Ca\textsuperscript{2+} Channel Regulation by a Conserved \(\beta\) Subunit Domain

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Summary

The \(\beta\) subunit is a cytoplasmic component that normalizes the current amplitude, kinetics, and voltage dependence of voltage-gated Ca\textsuperscript{2+} channels. Here, we identify a 30 amino acid domain of the \(\beta\) subunit that is sufficient to induce a stimulation and shift in the voltage dependence of activation of the Ca\textsuperscript{2+} channel currents. This domain is located at the amino terminus of the second region of high conservation among all \(\beta\) subunit gene products. Single point mutations within this region on the \(\beta_2\) subunit modified or abolished the stimulation of Ca\textsuperscript{2+} channel currents and the binding of the \(\beta\) subunit to the \(\alpha_\mathrm{1A}\) subunit. The binding of this domain is also required for the observed changes in kinetics and voltage dependence of steady-state inactivation induced by \(\beta\) subunits.

Introduction

The \(\beta\) subunit is an integral component of two purified Ca\textsuperscript{2+} channels, the skeletal muscle dihydropyridine receptor (Takahashi et al., 1987) and the neuronal \(\omega\)-conotoxin (\(\omega\)-CTX) GVIA receptor (Witcher et al., 1993). cDNAs encoding \(\alpha\) subunits from six different genes and \(\beta\) 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 \(\beta\) subunits may be present in most voltage-dependent Ca\textsuperscript{2+} channels. All expressed combinations of \(\alpha\) and \(\beta\) 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\textsuperscript{2+} channel currents (Lacerda et al., 1991; Varadi et al., 1991). The remarkable functional similarities shared by all \(\beta\) subunits have been well illustrated by expression with the cardiac \(\alpha_\mathrm{1C}\) 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 \(\beta\) 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 \(\beta\) subunits with different \(\alpha\) subunits and therefore strongly suggest that the mechanism whereby \(\beta\) subunits regulate the Ca\textsuperscript{2+} entry is largely conserved among all voltage-dependent Ca\textsuperscript{2+} channels. One molecular component of the \(\beta\) subunit-induced regulation has been described recently with the identification of a conserved amino acid motif present on \(\alpha_1\) subunits that is responsible for the binding of \(\beta\) subunits (Pragnell et al., 1994). We now report the minimum sequence of \(\beta\) subunits sufficient for current stimulation. This sequence is also required for the binding of \(\beta\) subunits to the \(\alpha_1\) subunit and, therefore, for all functional regulation by \(\beta\) subunits.

Results

\(\beta\) Subunits Regulate Four Major Biophysical Properties of the \(\alpha_\mathrm{1A}\) Subunit

Functional Ca\textsuperscript{2+} channels were expressed in Xenopus laevis oocytes by microinjection of in vitro transcribed RNAs encoding class A \(\alpha_1\) (\(\alpha_\mathrm{1A}\)) subunit and the neuronal 0.2 (\(\alpha_\mathrm{2}\)) subunit, with or without the neuronal \(\beta\) (\(\beta_3\)) subunit. Four biophysical changes occurred upon interaction of the \(\beta_3\) subunit with the \(\alpha_\mathrm{1A}\beta_2\) Ca\textsuperscript{2+} channel complex (Figure 1).

