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Ca^{2+} Channel Regulation by a Conserved β Subunit Domain

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Summary

The β subunit is a cytoplasmic component that normalizes the current amplitude, kinetics, and voltage dependence of voltage-gated Ca^{2+} channels. Here, we identify a 30 amino acid domain of the β subunit that is sufficient to induce a stimulation and shift in the voltage dependence of activation of the Ca^{2+} channel currents. This domain is located at the amino terminus of the second region of high conservation among all β subunit gene products. Single point mutations within this region on the β_{1b} subunit modified or abolished the stimulation of Ca^{2+} channel currents and the binding of the β subunit to the α_{1A} subunit. The binding of this domain is also required for the observed changes in kinetics and voltage dependence of steady-state inactivation induced by β subunits.

Introduction

The β subunit is an integral component of two purified Ca^{2+} channels, the skeletal muscle dihydropyridine receptor (Takahashi et al., 1987) and the neuronal ω-conotoxin (ω-CTx) GVIA receptor (Witcher et al., 1993). cDNAs encoding α_1 subunits from six different genes and β subunits from four different genes have now been isolated (Castellano et al., 1993b; Ellinor et al., 1993; Hullin et al., 1992; Mori et al., 1991; Pragnell et al., 1991; Snutch et al., 1990; Soong et al., 1993; Tanabe et al., 1987; Williams et al., 1992a, 1992b). Expression experiments provide further evidence that β subunits may be present in most voltage-dependent Ca^{2+} channels. All expressed combinations of α_1 and β subunits result in current amplitude stimulation (Ellinor et al., 1993; Hullin et al., 1992; Mori et al., 1991; Williams et al., 1992a, 1992b) and/or modifications of the kinetics and voltage dependence of inward Ca^{2+} channel currents (Lacerda et al., 1991; Varadi et al., 1991). The remarkable functional similarities shared by all β subunits have been well illustrated by expression with the cardiac α_{1C} subunit isoform (Castellano et al., 1993b; Perez-Reyes et al., 1992; Tomlinson et al., 1993; Wei et al., 1991). It has been demonstrated that all four β subunit gene products are not only capable of stimulating the current amplitude and the number of dihydropyridine-binding sites but also can modify the activation and inactivation kinetics and shift the voltage dependence of activation. These observations have been confirmed further with the expression of β subunits with different α_1 subunits and therefore strongly suggest that the mechanism whereby β subunits regulate the Ca^{2+} entry is largely conserved among all voltage-dependent Ca^{2+} channels. One molecular component of the β subunit-induced regulation has been described recently with the identification of a conserved amino acid motif present on α_1 subunits that is responsible for the binding of β subunits (Pragnell et al., 1994). We now report the minimum sequence of β subunits sufficient for current stimulation. This sequence is also required for the binding of β subunits to the α_1 subunit and, therefore, for all functional regulation by β subunits.

Results

β Subunits Regulate Four Major Biophysical Properties of the α_{1A} Subunit

Functional Ca^{2+} channels were expressed in Xenopus laevis oocytes by microinjection of in vitro transcribed RNAs encoding class A α_1 (α_{1A}) subunit and the neuronal 0.2 (a-n,) subunit, with or without the neuronal β (β_{1b}) subunit. Four biophysical changes occurred upon interaction of the β_{1b} subunit with the $\alpha_{1A}\alpha_{2b}$ Ca^{2+} channel complex (Figure 1).

β subunits induced a dramatic stimulation in current amplitude. $\alpha_{1A}\alpha_{2b}$ Ca^{2+} channels characteristically had a small current amplitude with an average I_{Ba} = –289 ± 141 nA (mean ± SEM; n = 16) at 20 mV. However, in the presence of the β_{1b} subunit, the peak current amplitude was 18-fold larger, with an average I_{Ba} = –5272 ± 762 nA (n = 13) at 10 mV. This stimulation in current amplitude was observed upon expression of three additional β subunits with factors ranging between 5.4 and 19.3 (Figure 1a). The β_{1b} subunit modified the decay of the inactivating current from a biexponential to a monoexponential mode (Figure 1b). $\alpha_{1A}\alpha_{2b}$ Ca^{2+} channel currents inactivated along two components. At 20 mV, the inactivating current had two time constants of τ_1 = 30 ± 1 ms (25% of total inactivating current) and τ_2 = 498 ± 42 ms (remaining 75% of the inactivating current; n = 14). The fast inactivating current was carried by the α_{1A} subunit and not by a small contaminating endogenous current, since both inactivating components were irreversibly blocked by ω-CTx MVIIC, a potent blocker of the α_{1A} subunit (data not shown). In contrast, the inactivating current always decayed monoexponentially in the presence of the β_{1b} subunit. The average time constant in the presence of β_{1b} was τ = 229 ± 17 ms (n = 13) at 10 mV. The β_{1b} subunit also induced a hyperpolarizing shift in the voltage dependence of activation of α_{1A} subunits. $\alpha_{1A}\alpha_2$ Ca2+ channel currents activated at –20 mV and reached peak level at 16 mV, whereas $\alpha_{1A}\alpha_{2b}\beta_{1b}$ Ca^{2+} channel currents activated at –30 mV and peaked at 6 mV (Figure 1c). This corresponds to a 10 mV hyper-

