. Studies were performed with *Xenopus* oocytes expressing α_{1A} , α_2/δ -1, and either β_{4a} or β_{4b} . Reference (I_R) and test current (I_T) traces were generated by 300 msec step depolarizations from various holding potentials to either 0 mV (β_{4b}) or +10 mV (β_{4a}). Maximum values from 300 msec I_R and I_T current traces were used to calculate I_T/I_R where indicated. Barium (5 mM) was used as the charge carrier. **A**, Influence of β_{4a} and β_{4b} on the development of slow inactivation at a conditioning potential (CP) of –40 mV. After a reference pulse (I_R) measured from a holding potential of –80 mV, oocytes were held at –40 mV for 5 min. During this time, 300 msec test pulses (I_T) were applied every 15 sec. Each point represents the mean value of I_T/I_R from 11 (β_{4a}) or 10 (β_{4b}) different recordings. The SEM is shown for each point unless the values were smaller than the symbol. The solid lines represent double-exponential fits to the data. **B**, Representative reference (I_R) and 5 min (I_5) current traces from Ca_v2.1 complexes containing either β_{4a} (top) or β_{4b} (bottom) generated as described in **A**. **C**, Voltage dependence of slow inactivation. The ratio of I_5 to I_R , generated as in **A**, plotted as a function of conditioning potential for Ca_v2.1 complexes containing either β_{4a} or β_{4b} . Data points represent the means of at least six determinations at a given membrane potential. Lines serve only to connect the data points. **D**, Influence of β_{4a} and β_{4b} on the time course of recovery from slow inactivation. After a 300 msec reference pulse (I_R) measured from a holding potential of either –80 or –100 mV, oocytes were held at –30 mV for 5 min. The membrane potential was then returned to either –80 or –100 mV, and sequential test pulses (I_T) were applied at 15 sec intervals for a total of 3 min. Each point represents the mean of at least seven different recordings. Solid lines represent the single-exponential fits of the data.

pulses from a conditioning potential of –40 mV for Ca_v2.1 complexes containing β_{4a} (top) and β_{4b} (bottom) are shown in Figure 1B. As seen in the figure and as described in our previous study, Ca_v2.1 complexes containing β_{4a} underwent open-state fast inactivation faster than did complexes containing β_{4b} . After 5 min at –40 mV, the rate of fast inactivation was unaltered for complexes containing β_{4a} , and slowed only somewhat for complexes containing β_{4b} . The absence of any appreciable tail-current indicated that deactivation was not affected by prolonged depolarization. The I_5/I_R ratio is plotted against the range of conditioning potentials (–100 to –20 mV) in Figure 1C. The figure illustrates that the voltage dependence of Ca_v2.1 slow inactivation is shifted to the left for complexes containing β_{4b} relative to those containing β_{4a} . Half-maximal inactivation occurred at approximately

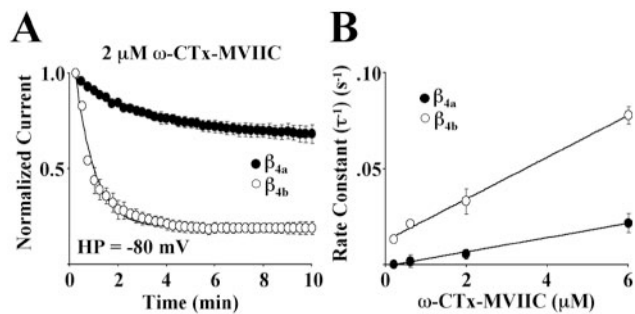


Figure 2. Effects of β_{4a} and β_{4b} on the blockade of $\text{Ca}_v2.1$ channels by $\omega\text{-CTX-MVIIIC}$. Studies were performed with *Xenopus* oocytes expressing α_{1A} , $\alpha_2/\delta-1$, and either β_{4a} or β_{4b} . **A**, Onset and degree of block by a 10 min exposure to $2 \mu\text{M}$ $\omega\text{-CTX-MVIIIC}$ for $\text{Ca}_v2.1$ subunit combinations at a holding potential (HP) of -80 mV. Each point represents the mean of seven (β_{4a}) or eight (β_{4b}) different recordings. The SEM is shown for each data point unless smaller than the symbol. Onset of block for both subunit combinations was fit (line) to a single-exponential time course plus a constant. **B**, The rate constants for the time course of the onset of toxin block were determined from steady-state degree of block from single exponential fits at four different toxin concentrations (0.2, 0.6, 2, and $6 \mu\text{M}$) for $\text{Ca}_v2.1$ complexes containing either β_{4a} or β_{4b} . The averaged rate constants were plotted as a function of toxin concentration (minimum of $n = 7$, \pm SEM). The line represents a linear fit to the data.

