Several Structural Domains Contribute to the Regulation of N-type Calcium Channel Inactivation by the β3 Subunit*

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Calcium channel β subunits are essential regulatory elements of the gating properties of high voltage-activated calcium channels. Co-expression with β3 subunits typically accelerates inactivation, whereas co-expression with β4 subunits results in a slowly inactivating phenotype. Here, we have examined the molecular basis of the differential effect of these two subunits on the inactivation characteristics of Cav2.2 + α2-δ, N-type calcium channels by creating a series of 22 chimeric β subunits that are based on various combinations of variable and conserved regions of the parent β subunit isoforms. Our data show that replacement of the N terminus region of β4 with a corresponding 14-amino acid stretch of β3 sequence accelerates the inactivation kinetics to levels seen with wild type. A similar kinetic speeding is observed by a concomitant substitution of the second conserved and variable region, but not when these regions are substituted individually, suggesting that 1) the second variable and conserved regions cooperatively regulate N-type calcium channel inactivation and 2) that there are two redundant mechanisms that allow the β3 subunit to accelerate N-type channel inactivation. In contrast with previous reports in Ca2.1 calcium channels, deletion of the C-terminal region of Ca2.2 did not alter the regulation of the channel by wild type and chimeric β subunits. Hence, the molecular underpinnings of β subunit regulation of voltage-gated calcium channels appear to vary with calcium channel subtype.

The influx of calcium ions through voltage-gated calcium channels triggers a range of intracellular responses, ranging from gene transcription (1) and activation of second messenger pathways (2, 3) to the release of neurotransmitters from presynaptic nerve termini (4). The amount of calcium entry is regulated by a number of factors, including the intrinsic ability of calcium channels to inactivate in response to prolonged membrane depolarization. It has been suggested that the inactivation process involves the occlusion of the inner mouth of the pore by the intracellular domain I–II linker region of the channel (5–8). This region physically interacts with ancillary calcium channel β subunits (9), which are subunit regulators of calcium channel inactivation. To date, four different types of calcium channel β subunits have been identified and shown to mediate a spectrum of modulatory actions on the function of high voltage-activated calcium channels. The β2a subunit uniquely slows the inactivation kinetics of the α1,β subunit through palmitoylation and membrane anchoring of its N terminus region (10–14), thus possibly restricting the mobility of the putative inactivation gate (for review, see Ref. 6). Despite the absence of palmitoylation sites, the remaining β subunit isoforms mediate differential effects on calcium channel inactivation. For example, channels co-expressed with β3 subunits typically undergo rapid inactivation, whereas channel complexes containing β2 tend to inactivate more slowly (12, 15). This suggests that regions other than the N terminus are important regulatory elements of β subunit function. Indeed, the C-terminal region of the β4 subunit has been implicated in the regulation of P/Q-type calcium channel inactivation (16). However, to date there has been no systematic approach toward delineating the β subunit structural determinants that are responsible for the differential effects mediated by β3 and β2.

The four calcium channel β subunits share a similar structural arrangement with two highly conserved regions (with 75 and 85% overall sequence identity, respectively) separated and flanked by a total of three variable regions of lower homology (35–55%; Ref. 17). Here, we report the creation and functional analysis of a series of chimeric β subunits in which variable and conserved domains were exchanged between wild type β3 and β4 subunits and co-expressed with rat Ca2.2 (N-type) calcium channels. Our data show that the transfer of any single domain was not sufficient to conclusively confer inactivation properties from β3 onto β4. Instead, multiple β subunit domains appear to be essential to facilitate the acceleration of N-type channel inactivation, with the V1 and C2 regions constituting critical structural determinants. We propose a model in which the intramolecular interactions among variable and conserved domains control the overall functional effect of β subunits on N-type channel gating.