\(\beta\) subunits induced a dramatic stimulation in current amplitude. \(\alpha_\mathrm{1A}\beta_2\) Ca\textsuperscript{2+} channels characteristically had a small current amplitude with an average \(I_{\text{Ba}} = -289 \pm 141\) nA (mean \pm SEM; \(n = 16\)) at 20 mV. However, in the presence of the \(\beta_3\) subunit, the peak current amplitude was 18-fold larger, with an average \(I_{\text{Ba}} = -5272 \pm 762\) nA (\(n = 13\)) at 10 mV. This stimulation in current amplitude was observed upon expression of three additional \(\beta\) subunits with factors ranging between 5.4 and 19.3 (Figure 1a). The \(\beta_3\) subunit modified the decay of the inactivating current from a biexponential to a monoeXponential mode (Figure 1b). \(\alpha_\mathrm{1A}\beta_2\) Ca\textsuperscript{2+} channel currents inactivated along two components. At 20 mV, the inactivating current had two time constants of \(\tau_1 = 30 \pm 1\) ms (25% of total inactivating current) and \(\tau_2 = 498 \pm 42\) ms (remaining 75% of the inactivating current; \(n = 14\)). The fast inactivating current was carried by the \(\alpha_1\) subunit and not by a small contaminating endogenous current, since both inactivating components were irreversibly blocked by \(\omega\)-CTX MVIIIC, a potent blocker of the \(\alpha_1\) subunit (data not shown). In contrast, the inactivating current always decayed monoeXponentially in the presence of the \(\beta_3\) subunit. The average time constant in the presence of \(\beta_3\) was \(\tau = 229 \pm 17\) ms (\(n = 13\)) at 10 mV. The \(\beta_3\) subunit also induced a hyperpolarizing shift in the voltage dependence of activation of \(\alpha_1\) subunits. \(\alpha_\mathrm{1A}\beta_2\) Ca\textsuperscript{2+} channel currents activated at \(-20\) mV and reached peak level at 16 mV, whereas \(\alpha_\mathrm{1A}\beta_2\beta_3\) Ca\textsuperscript{2+} channel currents activated at \(-30\) mV and peaked at 6 mV (Figure 1c). This corresponds to a 10 mV hyper-
polarizing shift in the current-voltage relation. Finally, the β2b subunit induced a hyperpolarizing shift in the voltage dependence of inactivation. Steady-state inactivation analyses revealed that half of the α1Aβ2s channels inactivated at an estimated potential of -42 mV (n = 4; Figure 1d). However, this inactivation occurred at -59 mV in the presence of β1b (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 β1b subunit induced a hyperpolarizing shift in the current-voltage relation. The shift was observed in every oocyte, independent of current amplitude. Fit to the data yield g = 0.02 (±β1b) and 0.023 nS (±β1b), E = 60.7 (+β1b) and 61.7 mV (+β1b), k = 6.5 (+β1b) and 6.1 mV (+β1b), and V½ = -6.2 (+β1b) and 6.1 mV (+β1b).

(d) Average steady-state inactivation curve for α1Aβ2s and α1Aβ1s Ca2+ 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 V½ = -42 mV (+β1b) or -59 mV (+β1b) and k = 6.1 mV (+β1b) or 10 mV (+β1b). Data are the average ± SE of n = 9 (+β1b) oocytes.

A Conserved β Subunit Sequence Interacts with the α1A Subunit
Since all β subunits from all four genes can stimulate, shift the voltage dependencies, and modulate the inactivation kinetics of the α1A subunit, it is likely that this regulation is performed by conserved β subunit sequences and α-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 β1b, whereas domain II encompassed amino acids 215-418 and exhibited 78% identity. To identify structural domains of the β subunit responsible for Ca2+ channel current modulation, we tested the functional and structural contributions of various truncated forms of the β1b subunit. We determined the ability of the shorter constructs to interact with α1A in vitro and to regulate (α1A Ba2+) currents in oocytes.

We have previously reported that β subunits bind to a conserved motif in the I-III cytoplasmic linker of all Ca2+ channel α subunits (Pragnell et al., 1994). We used 35S-labeled, in vitro translated wild-type and truncated β1b subunit probes to detect the interactions between various structural domains of the β subunit and the α1A binding epitope (Figure 3). We found that the 35S-labeled, in vitro translated β58-418 and β211-418 interact with the α1A 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 β211-418 was also detected with the conserved motif of α1S, α1B, and α1C (data not shown). No interaction could be detected with β1b, which comprises the first conserved domain, or β428-997, 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 β211-265 and β211-235 (data not shown), despite prolonged exposures and functional evidence of regulation (Figure 5; Figure 6).
The Amino-Terminal Region of the Second Conserved Domain of β Subunit Interacts with the α1A Subunit

Interaction of truncated β subunits with α1A 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, β58-418, with deletions at both the amino and the carboxyl termini of the molecule, and β211-418, which includes the entire second domain, still induced stimulation of the Ba2+ current (Figure 4), whereas the two constructs that expressed only the carboxyl terminus (β428-597) or the first conserved domain (β58-211) did not affect the properties of the α1Aα2b currents. All truncated probes that included amino acids 215-245 of the second conserved domain of β1b stimulated current and hence should interact with the α1A subunit. The effective constructs varied in their ability to stimulate the current from 3.2-fold (β211-245) to 18.2-fold (full-length β1b). As in the overlay assay, the lower stimulation factors of the shorter β1b 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 β1b subunit genes from 7 amino acids (β1b and β3) to 52 amino acids (β1a; Powers et al., 1992). Alignment of this β1b 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 Ca2+ channels that have been cloned.