-50 mV for complexes containing β_{4b} and -35 mV for β_{4a} . These values are ~ 10 mV (β_{4b}) and 5 mV (β_{4a}) more negative than were determined for inactivation in response to 20 sec conditioning prepulses (Helton and Horne, 2002). Figure 1D shows that recovery from 5 min of slow inactivation at -30 mV is nearly complete when the membrane potential is stepped back to -80 mV, and that there is no difference in the time course of recovery for $\text{Ca}_v2.1$ complexes containing either β_{4a} or β_{4b} . Recovery was somewhat faster and more complete when the membrane potential was stepped back to -100 mV. The recovery data at both potentials fit well to single exponentials. The time constants for recovery for β_{4a} and β_{4b} at -80 mV were 28.6 ± 2.0 and 27.8 ± 1.6 sec, respectively, and at -100 mV, 18.9 ± 0.5 and 17.2 ± 0.4 sec, respectively.

Alternative splicing of the β_4 subunit affects $\omega\text{-CTX-MVIIIC}$ block of $\text{Ca}_v2.1$ Ca^{2+} channels

The results to this point indicate that changes in the structure of the β_4 subunit N terminus impact α_{1A} subunit structures that are important for many aspects of gating, including activation, open-state inactivation, and fast and slow closed-state inactivation. Given that recent evidence indicates that cytosolic determinants of two-membrane-spanning K^+ channel gating are coupled to changes in outer vestibule structure (Perozo et al., 1999; Jiang et al., 2002a,b), we next sought to determine whether alternative splicing of the β_4 subunit would affect the block of $\text{Ca}_v2.1$ channels by a marine snail peptide conotoxin, $\omega\text{-CTX-MVIIIC}$. Conotoxin interactions with voltage-gated Ca^{2+} channels are entirely extracellular and occur through binding sites located near H5 (P) helices in several of the six helix transmembrane-spanning motifs (Ellinor et al., 1994). Figure 2A shows the effects of $2 \mu\text{M}$ $\omega\text{-CTX-MVIIIC}$ on $\text{Ca}_v2.1$ Ca^{2+} channel complexes expressed in *Xenopus* oocytes in the presence of either β_{4a} or β_{4b} . The oocytes were held at -80 mV for 10 min and stimulated every 15 sec. Under these conditions, $\omega\text{-CTX-MVIIIC}$ associated with $\text{Ca}_v2.1$ complexes containing β_{4b} at a faster rate ($\tau = 50 \pm 0.75$ sec) than complexes containing β_{4a} ($\tau = 200 \pm 16$ sec). The loss of Ca^{2+} current resulting from slow inactivation over 10 min at -80 mV

($<15\%$) was subtracted from the data plotted in the figure. Figure 2B demonstrates that as expected for a first-order reaction, the rate constants (τ^{-1}) for toxin block were linearly dependent on toxin concentration as described by the equation $(\tau)^{-1} = k_{\text{on}} [\text{Tx}] + k_{\text{off}}$. Slopes of linear fits to the data for $\text{Ca}_v2.1$ complexes containing either β_{4a} and β_{4b} were $3.7 \times 10^{-6} \text{ M}^{-1} \cdot \text{sec}^{-1}$ and $1.1 \times 10^{-5} \text{ M}^{-1} \cdot \text{sec}^{-1}$, respectively. This indicated that the on-rate (k_{on}) for toxin block was approximately threefold faster for $\text{Ca}_v2.1$ complexes containing β_{4b} than for those containing β_{4a} .

The molecular determinants of alternatively spliced β_4 subunit differential effects on gating and pharmacology are located within amino acids 10–20 of β_{4b}

Having characterized many of the functional consequences of alternative splicing of the β_4 A domain, we focused next on identifying the key structural determinants underlying the observed differences in effects. It was of particular interest to determine whether or not the effects of alternative splicing on gating and pharmacology could be assigned to separate structural entities. To accomplish this, we first created a series of β_{4b} deletion mutants in which the N terminus was shortened by multiples of 10 aa ($\beta_{4b} \Delta 1-10$ through $\beta_{4b} \Delta 1-49$) and characterized their effects on gating and pharmacology of $\text{Ca}_v2.1$ complexes. Figure 3A–D shows that, relative to full-length β_{4b} , deletion of the first 10 aa ($\beta_{4b} \Delta 1-10$) had no effect on the voltage dependence of activation (Fig. 3A), isochronal (20 sec prepulse) inactivation (Fig. 3B), onset into slow inactivation (Fig. 3C), or susceptibility to block by $2 \mu\text{M}$ $\omega\text{-CTX-MVIIIC}$ (Fig. 3D). However, when amino acids 1–20 were removed ($\beta_{4b} \Delta 1-20$), both the voltage dependence of activation (Fig. 3A) and inactivation (Fig. 3B) of $\text{Ca}_v2.1$ complexes shifted to more depolarized potentials. As shown in the figure, the acquired gating properties were essentially identical those for $\text{Ca}_v2.1$ complexes containing β_{4a} . $\text{Ca}_v2.1$ complexes containing $\beta_{4b} \Delta 1-20$ also had a slower onset into slow inactivation (Fig. 3C) and were less susceptible to block by $2 \mu\text{M}$ $\omega\text{-CTX-MVIIIC}$ (Fig. 3D). The effects of constructs $\beta_{4b} \Delta 1-30$, $\beta_{4b} \Delta 1-40$, and $\beta_{4b} \Delta 1-49$ were identical to those of $\beta_{4b} \Delta 1-20$ (data not shown). As a first attempt at determining whether the effects of $\beta_{4b} \Delta 1-20$ were simply the result of a decreased size of the β_{4b} N terminus, we reintroduced amino acids 1–10 to the N terminus of $\beta_{4b} \Delta 1-20$ to create the construct $\beta_{4b} \Delta 10-20$. As shown in Figure 3A,B, this did not restore the $V_{1/2}$ of either activation or inactivation to the hyperpolarized potentials characteristic of $\text{Ca}_v2.1$ complexes containing β_{4b} . Together, these results indicated that the molecular determinants responsible for the observed differences between $\text{Ca}_v2.1$ complexes containing β_{4a} versus β_{4b} were located in amino acids 10–20 of β_{4b} . Moreover, it was apparent that their influence extended to changes in both gating and pharmacology.