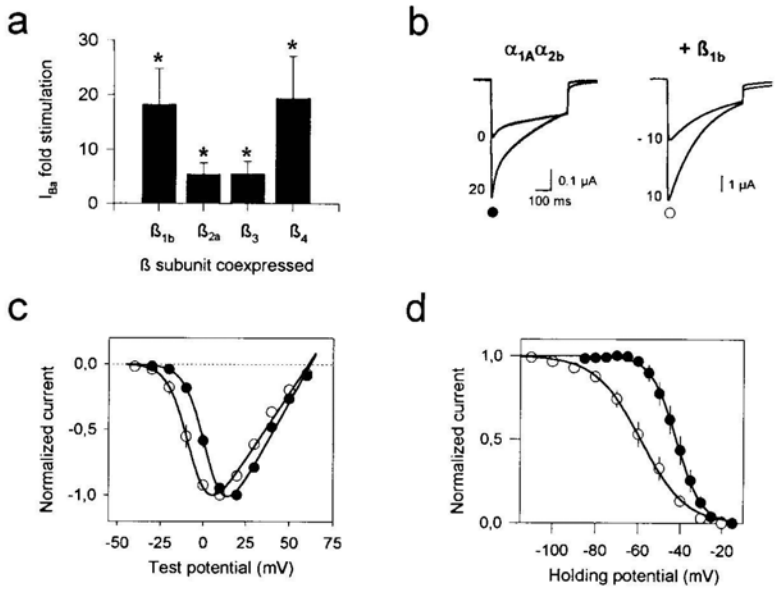

Figure 1. Effect of Different β Subunits on the Kinetics, Voltage Dependence, and Amplitude of the $\alpha_{1A}\alpha_{2b}$ Current

(a) Average Ba^{2+} current stimulation factor induced by the coexpression of all four β subunit gene products. Statistically significant stimulations ($p < .05$; Student's t test) are denoted by an asterisk.

(b) $\alpha_{1A}\alpha_{2b}Ba^{2+}$ current traces obtained with (open circles) and without (closed circles) coexpression of the β_{1b} subunit to illustrate the change in inactivation kinetics. Holding potential is -90 mV.

(c) Average normalized current-voltage relation for $\alpha_{1A}\alpha_{2b}$ with and without β_{1b} coexpression. The shift was observed in every oocyte, independent of current amplitude. Fit to the data yield g = 0.02 ($+\beta_{1b}$) and 0.023 nS ($-\beta_{1b}$), E = 60.7 ($+\beta_{1b}$) and 61.7 mV ($-\beta_{1b}$), k = 6.5 ($+\beta_{1b}$) and 6.1 mV ($-\beta_{1b}$), and $V_{½}$ = -6.2 ($+\beta_{1b}$) and 6.1 mV ($-\beta_{1b}$).

(d) Average steady-state inactivation curve for $\alpha_{1A}\alpha_{2b}$ and $\alpha_{1A}\alpha_{2b}\beta_{1b}$ Ca^{2+} channels. Peak current amplitudes are normalized to the maximum current amplitude reached during the protocol and plotted as a function of holding potential. Fits to the data yield $V_{½}$= -42 mV ($-\beta_{1b}$) or -59 mV ($+\beta_{1b}$) and k = 6.1 mV ($-\beta_{1b}$) or 10 mV ($+\beta_{1b}$). Data are the average ± SE of n = 4 ($-\beta_{1b}$) or n = 9 ($+\beta_{1b}$) oocytes.

polarizing shift in the current-voltage relation. Finally, the β_{1b} subunit induced a hyperpolarizing shift in the voltage dependence of inactivation. Steady-state inactivation analyses revealed that half of the $\alpha_{1A}\alpha_{2b}$ channels inactivated at an estimated potential of –42 mV (n = 4; Figure 1d). However, this inactivation occurred at -59 mV in the presence of β_{1b} (n = 13), which corresponds to an average hyperpolarizing shift of 17 mV. The functional similarities between β subunits were also illustrated by the observation that β_{2a}, β_3, and β_4 all had qualitatively similar effects on activation and inactivation (data not shown).

A Conserved β Subunit Sequence Interacts with the α_{1A} Subunit

Since all β subunits from all four genes can stimulate, shift the voltage dependencies, and modulate the inactivation kinetics of the α_{1A} subunit, it is likely that this regulation is performed by conserved β subunit sequences and α_1-β subunit interaction sites. The primary structures of all β subunits cloned so far indicated the presence of two structurally conserved domains (Figure 2). Domain I had 65% identity among the four β subunit genes and extended from amino acids 58 to 165 in the β_{1b}, whereas domain II encompassed amino acids 215-418 and exhibited 78% identity. To identify structural domains of the β subunit responsible for Ca^{2+} channel current modulation, we tested the functional and structural contributions of various truncated forms of the β_{1b} subunit. We determined the ability of the shorter constructs to interact with α_{1A} in vitro and to regulate (α_{1A} Ba^{2+} currents in oocytes.

We have previously reported that β subunits bind to a conserved motif in the I-II cytoplasmic linker of all Ca^{2+} channel α_1 subunits (Pragnell et al., 1994). We used ^{35}S-labeled, in vitro translated wild-type and truncated β_{1b} subunit probes to detect the interactions between various structural domains of the β subunit and the α_{1A} binding epitope (Figure 3). We found that the ^{35}S-labeled, in vitro translated $\beta_{58\text{-}418}$ and $\beta_{211\text{-}418}$ interact with the α_{1A} epitope expressed as a glutathione-S-transferase (GST) fusion protein. This localized the interaction site on the β subunit within the second most conserved domain of the subunit. A similar interaction of $\beta_{211\text{-}418}$ was also detected with the conserved motif of α_{1S}, α_{1B}, and α_{1C} (data not shown). No interaction could be detected with $\beta_{1\text{-}211}$, which comprises the first conserved domain, or $\beta_{428\text{-}597}$, which includes the carboxy-terminal portion of the subunit. The interaction of the second domain occurred with a lower affinity, suggesting that truncation induces a slightly modified structure of the β binding site. Also, this interaction was too weak to be detected with even shorter constructs within the second domain of the β subunit. For instance, no interaction could be seen with $\beta_{211\text{-}265}$ and $\beta_{211\text{-}235}$ (data not shown), despite prolonged exposures and functional evidence of regulation (Figure 5; Figure 6).