The interaction of truncated β1b subunits with the α1A 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 β1b structure (Figure 5). β58-418 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 β1b subunit. A similar change in kinetics was observed...
with an equivalent \( \beta_3 \) truncation, with an average inactivation rate of \( \tau = 287 \pm 7 \text{ ms} \) (n = 4) for \( \beta_{1b.365} \) compared with \( \tau = 112 \pm 5 \text{ ms} \) (n = 8) for the full-length \( \beta_3 \) at 10 mV (data not shown). In contrast, an additional deletion of the first conserved domain increased the inactivation, with an average time constant \( \tau = 92 \pm 5 \text{ ms} \) (n = 4) for \( \beta_{211-418} \) 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 \( \beta_{211-245} \), with \( \tau = 329 \pm 50 \text{ ms} \) (n = 9) compared with \( \tau = 11 \pm 1 \text{ ms} \) (n = 13) for the full-length \( \beta_{1b} \). Although a further removal of 10 amino acids (\( \beta_{211-235} \)) abolished the stimulation, this construct retained the ability to slow the time to peak of the current, suggesting that \( \beta_{211-235} \) could still interact with the \( \alpha_{1A} \) 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 \( \beta_{211-245} \), 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 \( \alpha_{1A} \) epitope fusion protein (data not shown).

**Mutations in the Stimulatory Domain Affect the Regulation by the \( \beta_{1b} \) Subunit**

The regulatory contribution of the \( \beta_{1b} \) Stimulatory domain and its importance in \( \alpha_{1A}-\beta_{1b} \) 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 (P221R), serine of the first potential PKC site at position 228 was

![Figure 4. Current Stimulation Factors Induced by Truncated Forms of \( \beta_{1b} \) Subunits](image)

Schematic of current stimulatory factors when progressive truncations of \( \beta_{1b} \) 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.

![Figure 5. Interaction of Truncated \( \beta \) Subunits Affect the Inactivation Kinetics of the \( \alpha_{1A}\alpha_{2b} \) Ca\(^{2+}\) Channel](image)

Representative Ba\(^{2+}\) current traces obtained upon coexpression of various stimulatory truncated \( \beta_{1b} \) subunits. Changes in current kinetics compared with \( \alpha_{1A}\alpha_{2b} \) current with and without \( \beta_{1b} \) subunit were also used as criteria to confirm \( \alpha_{1A}-\beta_{1b} \) interactions.
Figure 6. Mutations in the Stimulatory Region Perturb the \( \beta \) 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 \( \beta_{1b} \) (Wild) or mutated \( \beta_{1b} \) subunits (P221R, S228R, and P237R) when coexpressed with \( \alpha_{1A} \) and \( \alpha_{2b} \) 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 \( \beta_{1b} \) subunits were coexpressed with \( \alpha_{1A} \) and \( \alpha_{2b} \), and their ability to regulate the \( Ba^{2+} \) current was compared with the wild-type \( \beta_{1b} \) subunit in the same batch of oocytes.

P221 R and S228R differed from the wild-type \( \beta_{1b} \) 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 \( I_{Ba} = -14426 \pm 1961 \) nA (n = 5) and \( I_{Ba} = -3570 \pm 848 \) nA (n = 5), respectively, compared with \( I_{Ba} = -7453 \pm 1808 \) nA (n = 6) for coexpression of the wild-type \( \beta_{1b} \). 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 \( \beta_{1b} \).

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 \( \beta \) subunit coexpression. The average current amplitude of \( \alpha_{1A} \alpha_{2b}P237R \) was \( I_{Ba} = -116 \pm 36 \) nA (n = 5) compared with \( I_{Ba} = -283 \pm 99 \) nA (n = 4) for \( \alpha_{1A} \alpha_{2b} \) in the absence of \( \beta \) subunit. Also, similar to \( \alpha_{1A} \alpha_{2b} \) currents, \( \alpha_{1A} \alpha_{2b}P237R \) currents inactivated biexponentially at 10mV with \( \tau_{1} = 36 \pm 2 \) ms (22% of total current) and \( \tau_{2} = 773 \pm 116 \) ms (78% of total current; n = 5) compared with \( \tau_{1} = 30 \pm 1 \) ms (30% of total current) and \( \tau_{2} = 505 \pm 83 \) 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 \( \beta_{1b} \) (Figure 7b). In addition, the potential of half steady-state inactivation (\( V_{1/2} \)) of \( \alpha_{1A} \alpha_{2b}P237R \) was \( V_{1/2} = -38 \) mV, similar to \( V_{1/2} = -42 \) mV for \( \alpha_{1A} \alpha_{2b} \) currents (Figure 7c). In contrast with P237R, but like the wild-type \( \beta_{1b} \), the inactivation of \( \alpha_{1A} \alpha_{2b}P221R \) or \( \alpha_{1A} \alpha_{2b}S228R \) was monoexponential, with, respectively, \( \tau_{1} = 273 \pm 35 \) ms (n = 4) and \( \tau_{1} = 273 \pm 35 \) ms (n = 4).
250 ± 17 ms (n = 5) at 10 mV, compared with τ = 256 ± 14 ms (n = 5) for α1A(2b)1b. Analysis of the current-voltage relation of α1A2bP221R and α1A2bS228R showed peak currents at 1 and 0 mV, respectively, compared with 1 mV for α1A2b1b. Also, the potentials of half steady-state inactivation of α1A2bP221R and α1A2bS228R were -61 and -63 mV, respectively, compared with -59 mV for α1A2b1b. These results strongly suggest that the P237R mutation, but not the P221R or S228R mutations, affects the ability of the β1b subunit to interact with the α1A subunit. However, since α1A2bP237R Ca2+ channels had slightly different voltage dependence of activation and inactivation than α1A2b Ca2+ channels, second site interactions between the α1A and β subunits cannot be ruled out.