The β_4 A domain is a distant homolog of the third PDZ domain of PSD-95

With the results of the deletion experiments highlighting a specific location for the molecular determinants of β_{4b} gating and pharmacology effects, and with the observation that the β_{1b} A domain resembles PDZ domains (Hanlon et al., 1999), we began a systematic comparison of the β_{4b} sequence with similar regions of a number of PDZ domains. Unexpectedly, we found that the entire β_{4b} A domain was weakly homologous to the third PDZ domain of PSD-95 (Fig. 4A). Of the 87 aa that have been shown by x-ray crystallography to form the modular PDZ structure of PSD-95 (Doyle et al., 1996), 27 of these ($\sim 31\%$) are conserved in

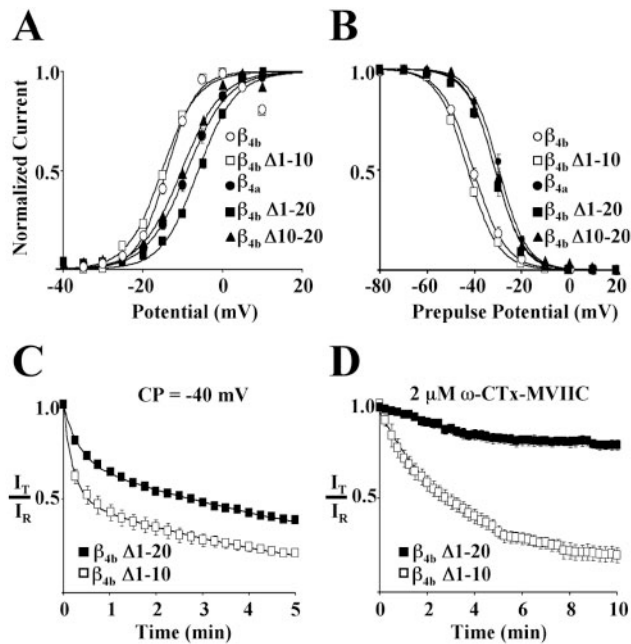


Figure 3. Localization of differential effects on $\text{Ca}_v2.1$ gating and pharmacology to β_{4b} N-terminal amino acids 10–20. The first 10 (β_{4b} Δ 1–10), first 20 (β_{4b} Δ 1–20), or second 10 (β_{4b} Δ 10–20) aa of the N terminus of the β_{4b} subunit were removed using PCR. The deletion mutants as well as β_{4a} or β_{4b} were expressed with α_{1A} and $\alpha_2\delta$ -1 in *Xenopus* oocytes. **A**, Effects of the N-terminal deletion mutants on the voltage dependency of activation of $\text{Ca}_v2.1$ channels. Plots were derived from averaged I - V data up to +10 mV for each β_4 subunit combination. *Data points* represent the means of the normalized data at a given membrane potential for a minimum of nine different recordings. *Smooth lines* represent single Boltzmann fits to the averaged data. **B**, Normalized, averaged isochronal inactivation curves for $\text{Ca}_v2.1$ complexes containing the various β_4 subunits. *Points* represent the means of the normalized data at a given membrane potential for a minimum of nine different recordings. *Smooth lines* represent single Boltzmann fits to the averaged data. **C**, Effects of β_4 N-terminal deletion mutants on the development of slow inactivation at a conditioning potential (CP) of -40 mV. Reference (I_R) and test (I_T) currents were generated as in Figure 1A. Each *point* represents the mean value of I_T/I_R from 13 (β_{4b} Δ 1–10) or nine (β_{4b} Δ 1–20) different recordings. The *solid lines* represent double-exponential fits to the data. **D**, Onset and degree of block by a 10 min exposure to $2 \mu\text{M}$ ω -CTX-MVIIIC for $\text{Ca}_v2.1$ complexes containing β_{4b} Δ 1–10 or β_{4b} Δ 1–20. Data were generated as in Figure 2A. Each *point* represents the average of a minimum of seven recordings. The *solid lines* represent single-exponential fits to the data.