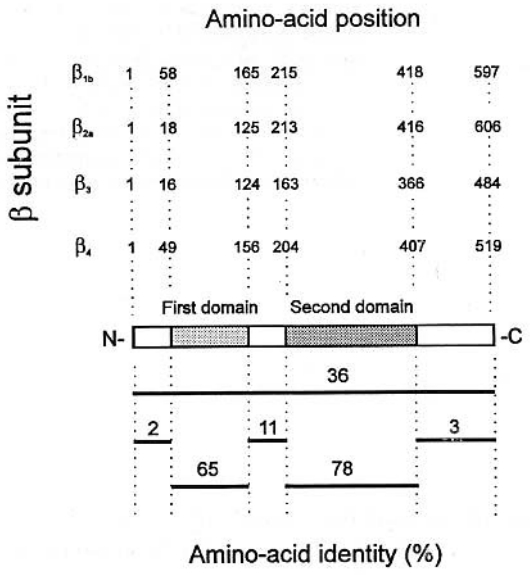

Figure 2. Sequence Similarities among Four β Subunit Gene Products

Light and dark shaded areas are regions of maximal amino acid identity among β subunits from four different genes. cDNA clones used for amino acid sequence comparisons are as follows: β_{1b} (GenBank accession number X61394; Pragnell et al., 1991), β_{2a} (X64297; Hullin et al., 1992), β_3 (M88751; Castellano et al., 1993b), and β_4 (L02315; Castellano et al., 1993a).

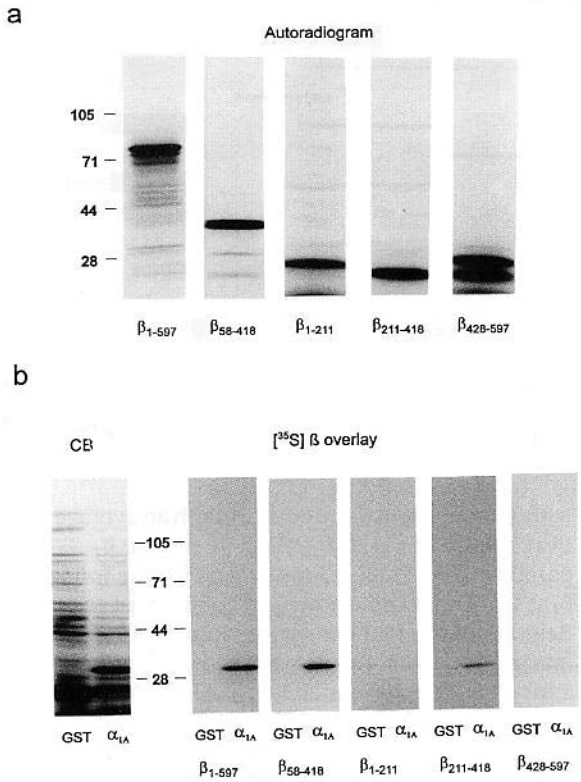

Figure 3. The β Subunit Interacts with the α_{1A} Subunit through the Second Conserved Domain

(a) Autoradiogram of an SDS-polyacrylamide gel of in vitro translated full-length or truncated [35S]methionine-labeled β_{1b} probes. (b) Coomassie blue-stained SDS-polyacrylamide gel of GST and α_{1A} epitope expressed as CST fusion proteins in total Escherichia coli lysate (left) and autoradiogram of corresponding overlay with full-length or truncated β_{1b} subunit probes on nitrocellulose immobilized GST fusion proteins (right). Lower molecular weight proteolytic fragments of α_{1A} fusion protein were also recognized by the probes. All overlay reactions were exposed for 1 hr. The probes were not equivalent in background labeling.

The Amino-Terminal Region of the Second Conserved Domain of β Subunit Interacts with the α_{1A} Subunit

Interaction of truncated β subunits with α_{1A} could be analyzed further by functional changes in the current stimulation, inactivation kinetics, and voltage dependence of the current, upon expression into Xenopus oocytes.

Consistent with the overlay experiments, $\beta_{58\text{-}418}$, with deletions at both the amino and the carboxyl termini of the molecule, and $\beta_{211\text{-}418}$, which includes the entire second domain, still induced stimulation of the Ba^{2+} current (Figure 4), whereas the two constructs that expressed only the carboxyl terminus ($\beta_{428\text{-}597}$) or the first conserved domain ($\beta_{58\text{-}211}$) did not affect the properties of the $\alpha_{1A}\alpha_{2b}$ currents. All truncated probes that included amino acids 215-245 of the second conserved domain of β_{1b} stimulated current and hence should interactwith the α_{1A} subunit. The effective constructs varied in their ability to stimulate the current from 3.2-fold ($\beta_{211\text{-}245}$) to 18.2-fold (full-length β_{1b}). As in the overlay essay, the lower stimulation factors of the shorter β_{1b} constructs probably arose because of small structural alterations relative to the full-length β subunit or because of an increased turnover rate. The stimulatory region is located immediately downstream of a splicing region that separates the first and second highly conserved domains and encodes exons varying in size in the four β_{1b} subunit genes from 7 amino acids (β_{1b} and β3) to 52 amino acids (β_{1a}; Powers et al., 1992). Alignment of this β_{1b} stimulatory sequence with the corresponding regions of all β subunits revealed a high degree of homology in this region, with at least 87% amino acid identity among all cloned β subunits. The structural complexity and potential regulatory importance of this domain is illustrated by the presence of 5 prolines and 2 consensus protein kinase C (PKC) phosphorylation sites (Ser/ThrXLys/Arg). A BLAST (Altschul et al., 1990) search with this sequence identified all β subunits of voltage-sensitive Ca^{2+} channels that have been cloned.