MATERIALS AND METHODS

β3/4 Chimeras—Unique restriction sites were introduced via the QuikChange site-directed mutagenesis kit (Stratagene) into the rat β3 (GenBank™ accession number M88751) and β4 (GenBank™ accession number L02315) cDNAs (both in the pMT2 expression vector) at exactly corresponding positions. The positions were chosen to align with the interfaces between previously identified regions of conservation and variability (Fig. 1 and Ref. 17). Specifically, we introduced NruI sites at

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neous mutations had been incorporated during PCR. T-easy constructs containing the 5' region of βs and βa were cut with Kpnl and BsiWI, and the insert was gel-isolated. The pMT2/βa and pMT2/βs constructs were similarly cut, and the fragments isolated from T-Easy were ligated. This resulted in four new cDNA constructs: βs and βa without the extensive 5'-untranslated region but containing novel 5' Kpnl sites and Kozak start sequences, as well as chimeric β3a2a and β3s2sa constructs. The 5' Kpnl and 3' NotI sites were removed from all constructs. The downstream antisense primers restricted the amplification region and introduced a novel 3' NotI site. The DNA and cDNA constructs were then cut with Ascl and NotI and a second series of cDNA constructs (βs, βas, β3sa2s, and β3sas2sa) were cut with the extensive 3'-untranslated region, as well as β3sas2as, β4asas2as, β4as2as, and β4sas2as) was created through ligation. Restriction diagnostics were carried out to identify appropriately ligated cDNA, and in each case the entire insert was sequenced. The βs, βas, β3sa2sa, β4asas2as, β3sas2sa, and β4sas2sa were cut further with Kpnl and SpeI to create more chimeras. The resulting DNA fragments were used for the next set of ligations, which produced β3sasas2sa, β4asas2as2sa, β4asas2as, β4asas2as, and β4asas2as2sa chimeras. A similar exchange of Kpnl-SpeI fragments produced the βsasas2sa, β4asas2as, β4asas2as, and β4asas2as chimeras. In all 22 chimeras were produced, analyzed through restriction digests, and sequenced.

**Transient Expression**—A human embryonic kidney tsA-201 cell line was used to express wild type and chimerical calcium channel complexes. The cells were grown in standard Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 0.5 mg/ml penicillin streptomycin. When the cells had grown to 90% confluency, they were split with trypsin-EDTA and plated on glass coverslips at a density of 10%. These cells were then transfected ~12 h later. Fresh medium was given to the cells just prior to transfection, which was performed according to the standard calcium phosphate protocol (19). The cells were transiently transfected with a combination of cDNA constructs encoding for the calcium channel α1sβ, and αsδ subunits (1 μg each) and 4 μg of enhanced green fluorescent protein (pEGFP, Clontech). After 2 h of incubation at 37°C in the presence of the calcium phosphate/DNA mixture, the cells were washed with Dulbecco's modified Eagle's medium and allowed to recover for an additional 12 h. The cells were then moved to a 28°C, 5% CO2 incubator to prevent the cells from dividing further. Electrophysiological recordings, or protein biochemistry, were carried out 3 days later.

**Immunoprecipitations**—Human embryonic kidney cells co-transfected with N-type calcium channels, plus the appropriate ancillary subunits were homogenized in 1 ml of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, and a mixture of protease inhibitors). The solution was incubated on ice for 1 h and centrifuged for 15 min at 15,000 × g, and the supernatant was collected for overnight dialysis against the dialysis solution (500 mM NaCl, 50 mM Tris, pH 7.5, and 0.1% Triton X-100). The protein concentration was determined with the modified Lowry assay, and 100 μg of the transfected human embryonic kidney cell lysate was mixed with the Ca2+2 antibody (1:500 dilution; Calbiochem). The mixture was incubated overnight at 4°C with rotation. 100 μl of precleared protein A-Sepharose (Amersham Biosciences) was added to the polyclonal antibody mixture, and the mixture was incubated at room temperature for 1 h. The solution was washed two times with Buffer B (0.2% Nonidet P-40, 10 mM Tris, pH 7.5, 0.15 mM NaCl, and 2 mM EDTA), one time with Buffer C (0.2% Nonidet P-40, 10 mM Tris, pH 7.5, 0.5 mM NaCl, and 2 mM EDTA), and one time with Buffer D (10 mM Tris, pH 7.5). The complex was centrifuged and resuspended in 25 μl of 5 μl of urea, 10 mM Tris, pH 7.5, plus 2 μM calmodulin. The solution was then incubated for 12 h at 4°C, and the supernatant was collected. The samples were separated and transferred to nylon membranes, and Western analysis was performed using the βs antibody (diluted 1:250, Exalapa Biologicals Inc.) and detected using ECL detection methods.