the β_{4b} sequence. Most importantly, these identities are conserved within key secondary structural elements, such as β -strand C and α -helix 2 of PSD-95. Also of note is the conservation of an RG(S/T)T motif in what would be the equivalent of the carboxylate binding loop (CBL) between β -strands A and B of PSD-95 and the loss of the GLGF motif that is extremely common among PDZ domain subtypes (Bezprozvanny and Maximov, 2001; Harris and Lim, 2001). Four of β_{4b} amino acids 10–20 (G10, D13, P15, and P18) were found in PSD-95. We used these as a starting point for further defining key β_{4b} residues involved in setting $\text{Ca}_v2.1$ gating parameters.

Figure 4B lists a series of site-directed mutants (*left*), along with their effects on the voltage dependence ($V_{1/2}$) of activation (*middle*) and inactivation (*right*) of $\text{Ca}_v2.1$ complexes. The $V_{1/2}$ values for complexes containing β_{4a} and β_{4b} are included for comparison. Interestingly, the first site-directed β_{4b} mutant tested, G10A, D13A, P15A, P18A, in which all four of the amino

acids in common with PSD-95 were altered, displayed activation and inactivation properties similar to that of β_{4a} . To determine whether this was a specific effect, we altered four different amino acids in the β_{4b} 10–20 sequence to create the mutant, T11A, S17A, T19A, S20A. As shown in Figure 4B, $\text{Ca}_v2.1$ gating properties changed little in response to these mutations. $\text{Ca}_v2.1$ complexes containing the G10A, D13A, P15A, P18A mutant also had β_{4a} -like slow inactivation and pharmacological properties (data not shown). This indicates that the conserved amino acids are playing a defining role in the gating motif. To delineate the structure in more detail, we subsequently characterized six of the possible G10, D13, P15, P18 amino acid pairs for their effects on gating. Surprisingly, none of the pairs were absolutely essential for maintaining wild-type β_{4b} gating behavior, although small but statistically significant hyperpolarizing effects on activation were noted for five of the six pairs. To complete the alanine substitution study, we created the mutant H16A, which had a small but statistically significant effect on inactivation but not activation.

One interpretation of these results is that β_{4b} amino acids 10–20 form a ligand motif that interacts with a binding pocket located somewhere either on the α_{1A} subunit or on the β_4 subunit itself. The affinity of the ligand motif for its receptor site could be defined, for example, by the sum of the interactions of amino acids G10, D13, P15, and P18 with their individual targets. Any given pair may be capable of maintaining a binding interaction under the conditions of our experiments. As a first step toward addressing this possibility, we created three-dimensional structural models of the β_{4a} and β_{4b} A domains (Fig. 5A,B) using the real-space optimization method used in the computer program MODELLER (Sali and Blundell, 1993). The models were initiated using the distance and dihedral angle restraints derived from alignments with portions of the sequence of the third PDZ domain of PSD-95. For β_{4b} , amino acids 10–96 were aligned with amino acids 303–390 of PSD-95. There is 31% sequence identity over this region, which is considered minimally acceptable for this type of comparative modeling (Martí-Renom et al., 2000). For β_{4a} , amino acids 10–49 of β_{4b} were deleted from the alignment. Thus, the models do not include the first 15 aa of β_{4a} and the first 9 aa of β_{4b} . Figure 5A (*left*) shows that β_{4a} models as a compact structure containing three β -sheets and 2 α -helices. Stereochemical quality assessment of the model using PROCHECK-NMR (Laskowski et al., 1993) identified 41 residues (87.7%) in most favored regions, 5 (10.6%) in additional and generously allowed regions, and 1 (2.1%) in a disallowed region. Calculation of the electrostatic surface potential using MOLMOL (Koradi et al., 1996) reveals that the face of the molecule as oriented in Figure 5A, *left*, contains a pocket of negative charge (red residues) between the two α -helices (Fig. 5A, *right*). Figure 5B, *right* and *left*, illustrates that the overall effect of alternative splicing to form β_{4b} is to bury the charged pocket beneath three additional β -sheets. Interestingly, the molecule acquires as the result of splicing a positively charged binding pocket (blue residues) in what would be the equivalent of the CBL in PSD-95 (Fig. 4A). The stereochemical quality of the β_{4b} model as shown is not quite as good as that for β_{4a} . PROCHECK-NMR identified 64 residues (79%) in most favored regions, 11 residues (13.6%) in additional allowed regions, and 3 residues (3.7%) each in generously allowed and disallowed regions. Two of the three disallowed residues (R30 and K34) flank what would be the equivalent PSD-95 β -sheet B. Together with the loss of the highly conserved PDZ GLGF sequence, these results are consistent with the notion that through evolution this region of the β_{4b} structure has evolved