The interaction of truncated β_{1b} subunits with the α_{1A} subunit was also analyzed by studying changes in the kinetics or voltage dependence of activation and inactivation of the corresponding currents. The rate of inactivation was particularly sensitive to changes in the β_{1b} structure (Figure 5). $\beta_{58\text{-}418}$ had much slower inactivation kinetics, with an average time constant τ = 702 ± 79 ms (n = 10) at 10 mV compared with an average τ = 229 ± 17 ms (n = 13) for the full-length β_{1b} subunit. A similar change in kinetics was observed

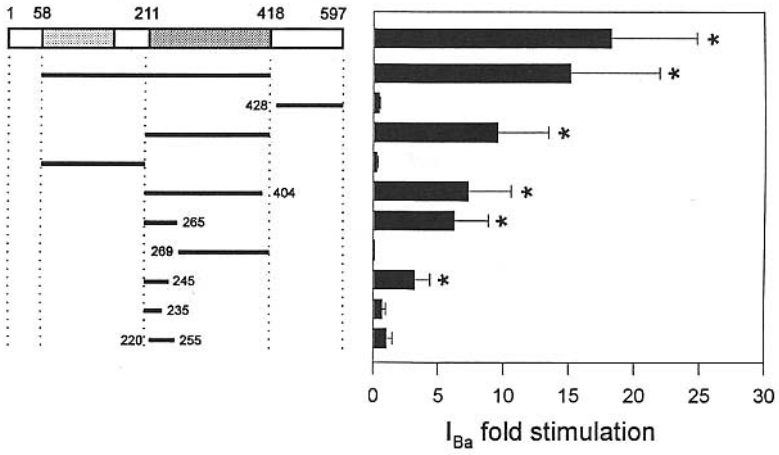

Figure 4. Current Stimulation Factors Induced by Truncated Forms of β_{1b} Subunits
Schematic of current stimulatory factors when progressive truncations of β_{1b} were coexpressed with $\alpha_{1A}\alpha_{2b}$ (n = 4-13 oocytes in each condition; n = 92 total). Error bars represent SE, and asterisks represent significant stimulations over $\alpha_{1A}\alpha_{2b}$ currents.

with an equivalent β_3 truncation, with an average inactivation rate of τ = 287 ± 7 ms (n = 4) for $\beta_{16\text{-}365}$ compared with τ = 112 ± 5 ms (n = 8) for the full-length β_3 at 10 mV (data not shown). In contrast, an additional deletion of the first conserved domain increased the inactivation, with an average time constant τ = 92 ± 5 ms (n = 4) for $\beta_{211\text{-}418}$ at 10 mV. Deleting the amino acid sequence between 245 and 265 resulted in a dramatic slowing of the activation kinetics. At -10 mV, the average time to peak was increased for $\beta_{211\text{-}245}$, with τ = 329 ± 50 ms (n = 9) compared with τ = 11 ± 1 ms (n = 13) for the full-length β_{1b}. Although a further removal of 10 amino acids ($\beta_{211\text{-}235}$) abolished the stimulation, this construct retained the ability to slow the time to peak of the current, suggesting that $\beta_{211\text{-}235}$ could still interact with the α_{1A} subunit (data not shown).

All the constructs that stimulated current also shifted the voltage dependencies of activation and inactivation (data not shown). The only exception to this observation was $\beta_{211\text{-}245}$, which was unable to shift the voltage dependence of steady-state inactivation. No regulation was observed for constructs that failed to stimulate or to interact with the α_{1A} epitope fusion protein (data not shown).

Mutations in the Stimulatory Domain Affect the Regulation by the β_{1b} Subunit

The regulatory contribution of the β_{1b} Stimulatory domain and its importance in α_{1A}-β interaction was analyzed by mutating potential important amino acids in the domain. The mutations performed were aimed at disrupting the tertiary structure of this site. Within the 30 amino acid Stimulatory region, 5 prolines and 2 serines of consensus PKC phosphorylation sites were identified as candidate sites for mutagenesis. Proline at position 221 was changed to arginine (P221 R), serine of the first potential PKC site at position 228 was

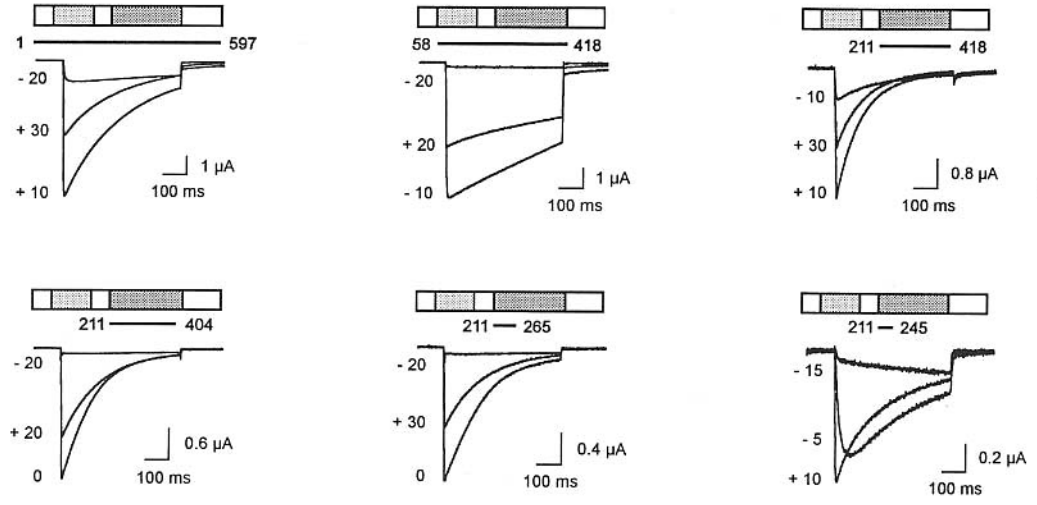

Figure 5. Interaction of Truncated β Subunits Affect the Inactivation Kinetics of the $\alpha_{1A}\alpha_{2b}$ Ca^{2+} Channel
Representative Ba^{2+} current traces obtained upon coexpression of various stimulatory truncated β_{1b} subunits. Changes in current kinetics compared with $\alpha_{1A}\alpha_{2b}$ current with and without β_{1b} subunit were also used as criteria to confirm α_{1A}- β_{1b} interactions.