**Electrophysiology**—Wild type, chimeric, and mutant calcium channel complexes were screened at room temperature for macroscopic currents using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with either pCLAMP version 6.0 or 9.0. Patch pipettes (Sutter borosilicate glass, BP150-66-15) were pulled using a Sutter P-97 micropipette puller to a typical resistance 3–4 MΩ when filled with 1 M KCl. All were filled with a N-type solution. For cells with currents greater than 1 nA or a series resistance greater than 12 MΩ, cell capacitance was measured, and series resistance was compensated to minimize contamination of records by voltage errors. The internal pipette solution consisted of 108 mM cesium methanesulfonate,
TABLE I

Sense primers sequences used to generate unique and silent restriction sites in the \( \beta_2 \) and \( \beta_3 \) sequences

- Changed nucleotides are noted in bold lettering, and italics highlight the presence of the unique restriction sites. Only forward primer sequences are shown, because the reverse mutagenesis primers are simply the reverse complement of the shown sequences.

| Primer name | Primer sequence 5’ to 3’ |
|-------------|-------------------------|
| \( \beta_2 \) NruI | GCGGACTCTTACACCCTCGACCGCCCTCTCTGG |
| \( \beta_2 \) BsiWI | CATGTCCTCCCGATGATCTGAGTGGTGG |
| \( \beta_2 \) AscI | CTGGCGCGCGACACCCACACC |
| \( \beta_3 \) NruI | GGTTGATACCGAGACTAGATGAGGAGGGCTG |
| \( \beta_3 \) BsiWI | GGACATTCTCCTGACGTGATGTGGC |
| \( \beta_3 \) AscI | GGAGGCATATGGGCGCCACACACACACazzz |

TABLE II

PCR primers used to alter the 5’ and 3’-UTR of the \( \beta_3 \) and \( \beta_2 \) subunits

- The restriction sites are shown in bold lettering, and the Kozak sequences are in italics.

| Primer name | Primer sequence 5’ to 3’ |
|-------------|-------------------------|
| \( \beta_2 \) KpnI sense | GGTACCCCAGCCATATCGACGACTCTAGC |
| \( \beta_2 \) BsiWI antisense | GTGACCTGCTCCGACATGCTCTAGC |
| \( \beta_2 \) NotI antisense | CCGGCGGCTAGTCTGCTCTTTAGGCCAAGG |
| \( \beta_2 \) KpnI sense | GTTCTACTGCGGGGCCACCCGACACCCACACG |
| \( \beta_3 \) BsiWI antisense | GCATATTGGGCGCCACACACACACAGC |
| \( \beta_3 \) AscI sense | GCGGCGGCTCACAGCTGCTCCGACGATGTC |

4 mM MgCl₂, 9 mM EGTA, and 9 mM HEPES (pH 7.2 with CsOH). The external bath solution consisted of 20 mM BaCl₂, 1 mM MgCl₂, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 65 mM CsCl (pH 7.2 with tetraethylammonium hydroxide). For experiments examining calcium-dependent inactivation (Fig. 2, F and G), the internal solution was 118 mM cesium methanesulfonate, 1 mM MgCl₂, 0.5 mM EGTA, 9 mM HEPES, 4 mM MgATP (pH 7.4 with CsOH). Solutions were exchanged via a home built gravity-driven micropipet system. Current-voltage (I-V) relations were obtained by stepping the membrane potential from a holding potential of -100 mV to various test potentials. Steady state inactivation curves were obtained by holding cells at various conditioning potentials for 4 s prior to a test depolarization to +20 mV.