Figure 4. The β_4 subunit is a distant homolog of PSD-95. Identification of a conserved GXXDXPXXP motif critical to $\text{Ca}_v2.1$ gating. *A*, Amino acid alignment of the A domains of the human spinal cord β_{4a} (amino acids 1–63) and β_{4b} (amino acids 1–97) subunits with the third PDZ domain (amino acids 294–391) of PSD-95. Vertical bars denote identical amino acids between β_{4b} and PSD-95. Important amino acids involved in modulating the leftward shift in the voltage dependence of activation and inactivation of β_{4b} (GXXDXPXXP) are highlighted. Arrows and hatched bars represent predicted α -helices and β -strands of the third PDZ domain of PSD-95, respectively. *B*, Differences in the $V_{1/2}$ values of activation and inactivation of β_{4a} , β_{4b} , and β_{4b} N-terminal amino acid mutants. Solid bars represent average $V_{1/2}$ values of a minimum of nine different recordings for each β_4 subunit variant. Positive or negative shifts in the $V_{1/2}$ values (in millivolts) of activation and inactivation of β_{4a} and β_{4b} mutants are relative to the $V_{1/2}$ values of activation and inactivation of β_{4b} . Currents were generated as described in Figure 3, *A* and *B*. The SEM for each bar is shown. Asterisks denote statistical significance ($p < 0.05$) by a Student's two-sample equal variance *t* test.

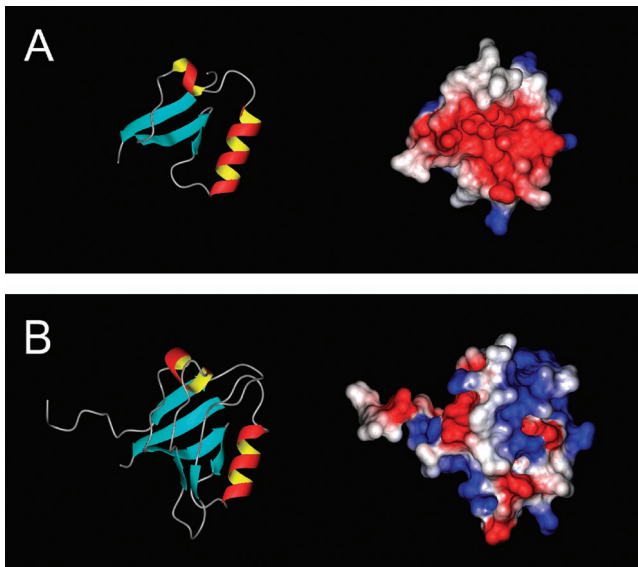
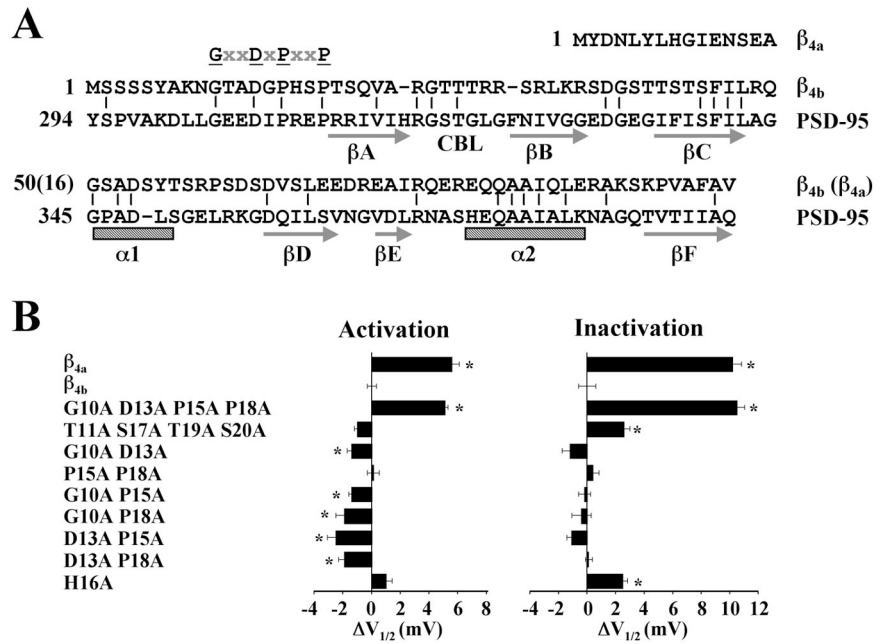


Figure 5. Real-space optimization structural models of the A domains of β_{4a} (*A*) and β_{4b} (*B*) based on sequence identities with the third PDZ domain of PSD-95. Ribbon (*left*) and electrostatic surface potential (*right*) diagrams were created using MOLMOL (Koradi et al., 1996). For ribbon diagrams, arrows indicate β -strands, and helices indicate α -helices. For surface potential diagrams, red, white, and blue regions indicate negatively charged, hydrophobic, and positively charged amino acids, respectively.

away from the capacity to bind C-terminal peptide motifs. Of most importance to our present results, however, is the observation that β_{4b} amino acids 10–20 model as an extended arm (pointing to the left in Fig. 5*B*, right, left) that may serve as a ligand motif. Interestingly, the orientation of the arm appears to be dictated by the isomerization state of proline18 (data not shown).