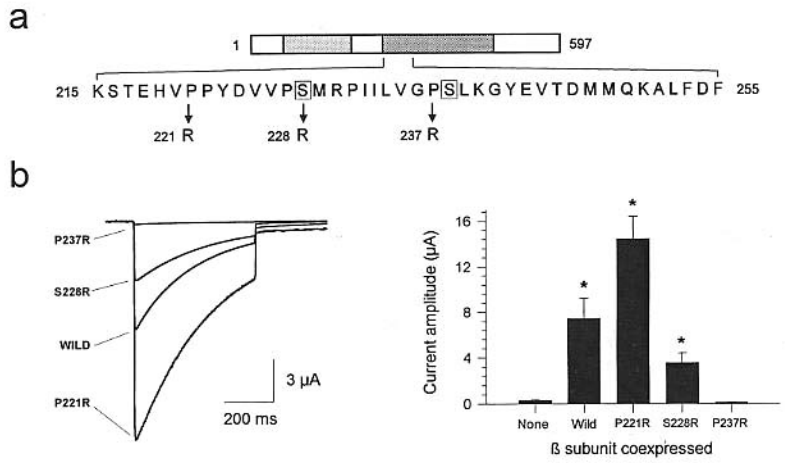

Figure 6. Mutations in the Stimulatory Region Perturb the β Subunit Stimulation of Ca^{2+} Currents

(a) Schematic representation of the point mutations performed. Boxed serines are conserved consensus PKC phosphorylation sites.

(b) Ba^{2+} currents induced by β_{1b} (Wild) or mutated β_{1b} subunits (P221R, S228R, and P237R) when coexpressed with α_{1A} and α_{2b} subunits. Left panel shows superimposed representative traces of Ba^{2+} currents evoked from a holding potential of --90 mV to a test potential of 10 mV (Wild, P221R, and S228R) or 20 mV (P237R). Right panel shows average peak currents obtained (n = 4-6 oocytes in each condition; n = 25 total). Error bar represent SE, and asterisks represent significant stimulation over $\alpha_{1A}\alpha_{2b}$ currents.

changed to arginine (S228R), and proline at position 237 was changed to arginine (P237R; Figure 6a). The mutated β_{1b} subunits were coexpressed with α_{1A} and α_{2b}, and their ability to regulate the Ba^{2+} current was compared with the wild-type β_{1b} subunit in the same batch of oocytes.

P221 R and S228R differed from the wild-type β_{1b} only in their ability to stimulate the current amplitude (Figure 6b). Maximum average current amplitudes obtained by coexpression of P221R or S228R with $\alpha_{1A}\alpha_{2b}$ were I_{Ba}, = –14426 ± 1961 nA (n = 5) and I_{Ba} = –3570 ± 848 nA (n = 5), respectively, compared with I_{Ba} = –7453 ± 1808 nA (n = 6) for coexpression of the wild-type β_{1b}. These values represent a 2-fold enhancement for P221R and a 2.1-fold reduction for S228R in current stimulation, compared with the wild-type β_{1b}.

In contrast with P221 R and S228R, the coexpression of P237R with $\alpha_{1A}\alpha_{2b}$ yielded current levels and properties reminiscent of currents obtained in the absence of any β subunit coexpression. The average current amplitude of $\alpha_{1A}\alpha_{2b}$P237R was I_{Ba} = –116 ± 36 nA (n = 5) compared with I_{Ba} = –283 ± 99 nA (n = 4) for $\alpha_{1A}\alpha_{2b}$ in the absence of β subunit. Also, similar to $\alpha_{1A}\alpha_{2b}$ currents, $\alpha_{1A}\alpha_{2b}$P237R currents inactivated biexponentially at 10mV with τ_1 = 36 ± 2ms (22% of total current) and τ_2 = 773 ± 116 ms (78% of total current; n = 5) compared with τ_1 = 30 ± 1 ms (30% of total current) and τ_2 = 505 ± 83 ms (70% of total current; n = 4) in the absence of P237R (Figure 7a). Like $\alpha_{1A}\alpha_{2b}$ currents, which peaked at 17 mV, $\alpha_{1A}\alpha_{2b}$P237R currents peaked at 24 mV, which is significantly more depolarized than currents achieved with the wild-type β_{1b} (Figure 7b). In addition, the potential of half steady-state inactivation ($V_{½}$) of $\alpha_{1A}\alpha_{2b}$P237R was $V_{½}$ = –38 mV, similar to $V_{½}$ = –42 mV for $\alpha_{1A}\alpha_{2b}$ currents (Figure 7c). In contrast with P237R, but like the wild-type β_{1b}, the inactivation of $\alpha_{1A}\alpha_{2b}$P221R or $\alpha_{1A}\alpha_{2b}$S228R was monoexponential, with, respectively, τ = 273 ± 35 ms (n = 4) and τ =