**RESULTS**

\( \beta_2 \) and \( \beta_3 \) Mediate Distinct Effects on Ca\(_{\text{v}2.2}\) Gating—Fig. 2 highlights the differential effects of the co-expression of \( \beta_2 \) and \( \beta_3 \) on the biophysical properties of Ca\(_{\text{v}2.2} + \alpha_2 - \delta_1 \) channels. In response to membrane depolarization, the Ca\(_{\text{v}2.2} \beta_3 \) channel complex quickly activates, followed by rapid inactivation. (Fig. 2A, left panel). In contrast, the Ca\(_{\text{v}2.2} \beta_4 \) channels display slower activation and inactivation kinetics (Fig. 2A, right panel), resulting in a much smaller fraction of inactivated channels at the end of the test pulse (i.e. inactivation at 125 ms = 48.5 ± 4.0, \( n = 16 \) for Ca\(_{\text{v}2.2} + \beta_2 \) and 83.4 ± 2.8, \( n = 11 \) for Ca\(_{\text{v}2.2} + \beta_3 \)). Inactivation time constants observed in the absence of any \( \beta \) subunit were similar to those seen with \( \beta_2 \) (not shown), indicating that \( \beta_3 \) subunits do not significantly modulate Ca\(_{\text{v}2.2} \) channel inactivation. Fig. 2 (B-G) quantitatively examines the distinct effects of the two \( \beta \) subunits on activation and inactivation gating. As seen in the figure, the activation time constants (Fig. 2B) and inactivation time constants (Fig. 2D) differ significantly at a range of different test potentials (Student’s t test, \( p < 0.05 \)). The half-inactivation potential was ~5 mV more depolarized for channels containing the \( \beta_3 \) subunit (Fig. 2E), whereas the half-activation potential was not significantly different (Fig. 2C). Although statistically significant (\( p < 0.05 \)), the difference in half-inactivation potential was insufficiently large to allow for an investigation into the underlying \( \beta \) subunit structural determinants. However, as seen in Fig. 2D, the time constants of inactivation differ by ~500%, thus providing a suitable base line for a chimeric approach.

It was recently reported by Liang et al. (20) that Ca\(_{\text{v}2.2} \) calcium channels co-expressed with \( \beta_3 \) undergo significant calcium-dependent inactivation that can be unmasked when intracellular calcium buffering is reduced. To determine what extent calcium ions affect the difference in inactivation kinetics observed upon co-expression of \( \beta_2 \) and \( \beta_3 \) (Fig. 2D), we examined the effects of switching from 20 mM external barium to 20 mM external calcium. In this set of experiments, the EGTA concentration in the intracellular recording solution was

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\(^{1}\) The abbreviation used is: ANOVA, analysis of variance.
dropped to 0.5 mM, and ATP was added such that our internal solution matched that of Liang et al. (20). Fig. 2 (F and G, respectively) shows the inactivation time constants obtained in barium and calcium containing recording solution for Ca_{2.2} channels co-expressed with β_3 (Fig. 2F, n = 6) or the β_4 (Fig. 2G, n = 6) subunit. In each case, the time constants in barium and calcium were determined from the same cell. In the low EGTA solution, inactivation kinetics obtained in barium were slightly faster than those observed in high EGTA, but this was statistically significant only at 0 mV for β_3, and at +30 mV for β_4. Surprisingly, however, calcium did not significantly affect inactivation time constants at any test potential (paired t test between barium and calcium conditions). Moreover, the percentage of inactivation 300 ms into the test pulse was not different in barium and calcium (not shown). In light of the work of Liang et al. (20), these data suggest the intriguing possibility that calcium-dependent inactivation of N-type calcium channels may be dependent on the type of calcium channel β subunit. Moreover, our data suggest that inactivation of N-type channels complexes containing β_3 and β_4 is governed predominantly by a voltage-dependent process. Hence, for the remainder of the manuscript, we shall focus on voltage-dependent inactivation, using 20 mM barium as the external charge carrier and internal solution with 10 mM EGTA.
Fig. 3A, time constants of inactivation for Ca,2.2 + α₁δ₁ co-expressed with chimeric and wild type β subunits. The test potential was +50 mV in each case. The number of experiments in each data set ranged between 6 and 16 cells (from left to right: β₂, n = 11; β₃4433, n = 12; β₃4433, n = 13; β₃4433, n = 14; β₃4434, n = 15; β₃4434, n = 16; β₃4434, n = 17; β₃4434, n = 18; β₃4434, n = 19; β₃4434, n = 20; β₃4434, n = 21; β₃4434, n = 22; β₃4434, n = 23; β₃4434, n = 24; β₃4434, n = 25; β₃4434, n = 26; β₃4434, n = 27; β₃4434, n = 28). An ANOVA on ranks was conducted on the entire data set to determine which chimeras had currents that inactivated slower or faster than β₃ and β₄, respectively. Chimeras with mean inactivation time constants above the dotted line were significantly slower than that of β₃, containing channels (ANOVA, Dunn’s method; p < 0.05). Chimeras with mean inactivation time constants below the dashed line were significantly faster than that seen with β₄ (ANOVA, Dunn’s method; p < 0.05), and chimeras shown in bold type are of particular interest. Note that only the β₃4434 chimera results in inactivation time constants that are significantly different from both wild type subunits. B. Western blot analysis of a co-immunoprecipitation experiment using a Ca,2.2 antibody for the immunoprecipitation and a β₄-selective antibody for detection of the calcium channel β subunit.