Differential distribution of alternatively spliced β_4 subunit mRNA

We noted in our previous study (Helton and Horne, 2002) that, based on extensive cDNA library screening, β_{4a} was the predom-

inant alternatively spliced variant of the β_4 subunit expressed in human spinal cord. To confirm this observation, we performed a comparative Northern blot analysis using a commercially available multiple tissue Northern blot (Human Brain II; Clontech) and a β_4 cDNA probe containing sequence common to both β_{4a} and β_{4b} . The mRNAs for β_{4a} and β_{4b} can be readily distinguished by their distinct migration pattern in agarose-formaldehyde gels (Fig. 6*A*). The results of the Northern blot analysis, shown in Figure 6*B*, were striking, revealing that not only was β_{4a} the predominant form of β_4 subunit in the spinal cord, but also in other “reptilian” portions of the human CNS such as the medulla and putamen. Moreover, β_{4a} was the predominant form of β_4 subunit expressed in evolutionarily older regions of the cerebrum, the temporal lobe, and occipital pole. In marked contrast, β_{4b} was highly expressed in the most recent and most highly integrative region of the cerebrum, the frontal lobe. The two forms of the β_4 subunit were equally expressed in the cerebellum.

A basic local alignment search tool search of the human genome (Altschul et al., 1990) with β_4 sequences revealed that the exons coding for alternatively spliced forms of the β_4 subunit A domain are distributed widely on human chromosome 2. Figure 6*C* shows that, depending on the splice variant, the coding sequence for the β_4 PDZ domain is contained within three (β_{4a}) or four (β_{4b}) exons spread out over ~218,000 bases. The coding sequence for the GXXDXPXXP motif is included in the 5'-most exon of a pair of short exons that code for β_{4b} amino acids 1–49. Assembly of the β_{4b} mRNA requires that three RNA segments (536 bases, 214,959 bases, and 2329 bases) be spliced out. The short exon coding for the first 15 aa of β_{4a} lies between the β_{4b} N-terminal exons and the exon coding amino acids 50–89 and 16–55 of β_{4b} and β_{4a} , respectively. By comparison, the third PDZ domain of PSD-95 is encoded by two exons separated by a 200 bp intron (data not shown).

DISCUSSION

We have identified an alternatively spliced proline-rich motif in the Ca^{2+} channel β_4 subunit that has considerable influence over gating of neuronal $\text{Ca}_v2.1$ Ca^{2+} channels. Given that the motif also affects extracellular toxin binding, it is likely that the inter-