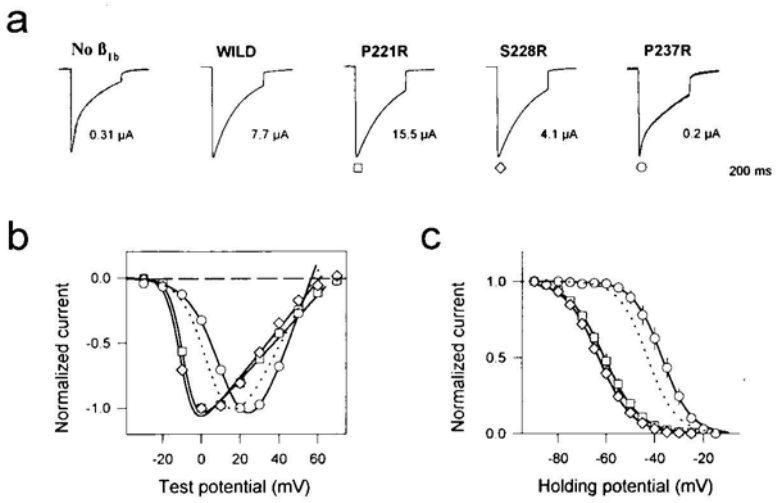

Figure 7. Changes in Inactivation Kinetics and Shifts in Voltage Dependence by Mutant β_{1b} Subunits

(a) Same representative current traces as in Figure 5b compared with an $\alpha_{1A}\alpha_{2b}$ current trace, to illustrate changes in inactivation kinetics.

(b) Average voltage dependence of activation of $\alpha_{1A}\alpha_{2b}\beta_{1b}$ mutants. Fit to the data yield g = 0.016, 0.018, or 0.02; E = 68.3, 61.3, or 56.7 mV; k = 3.6, 3.7, or 8.3 mV; and $V_{½}$ = -9.1, -10.3, or 15.2 mV for P221R (n = 5),S228R(n = 5), or P237R (n = 5), respectively.

(c) Average voltage dependence of steady-state inactivation. Fit to the data yield $V_{½}$ = -60.7, -63, or -37.1 mV and k = 7.4, 6.9, or 6 mV for P221R (n = 5), S228R (n = 5), or P237R (n = 2), respectively. The data obtained for "No β_{1b}" were represented by dotted curves to allow comparison with P237R.

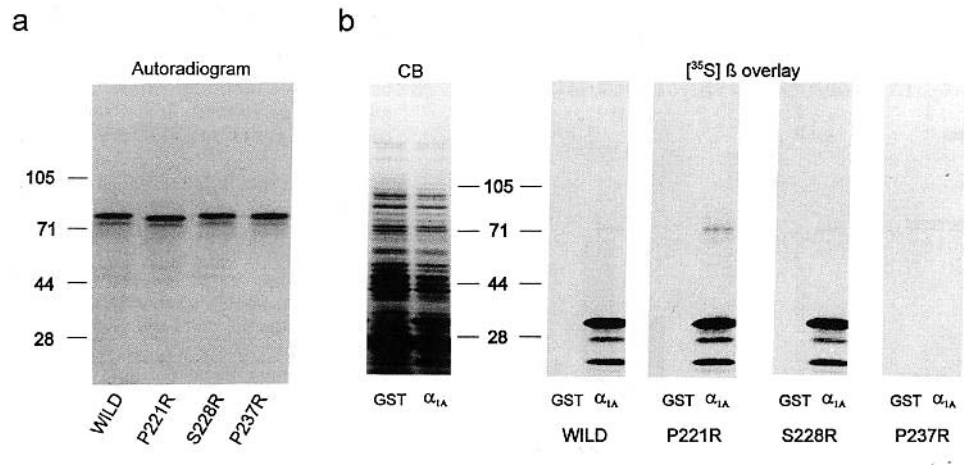

Figure 8. Mutations in the β Stimulatory Region Perturb the β Subunit Interaction with the I-II Cytoplasmic Linker of α_{1A}
(a) Autoradiogram of an SDS-polyacrylamide gel of in vitro translated wild-type or mutant [^{35}S]methionine-labeled β_{1b} probes.
(b) Coomassie blue-stained SDS-polyacrylamide gel of GST and α_{1A} epitope expressed as GST fusion proteins in total E. coli lysate (left) and autoradiogram of corresponding overlay with wild-type or mutant [^{35}S]methionine-labeled β_{1b} subunit probes on nitrocellulose immobilized GST fusion proteins (right). The overlay reactions were exposed for 1 hr.

250 ± 17 ms (n = 5) at 10 mV, compared with τ = 256 ± 14 ms (n = 5) for $\alpha_{1A}\alpha_{2b}\beta_{1b}$. Analysis of the current-voltage relation of $\alpha_{1A}\alpha_{2b}$P221R and $\alpha_{1A}\alpha_{2b}$S228R showed peak currents at 1 and 0 mV, respectively, compared with 1 mV for $\alpha_{1A}\alpha_{2b}\beta_{1b}$. Also, the potentials of half steady-state inactivation of $\alpha_{1A}\alpha_{2b}$P221R and $\alpha_{1A}\alpha_{2b}$S228R were -61 and -63 mV, respectively, compared with -59 mV for $\alpha_{1A}\alpha_{2b}\beta_{1b}$. These results strongly suggest that the P237R mutation, but not the P221R or S228R mutations, affects the ability of the β_{1b} subunit to interact with the α_{1A} subunit. However, since $\alpha_{1A}\alpha_{2b}$P237R Ca2* channels had slightly different voltage dependence of activation and inactivation than $\alpha_{1A}\alpha_{2b}$ Ca^{2+} channels, second site interactions between the α_{1A} and β subunits cannot be ruled out.