Multiple β Subunit Structural Determinants Contribute to the Regulation of N-type Channel Inactivation—To investigate the molecular mechanisms underlying the differential effects of the β subunits on the voltage-dependent inactivation rates of Ca,2.2, a series of 22 chimeras were created that combined structural features of parent β₃ and β₄ subunits. The chimera nomenclature denotes the identity of the variable and conserved regions (Fig. 1). For example, β₃4434 contains β₄ sequence in V1, C1, and C2, whereas V2 and V3 correspond to the β₃ sequence. All of the 22 chimeras were then individually co-expressed with Ca,2.2 + α₁δ₁, and the biophysical properties of the resulting calcium channel populations were assessed. In each case, the half-activation potentials closely aligned with those observed in the presence of the two wild type β subunits (not shown); hence, any putative effects of the chimeras on inactivation kinetics would unlikely be due to altered voltage dependence of activation gating. In addition, there was no significant difference in half-inactivation potentials (not shown). Whole cell conductances observed in the presence of the various chimeras were highly variable, ranging from −10 to 40 nS.

Fig. 3A summarizes the time constants of inactivation obtained with Ca,2.2 channels in the presence of the wild type and chimeric β subunits. As seen in the figure, the co-expression of the chimeric β subunits results in inactivation time constants that span virtually the entire range of values observed with the two wild type constructs. Statistical analysis of the data set indicates that the inactivation time constants of seven chimeras (data above the dotted line in Fig. 3A) are different from wild type β₃, whereas sixteen chimeras (data below the dotted line in Fig. 3A) yielded inactivation time constants that differed significantly from those of β₄-containing channels (ANOVA, p < 0.05). The time constant observed with the chimera β₄4434 differed significantly from those mediated by both wild type β subunits. Closer inspection of the data reveals that individually replacing the C1, V2, C2, and V3 domains of β₃ with β₄ sequence did not significantly accelerate Ca,2.2 channel inactivation. Only the exchange of the V1 domain resulted in inactivation kinetics that resembled those seen with wild type β₃. Indeed, with one exception (β₃4434), any chimera containing β₄ sequence in the V1 domain yielded inactivation kinetics statistically indistinguishable from wild type β₃. However, although these data implicate the V1 domain as a crucial structural element in β₄ subunit function, replacement of the V1 domain in wild type β₃ (β₃4434) did not produce a slowing of inactivation kinetics. Concomitant substitution of the C1 domain (β₃4433), the C2 domain (β₃4434), or the C2 + V3 domains (β₄4434) still maintained the rapidly inactivating phenotype. These data suggest that there is a second structural entity that is capable of conferring rapid inactivation kinetics independently of the V1 region. Yet, as stated above, no other single domain was capable of inducing rapid inactivation characteristics, thus suggesting that regions outside of V1 must work cooperatively to promote rapid inactivation. Indeed, in chimeras where V1 was of β₄ origin, the presence of β₄ sequence in the V2 + C1 regions or in the V2 + C2 regions resulted in rapid β₄-like inactivation. In contrast, the C2 + V3 or the V2 + V3 combinations were ineffective. These data suggest that although the V1 domain appears to be a major determinant of Ca,2.2 calcium channel inactivation, the β₃ subunit V2 region in combination with one of the conserved domains can also contribute significantly to regulating Ca,2.2 inactivation.