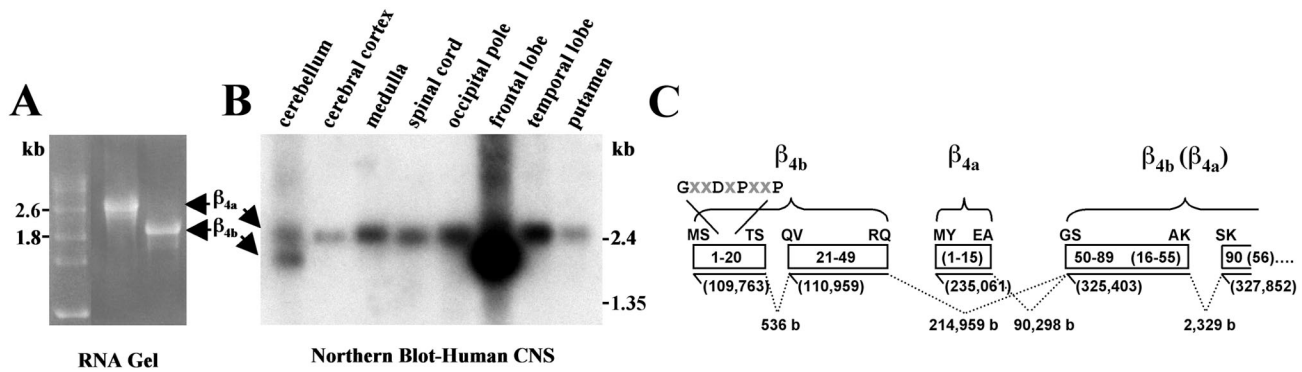


Figure 6. Differential distribution of β_{4a} and β_{4b} mRNA in the human CNS. *A*, Electrophoresis of full-length β_{4a} (left) and β_{4b} (right) cRNAs (includes 5' and 3' untranslated) in a 1% agarose formaldehyde denaturing gel. RNA markers (in kilobases) are indicated on the left. *B*, Northern blot analysis performed with human multiple tissue blot (Human Brain II; Clontech) and a ^{32}P -labeled β_4 subunit probe (coding nucleotides 215–1628 plus ~300 bp 3' untranslated sequence). Molecular masses on the right correspond to labeled blot markers. *C*, A human β_4 subunit genome map depicting the lengths of intron sequences (b, bases) between alternatively spliced β_{4a} and β_{4b} N-terminal exons and the beginning of exon 2. *Solid lines* represent exons, and *dashed lines* represent introns. *Numbers in parentheses* below *solid lines* indicate position on chromosome 2. *Boxes* indicate protein sequence (β_{4a} in parentheses). The first and last two amino acids of each sequence are indicated above each box.

action of this motif with its binding site has wide-reaching impact on resting and open-state Ca^{2+} channel conformations. This notion is supported by recent images of the conformational changes that occur with gating of bacterial two-membrane-spanning K^+ channels (Liu et al., 2001; Jiang et al., 2002a,b). Like eukaryotic six-membrane-spanning K^+ channels, KcsA and MthK channels are tetramers that pack with fourfold symmetry around a central pore (Doyle et al., 1998; Jiang et al., 2002a). The principal structural elements of KcsA and MthK from N to C terminus include an outer transmembrane helix (M1), a pore helix (P), and an inner transmembrane helix (M2). These correspond to S5, H5, and S6 segments of voltage-gated Ca^{2+} channels, respectively. In the closed conformation of the KcsA structure, the four M2 helices are straight and arranged such that they form the walls of an inverted teepee that narrows from a 12 Å diameter at its center to a 4 Å pore at its tip (Doyle et al., 1998). After opening, the KcsA M2 helices tilt away from the permeation pathway and rotate about their helical axis (Liu et al., 2001). In MthK, bending and splaying of the inner helices after opening expands the diameter of the pore threefold (Jiang et al., 2002a,b). The nearly 30° bend occurs at a “gating hinge” corresponding to a glycine residue just below the selectivity filter. Applying a radial-outward force on the intracellular aspects of the inner helices places a torque on the gating hinge such that a conformational change is transmitted the full length of the M2 helix. This results in a widening of the outer vestibule [Jiang et al. (2002b), see supplementary information (movie)]. It has been hypothesized that similar mechanical forces are at work in the gating of voltage-gated Ca^{2+} channels (Jiang et al., 2002b).

Considered in the context of this mechanical framework, our results suggest that an interaction of the β_{4b} ligand motif with an inner aspect of the $\text{Ca}_v2.1$ complex either directly or indirectly fine-tunes the torque experienced by α_{1A} S6 segments. In so doing, the interaction alters the conformation of the voltage sensor or gate (or both) as well as the outer vestibule. This would explain why alternative splicing of the β_4 subunit would affect both gating and toxin sensitivity. Given the potential for the ligand motif interaction to occur over a wide range (Helton and Horne, 2002), it is not possible from our results to pinpoint which S6 helices might be most affected. And although our ω -CTX-MVIIC binding results might be providing some direction, a

previous study has shown that ω -CTX-GVIA binding to α_{1B} is affected by alterations in any of the four P loops that make up the outer vestibule (Ellinor et al., 1994). A case can be made for an indirect effect on the IS6 helix, because the primary α_1 - β_4 subunit interaction occurs on the intracellular loop between homology domains I and II (I-II loop) (Pragnell et al., 1994). This is consistent with a previous study showing that IS6 is a critical determinant of voltage-dependent inactivation in $\text{Ca}_v2.1$ and $\text{Ca}_v2.3$ channels (Zhang et al., 1994). However, site-directed mutagenesis and domain-swapping studies have highlighted the equal importance of IIS6, IIS6, and IVS6 in Ca^{2+} channel gating (for review, see Stotz and Zamponi, 2001; Shi and Soldatov, 2002), making the case for direct effects on these S6 helices equally plausible. It is interesting that many of the proteins that have evolved to modulate Ca^{2+} channel gating target α_1 I-II (β subunits, $G_{\beta\gamma}$ subunits, protein kinase C) and II-III loops (syntaxin, synaptotagmin, and synaptosomal-associated protein-25). The IS6 and IIS6 (but not IIS6 or IVS6) helices of $\text{Ca}_v2.1$, 2.1, and 2.3 Ca^{2+} channels have glycines in hinge positions comparable with those present in KcsA and MthK (see alignments in Horne et al., 1993).