Mutations in the Stimulatory Domain Affect Binding of the β_{1b} Subunit to the α_{1A} Subunit

We tested the interaction of mutated β subunits with the β subunit-binding α_{1A} epitope in our overlay assay. ^{35}S -labeled, in vitro translated wild-type and mutated β_{1b} subunit probes were used to detect the interaction between the β subunit and the α_{1A} binding epitope. All four β subunits could be synthesized to their full length (Figure 8a). Consistent with the results of the expression experiments, we found that, like the wild-type β_{1b}, P221R and S228R were still capable of interacting with the α_{1A} binding epitope, whereas binding of the P237R probe was completely abolished (Figure 8b). Additionally, the P237R probe did not interact with the α_{1S} of the purified dihydropyridine receptor immobilized on nitrocellulose, nor did it identify any positive clones on screening of an α_{1S} epitope library, suggesting the absence of any other equivalent interaction with α_1 subunits.

Discussion

Although α_1 subunits carry all the essential properties of voltage-gated Ca^{2+} channels (gating, permeability, voltage dependence, and pharmacology), the expression of these properties is modulated by the association of ancilliary subunits. The functional contribution of the β subunit is most dramatic. The β subunit is required to normalize the amplitude, the voltage dependence of activation and inactivation, and the kinetics of Ca^{2+} entry through the α_1 subunit. An intriguing property of all cloned β subunits is their ability to stimulate dramatically the functional expression of the six α_1 subunit genes reported so far. This stimulation occurs upon coexpression of any α_1-β subunit combination. This observation strongly suggests that the mechanism whereby β subunits stimulate current amplitude is largely conserved among all voltage-gated Ca^{2+} channels.

Expression of truncated forms of the brain β_{1b} subunit with the neuronal α_{1A} and α_2 subunits allowed us to make important deductions about the structural requirements of this subunit within Ca^{2+} channels. Our data show that most of the β subunit-induced regulation can be localized to a very small domain. The region between amino acids 215 and 265 of β_{1b} is sufficient to induce the current stimulation and the hyperpolarizing shifts in voltage dependency of activation and inactivation. The Ca^{2+} current stimulation and the shift in voltage dependence of activation were even more closely localized between amino acids 215 and 245, which encompasses less than 1/20 of the β_{1b} subunit sequence. The second important finding of our work is that the region responsible for Ca^{2+} current stimulation in β subunits is, coincidentally, also important for anchoring the β subunit to the α_1 sub-

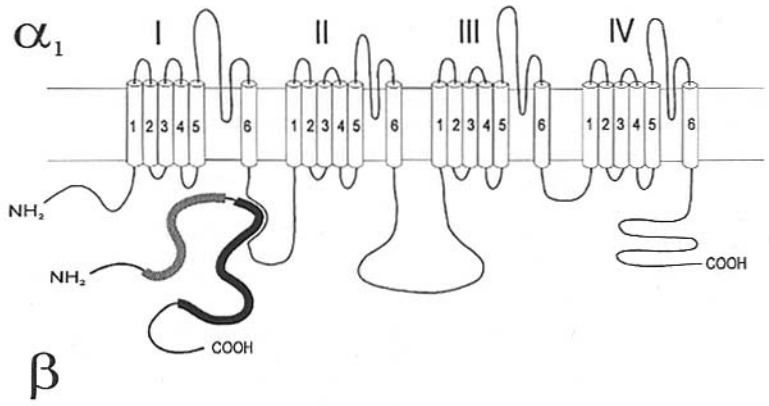

Figure 9. β Subunit Regulation Occurs via Binding of the Amino-Terminal Region of the Second Conserved Domain to the Cytoplasmic Linker between Repeats I and II of the α_1 Subunit

unit (Figure 9). This binding occurs on a conserved motif previously identified on the 1-11 cytoplasmic linker of the α_1 subunit. A mutational analysis of the β stimulatory domain further confirms the functional importance of this region and demonstrates that binding of the β subunit is required for the observed changes in the kinetics and voltage dependence of activation and inactivation. The two complementary sites (α_1 and β sites) are essential for current stimulation, since mutations, within either the β stimulatory region or the α_1 cytoplasmic linker (Pragnell et al., 1994), that do not alter the ability of β to bind to its α_1 site all result in changes in the amplitude of the current.

All the truncated β subunits that were capable of stimulating the $\alpha_{1A}\alpha_{2b}$ Ca^{2+} channel currents also changed the inactivation kinetics. However, these changes were not similar to those induced by the full-length β_{1b} subunit. The apparent nonspecificity of the changes in inactivation kinetics may be due to an altered conformation of the interaction site we describe or to the loss of potential secondary interaction sites; these two hypotheses are not mutually exclusive. Reduction in affinity for the α_{1A} epitope fusion protein and progressive reduction in stimulation efficiency of the truncated β_{1b} subunits would be in favor of an altered conformation of this single interaction site. In contrast, the great degree of structural conservation among β subunits, which goes well beyond the small sequence necessary to trigger Ca^{2+} current stimulation, would be in favor of the existence of additional interaction sites between α_{1A} and β subunits. In this respect, the rest of the second highly conserved domain and the entire first domain are intriguing, as are the unique amino and carboxyl termini among β subunits. The possible existence of secondary interaction sites is favored by the following observations: similar truncations on β_{1b} and β_3 resulted in similar changes in inactivation kinetics, all truncated β constructs shifted the voltage dependence of activation and inactivation to the same extent until deletion of a particular sequence (i.e., shift in steady-state inactivation is lost upon deletion of the amino acid sequence 245-265 of β_{1b}), and mutations in the primary interaction site (on either (α_{1A} or β_{1b}) that did not affect the ability of β subunits to bind to their site affected only the ability of β to stimulate current amplitude, leaving intact all the shifts in voltage dependence and the change in inactivation kinetics. Three lines of evidence suggest that additional interactions would be contingent upon interaction of the stimulatory region of β subunits with the α_1 binding site. It was found that truncated forms of the β_{1b} subunit that do not include the stimulatory domain fail to modify all $\alpha_{1A}\alpha_{2b}$ current properties. Additionally, point mutations in either β_{1b} or an motif (Pragnell et al., 1994) that inhibit binding of β to α_1 also fail to modify $\alpha_{1A}\alpha_{2b}$ current properties.