In our hands, co-expression of the Ca,2.2 + α₁δ₁ subunit with either wild type β₃, wild type β₄, or chimeric β subunits did not result in a consistent increase in current densities.
compared with channels lacking the β subunit altogether. Hence, for β subunits that did not significantly alter the inactivation properties of the channel (i.e. βα, β34444, β43443, β44434, β43444, β43434, β43444, and βp), it is difficult to rule out by electrophysiological means the possibility that the lack of effect on channel gating might perhaps have arisen from a lack of functional association between the channel and these β subunits. Hence, we transfected Ca2.2 α1 subunits, α2-δ1, and several of the β subunit constructs that mediated a slowly inactivating phenotype and carried out co-immunoprecipitations using a Ca2.2-selective antibody for the immunoprecipitation and a β1 subunit-selective antibody for Western blot detection. As shown in Fig. 3B, the wild type and chimeric β subunits were effectively immunoprecipitated by the N-type calcium channel α1 subunit, whereas no β subunit signal was detected in cells in which β subunit cDNA was omitted during transfection. These data show that β subunit constructs that do not alter the inactivation characteristics of the channel are indeed properly expressed in tsA-201 cells and that they associate with the Ca2.2 calcium channel α1 subunit.

The above considerations concerning β subunit structural determinants of inactivation were based exclusively on the examination of the effects of placing β3 sequence into the overall wild type β1 subunit backbone. To examine in greater detail the effects of individual variable and conserved β subunit regions, we extended our analysis across the whole set of chimeras, such that inactivation time constants were compared among chimera pairs that differed only in the identity of the V1 (Fig. 4A), C1 (Fig. 4B), V2 (Fig. 4C), C2 (Fig. 4D), or V3 (Fig. 4E) domains. As indicated in the figure, the substitution of the V1, V2, and C2 domains into chimeric β subunits tended to further accelerate inactivation kinetics, consistent with the considerations raised earlier. However, in some cases, the C1 domain also appeared to be able to affect inactivation rates. To obtain a semi-quantitative measure of these trends, the change in inactivation time constants that resulted from the replacement of β4 sequence with corresponding β3 sequence in a particular domain (\(\pi(D4) - \pi(D3)\)) was normalized to the difference in the inactivation time constants between a given chimera and wild type β2 (\(\pi(D4) - \pi(B3)\)). The data for all existing chimera pairs were averaged for each individual domain and plotted in Fig. 5. It is important to note that this analysis is merely designed to reveal whether inactivation is accelerated further with the introduction of additional β3 sequence into a chimera that already has accelerated kinetics. Fig. 5 confirms the notion that placing β3 sequence into the V1, V2, and C2 domains promoted additional acceleration of inactivation kinetics, whereas the C1 and V3 domains appeared to be substantially less important. When V1, V2, and C2 were swapped in combination, the speeding effect was augmented. The observation that the V2 and C2 domains individually appear to produce robust speeding may seem at odds with the considerations presented earlier. It is important to note that the bars representing V2 (or C2) include a number of chimera pairs in which C2 (or V2, respectively) was already of β3 origin; hence, the magnitude of the V2 and C2 bars in Fig. 5 is in part a reflection of the additive effect of the two regions. Taken together, the data further support the notion that the V1, V2, and C2 domains are important regulatory elements of β3-mediated speeding of Ca2.2 channel inactivation.