Despite extensive binding studies with β_4 subunits, there is currently no evidence to support the notion that the β_{4b} N terminus binds directly to α_{1A} subunits (Walker et al., 1998, 1999). One possible explanation for this is that the interaction is too weak to be detected in solution binding assays. The other possibility is that the α_{1A} subunit is not the primary binding target. The sequence of the ligand motif (GXXDXPXXP) may provide an important clue as to the nature of its own binding site. Proline-rich motifs are common within the primary structures of many ligands important for protein–protein interactions (for review, see Kay et al., 2000; Macias et al., 2002). Src homology 3 (SH3) and WW domains, for example, recognize proline-rich sequences containing a core PXXP, where X denotes any amino acid. These sequences adopt a PPII helix conformation that presents a hydrophobic surface as well as backbone carbonyls that are ideal for hydrogen bonding. Proline-rich ligands bind with low affinity, allowing for rapid modulation and added versatility in signaling pathways. In many respects, the β_{4b} ligand motif resembles the serine/threonine proline motifs recognized by group IV WW domains (Sudol and Hunter, 2000). As is the case for the β_{4b}

motif, the structural basis for recognition of these motifs is based on the summed contributions of a series of side-chain interactions, none of which is absolutely essential for ligand binding (Verdecia et al., 2000). It is possible that the β_{4b} proline-rich motif binds to its own B domain, which is structurally similar to SH3 and WW domains (Hanlon et al., 1999). This possibility is supported by what is known about related membrane-associated guanylate kinase family proteins in which intramolecular interactions are a key aspect of their functional diversity (Dimitratos et al., 1999).

To date, no specific function has been assigned to the GXXDXPXXP motif of the third PDZ domain of PSD-95. Most attention has been paid to the structure of the carboxylate binding loop to which it is immediately adjacent (Doyle et al., 1996) (Fig. 4, CBL). A partial list of the proteins that interact specifically with the third PDZ domain of PSD-95 include the cell-surface neuroligins (Irie et al., 1997), the microtubule binding protein cysteine-rich interactor of PDZ 3 (Niethammer et al., 1998), the Rho effector protein citron (Zhang et al., 1999), and the β_1 adrenergic receptor (Hu et al., 2000). It would be interesting to determine whether the GXXDXPXXP motif of PSD-95 plays a role maintaining the structure of the CBL. Such a role could also be considered for the GXXDXPXXP motif of β_{4b} . As is apparent in the β_{4b} A domain model structure (Figs. 4A, 5B), the sequences of β_{4b} corresponding to the hydrophobic CBL and β -strand B of PSD-95 are highly positively charged. It is possible, as is true for some PDZ domains (Cuppen et al., 1998), that this region binds to an internal (as opposed to a C-terminal) negatively charged domain. Interestingly, the immediate 5' sequence of the II-III loop of $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ Ca^{2+} channels fits this description, because it is densely packed with glutamate residues. Moreover, several lines of evidence point to the II-III loop as a critical determinant of channel gating and pharmacology. Similar to the effects of β_{4b} on α_{1A} , binding of syntaxin to a "synprint site" just downstream from this region in the II-III loop of α_{1B} shifts the $V_{1/2}$ of inactivation to more hyperpolarized potentials (Bezprozvanny et al., 2000) and accelerates entry into slow inactivation (Degtiar et al., 2000), and an α_{1B} splice variant that lacks a large portion of the II-III linker region ($\Delta 1$) (Kaneko et al., 2002) inactivates at more depolarized potentials and is less sensitive to ω -CTx-MVIIA than is the full-length $\text{Ca}_v2.2$ variant.

Viewed from the perspective of changing Ca^{2+} channel function, the differential distribution of β_{4a} and β_{4b} subunit mRNA displayed in Figure 6B provides an unexpected snapshot of the evolution of forebrain synapses. It raises the possibility that with the introduction of β_{4b} to the genome, synapses acquired properties that fit better with the overall demand to organize complex neural networks. It could be that with the advent of $\text{Ca}_v2.1$ complexes that enter more readily into closed inactivation states (Fig. 1A,C) without perceptible gain in time required for recovery (Fig. 1D), synapses inherited an enhanced mechanism for synaptic plasticity. In this regard, short-term synaptic depression has been linked to Ca^{2+} channel inactivation (Forsythe et al., 1998) through mechanisms shown to be β subunit dependent (Patil et al., 1998). This form of short-term synaptic plasticity has been implicated in cortical gain control (Abbott et al., 1997) and low-pass temporal filtering (Fortune and Rose, 2001). In addition, long-lasting $\text{Ca}_v2.1$ channel inhibition has been identified as the mechanism underlying certain forms of long-term synaptic depression (Robbe et al., 2002). Accordingly, our future studies will be directed toward characterizing the responsiveness of Ca_v2 Ca^{2+} channels containing alternatively spliced β_4 subunits to

changes in more dynamic regulatory inputs, such as neuronal firing frequency and action potential waveform.

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