Experimental Procedures

Preparation of Xenopus Oocytes and cRNA Injections

Mature female Xenopus frogs (NASCO) were anesthetized with 0.03% ethyl-p-aminobenzoate (Sigma), and their ovaries were surgically removed. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (type IA, Sigma) in Ca^{2+}-free Barth's solution containing 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4 \cdot 7H_2O$, 2.4 mM $NaHCO_3$, and 15 mM HEPES (pH 7.4 with NaOH). Stage V and VI oocytes were incubated at 18°C overnight before RNA injections. RNAs were transcribed in vitro using T7 (β_{1b} and α_{2b} cDNA) or SP6 polymerase (pSPCBI-2 cDNA). Various subunit compositions (50 nl) were injected into each oocyte at the following concentrations: 0.4 μg/μl (α_{1A}), 0.4 μg/μl (α_{2b}), and 0.1 μg/μl (β).

Electrophysiological Recording and Data Analysis

Ba^{2+} currents were recorded using a Dagan two microelectrode voltage clamp (TEV-200). Voltage and current electrodes (0.5-1 MΩ tip resistance) were filled with 3 M KCl. Extracellular solution was 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KCl, 1 mM niflumic acid, 0.1 mM EGTA, 5 mM HEPES (pH 7.4 with methanesulfonic acid). Records were filtered at 0.2-0.5 kHz and sampled at 1-2 kHz. Leak and capacitance currents were subtracted off-line by a P/4 protocol. Voltage pulses were delivered every 10 s (activation data) or 30 s (inactivation data). For activation data, smooth curves were generated, assuming a Boltzmann function with $I_{Ba} = [g(TP - E)]/\{1 + \exp[-(TP - V_{½})/k]\}$, where g = normalized conductance, E = reversal potential, and k = range of potential for an e-fold change around $V_{½}$. The steady-state inactivation curves were also described by a Boltzmann equation: $I_{Ba} = \{1 + \exp[(V - V_{½})/k]\}^{-1}$, where the current amplitude I_{Ba} has decreased to half-amplitude at $V_{½}$ with an e-fold change over k mV. Endogenous Ba^{2+} current was less than 10 nA (n = 8). Injection of cRNAs

coding for α_{2b} (n = 7), β (n = 10), or α_{2b} and β (n = 5) yielded maximum average inward currents of 10-60 nA.

cDNA Constructions of the Truncated β_{1b} Subunit Forms

Truncated β_{1b} subunits were amplified by polymerase chain reaction from cDNA encoding β_{1b}, using forward primers that contained an NcoI restriction site encoding the translation initiation codon and reverse primers with an XbaI restriction site encoding the termination codon. The amplified fragments were purified by QIAEX extraction (Qiagen) and subcloned into pCEM-3 vector (Promega) that was modified to contain a 5' alfalfa mosaic virus consensus initiation site and a 3' poly(A)$^+$ tail for enhanced expression in oocytes. These constructs were verified on an automated sequencer (Applied Biosystems, Inc.).

Mutations of the β_{1b} Subunit

Site-directed mutagenesis was performed on the full-length β_{1b} using the Transformer Site-Directed Mutagenesis System (Clontech). The following mutagenic primers were used: 5'-GTCGACAGAGCACGTGCGGCCGTATGACGTGGTGCCTTC-3' (P221R), 5'-CCTATCACGTGGTGCCTAGGATGAGGCCCATCATCC-3' (S228R), and 5'-CATCATCCTGGTGGGAAGATCTCTCAAGGGCTATGAGG-3' (P237R). The selection primer was 5'-TTAGCCCAGCTAGAGAAAGCCAAGA-3'. All mutations were verified by sequence analysis.

In Vitro Translation of the β_{1b} cDNA Constructions and Overlay Experiments

The wild-type and mutant [^{35}S]methionine-labeled β_{1b} subunit probes were synthesized by coupled in vitro transcription and translation in the TNT system (Promega), in the presence of a protease inhibitor cocktail containing pepstatin A (0.1 >μg/ml), chymostatin (0.1 μg/ml), aprotinin (0.1 μg/ml), leupeptin (0.1 μg/ml), and calf liver tRNA (40 μg/ml) to minimize proteolysis and reduce background translation. Specific incorporation of [^{35}S]methionine and total amount of protein synthesized were determined bytrichloroacetic acid protein precipitation to normalize for the amount of probe used. The fusion protein epitope of the α_{1A} subunit was constructed and induced as previously described (Pragnell et al., 1994). Equivalent quantities of GST control and α_{1A} fusion protein epitopes in crude E. coli lysates were electrophoretically separated on 3%-12% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were blocked with 5% nonfat dry milk in 150 mM NaCl, 50 mM sodium phosphate (PBS), followed by an overlay buffer of 5% bovine serum albumin, 0.5% nonfat dry milk in PBS. The translation reactions were added with equal amounts of probe at 1 μl/ml (wild-type β_{1b}), 1.2 μl/ml (P221R), and 0.9 μl/ml (S228R and P237R) overlay buffer and incubated overnight at 4°C with gentle mixing. The transfers were washed 1 hr with 5% bovine serum albumin in PBS at room temperature, air dried, and exposed 1 hr to film (X-OMAT AR, Kodak).

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