β3 Modulation of N-type Channel Inactivation Occurs Independently of the Ca2.2 C-terminal Region—It has been shown that besides binding to the alpha interaction domain in the I-II linker region of the calcium channel α1 subunit (9), β subunits also physically interact with the C terminus regions of the α1 subunit (21, 22). Moreover, an interaction between the C terminus region of the α1 subunit and the V3 domain of β3 has been implicated in regulation of Ca2.1 (P/Q-type) channel inactivation (16). To determine whether the differential modulation of N-type channel activity by β3 and β4 subunit involves the C-terminal region of the Ca2.2 α1 subunit, we assessed the inactivation characteristics of a mutant Ca2.2 construct (Ca2.2ΔXba; see Ref. 23) in which the last 409 of the 653 residues of the C terminus, including the putative β subunit binding site, were deleted. In the absence of a β subunit, the mutant Ca2.2ΔXba construct did not yield functional channels. However, in the presence of wild type or chimeric β subunits, robust current activity could be recorded. The inactivation kinetics of the mutant channels did not differ significantly in the presence of β3. In the presence of β3, however, the mutant channels inactivated significantly slower than the wild type channels, but only at potentials more negative than +30 mV (Student’s t test, \(p < 0.05\); compare Fig. 6 with Fig. 2). Yet, the differential effects of the two β subunits were preserved in the mutant channel at every test potential examined (Fig. 6).


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Hence, even if one of the two mechanisms (for example that mediated by V1) were to depend on the presence of the N-type channel C terminus, the effect of its deletion might be masked by the presence of the second mechanism (i.e., the presence of β4 sequence in V2 + C2). To test for this possibility, we co-expressed the β36444 and the β44334 chimeras with the Ca_{2.2}∆Xba clone and examined the effect on inactivation kinetics. As shown in Fig. 6, both of the two chimeric constructs accelerated the inactivation kinetics of the Ca_{2.2}∆Xba construct, indicating that neither the V1-mediated speeding nor that caused by substitution of domains V2 + C2 relies on the C terminus of the N-type calcium channel α1 subunit.

**FIG. 5. Average change in inactivation time constants.** The average change in inactivation time constants observed upon replacement of a given variable or conserved domain alone or in certain combinations and expressed in form of the equation,

\[
\text{Ratio } \tau_{\text{max}} = \frac{\tau(D4) - \tau(D3)}{\tau(D4) - \tau(D3)} \quad \text{(Eq. 3)}
\]

where \(\tau(D4)\) and \(\tau(D3)\) are, respectively, the inactivation time constants for a given chimera pair carrying \(\beta_3\) and \(\beta_4\) sequence in a particular region, and \(\tau(D3)\) is the inactivation time constant seen with the wild type \(\beta_3\) subunit. Unlike in the case of simple ratios of inactivation time constants for a give chimera pair, the analysis presented in this figure isolates the role of any given domain from speeding effects mediated by other regions. The number of chimera pairs included in the analysis is indicated in parentheses, and the error bars have been omitted for clarity. The dashed line represents the ratio obtained when replacing wild type \(\beta_3\) for \(\beta_4\).

**FIG. 6. Deletion of the Ca_{2.2} C terminus does not alter the differential modulation of Ca_{2.2} inactivation by wild type β3 and β4 subunits and by key chimeras.** The panel shows the inactivation time constants observed with Ca_{2.2}∆Xba (+ α_{2χδ1}) channels in the presence of wild type \(\beta_3\) (n = 8) and \(\beta_4\) (n = 14) subunits and in the presence of the β36444 (n = 14) and β34433 (n = 13) chimeras. Note that channels containing the \(\beta_3\) subunit or the two chimeric \(\beta\) subunits inactivate significantly faster than those containing \(\beta_4\) at every test potential examined (ANOVA, p < 0.05).

These data indicate that the β subunit regulation of N-type calcium inactivation occurs independently of the C-terminal region of the α1 subunit, suggesting that the regulation of inactivation of Ca_{2.1} and Ca_{2.2} channels is governed by different molecular mechanisms.

As outlined earlier, the V1 region of \(\beta_3\) and the combination of the V2 + C2 region of \(\beta_4\) appear to be independently capable of accelerating inactivation kinetics of the wild type channels.

**DISCUSSION**

Our data provide novel insights into the mechanisms by which the calcium channel β3 subunit regulates inactivation kinetics of N-type calcium channels. Our data hint at a complex interplay among conserved and variable domains in regulating inactivation kinetics. Simple insertion of the V1 domain of \(\beta_3\) into the wild type \(\beta_4\) subunit conferred the functional properties of \(\beta_3\) onto \(\beta_4\). Thus, the V1 region is a major determinant of \(\beta_3\) subunit regulation of N-type channels. However, although the \(\beta_3\) V1 domain was sufficient, it did not appear to be unequivocally necessary. Although the remaining variable and conserved domains had no effect in isolation, the V2 and C2 domains could cooperatively regulate β subunit effects on the channel. Indeed, any chimera carrying \(\beta_3\) sequence in both the V2 and C2 domains resulted in rapid inactivation of Ca_{2.2} channels. The V1 region in \(\beta_3\) is comprised of only a short 14-amino acid stretch compared with the much longer V1 region of \(\beta_4\). This suggests a highly localized structural basis for the observed effects. It is possible that the unique amino acid sequence of the V1 region directly regulates inactivation kinetics by interactions with cytoplasmic linkers implicated in calcium channel inactivation, such as the N terminus (24), or the III-IV linker (25), but further experiments using pull-down assays will be required to test this hypothesis. It is unlikely that the C terminus is involved, because the Ca_{2.2}∆Xba construct was still differentially regulated by the different β subunit isoforms and showed rapid inactivation in the presence of \(\beta_3\). The V1 region has been previously implicated in being important for the \(\beta_{3\alpha2}\) subunit-mediated slowing effects on the inactivation characteristics of Ca_{2.1}, Ca_{2.3}, and Ca_{1.2} calcium channel inactivation (10, 12–14). However, the \(\beta_{3\alpha2}\)-mediated effects are primarily due to palmitoylation of two cysteine residues in the \(\beta_3\) V1 region that are present in none of the other β subunits. Hence, the V1 region of \(\beta_3\) appears to act via a uniquely different mechanism.

In contrast, the C2 region and the V2 regions appear to act cooperatively in the regulation of inactivation kinetics. The N-terminal region of the C2 domain contains the interaction site for binding to the I–II linker region of the calcium channel α1 subunit (26), a region that has been suggested to act as the putative inactivation gate of the channel (6). Among the 183 residues of the C2 region, there are relatively few (nineteen) amino acid differences among \(\beta_3\) and \(\beta_4\), and they are scattered throughout the entire C2 region (Fig. 1). Hence, although the functional differences can be attributed to as little as 10% of the entire C2 domain sequence, there does not appear to be a clearly identifiable locus within the C2 region that can account for the observed effects. The V2 region has previously been implicated in regulating inactivation characteristics of Ca_{2.3} calcium channels (11), with β subunits carrying a shorter form of V2 producing more rapid inactivation kinetics compared with β subunits containing a longer V2 sequence. Our data are qualitatively consistent with this notion because the β3 subunit lacks a total of nine residues compared with the β4 subunit in...
the V2 region. However, our data also reveal that transfer of the shorter V2 region of β3 alone is not sufficient to accelerate Cav2.2 calcium channel inactivation characteristics. Hence, for Cav2.2 calcium channels, it is conceivable that intramolecular interactions between the one or more of the nineteen distinct residues in the C2 domain and the shorter V2 region allosterically control the function of the inactivation gate (i.e. I–II linker) per se, thus regulating inactivation kinetics.

Finally, we note that the V3 region of β3 has been implicated in the inactivation of Cav2.1 calcium channels through interactions with the C terminus of the α1 subunit (15). In contrast, as we report here, for Cav2.2 calcium channels, the V3 region appears to be relatively unimportant. Furthermore, as stated earlier, deletion of the C terminus of the Cav2.2 calcium channel α1 subunit did not affect β subunit regulation. Thus, the molecular basis of β subunit regulation may be different for Cav2.1 and Cav2.2 calcium channels.

In summary, our data indicate that the molecular details underlying β subunit regulation of voltage-gated calcium channels appear to vary with calcium channel subtype. Although previous studies have emphasized the role of individual regions, we present evidence that the modulation of the inactivation characteristics of Cav2.2 channel current by β3 subunits is the result of two distinct and redundant mechanisms: one that appears to result from a concerted action of C2 and V2 and a second one that involves the first 14 amino acid residues. This redundancy highlights the importance of the inactivation process in controlling calcium entry into excitable cells.

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