Mutations in the Stimulatory Domain Affect Binding of the β1b Subunit to the α1A Subunit
We tested the interaction of mutated β subunits with the β subunit-binding α1A epitope in our overlay assay. 35S-labeled, in vitro translated wild-type and mutated β1b subunit probes were used to detect the interaction between the β subunit and the α1A 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 β1b, P221R and S228R were still capable of interacting with the α1A binding epitope, whereas binding of the P237R probe was completely abolished (Figure 8b). Additionally, the P237R probe did not interact with the α1S of the purified dihydropyridine receptor immobilized on nitrocellulose, nor did it identify any positive clones on screening of an α1S epitope library, suggesting the absence of any other equivalent interaction with α1 subunits.

Discussion
Although α1 subunits carry all the essential properties of voltage-gated Ca2+ 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 Ca2+ entry through the α1 subunit. An intriguing property of all cloned β subunits is their ability to stimulate dramatically the functional expression of the six α1 subunit genes reported so far. This stimulation occurs upon coexpression of any α1-β subunit combination. This observation strongly suggests that the mechanism whereby β subunits stimulate current amplitude is largely conserved among all voltage-gated Ca2+ channels.

Expression of truncated forms of the brain β1b subunit with the neuronal α1A and α2 subunits allowed us to make important deductions about the structural requirements of this subunit within Ca2+ 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 α1 subunit sequence. The second important finding of our work is that the region responsible for Ca2+ current stimulation in β subunits is, coincidentally, also important for anchoring the β subunit to the α1 sub-
unit (Figure 9). This binding occurs on a conserved motif previously identified on the 1-11 cytoplasmic linker of the \( \alpha_1 \) subunit. A mutational analysis of the \( \beta \) stimulatory domain further confirms the functional importance of this region and demonstrates that binding of the \( \beta \) subunit is required for the observed changes in the kinetics and voltage dependence of activation and inactivation. The two complementary sites (\( \alpha_1 \) and \( \beta \) sites) are essential for current stimulation, since mutations, within either the \( \beta \) stimulatory region or the \( \alpha_2 \) cytoplasmic linker (Pragnell et al., 1994), that do not alter the ability of \( \beta \) to bind to its \( \alpha_1 \) site all result in changes in the amplitude of the current.

All the truncated \( \beta \) subunits that were capable of stimulating the \( \alpha_1 \beta_2 \) current also changed the inactivation kinetics. However, these changes were not similar to those induced by the full-length \( \beta_2 \) 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 \( \alpha_1 \) subunit, \( \beta_2 \) subunits with the \( \alpha_1 \) binding site. It was found that truncated forms of the \( \beta_1 \) subunit that do not include the stimulatory domain fail to modify all \( \alpha_1 \beta_2 \) current properties. Additionally, point mutations in either \( \beta_2 \) subunit, which goes well beyond the small changes in the kinetics and voltage dependence of activation and inactivation to the same extent until deletion of the amino acid sequence necessary to trigger Ca\(^{2+}\) current stimulation, would be in favor of the existence of additional interaction sites between \( \alpha_1 \) and \( \beta_2 \) subunits.

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\(_4\) • 7H\(_2\)O, 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 (\( \beta_2 \) and \( \beta_9 \) cDNA) or SP6 polymerase (pSPCBI-2 cDNA). Various subunit compositions (50 nl) were injected into each oocyte at the following concentrations: 0.4 \( \mu \)g/\( \mu \)l (\( \alpha_2 \)), 0.4 \( \mu \)g/\( \mu \)l (\( \beta_2 \)), and 0.1 \( \mu \)g/\( \mu \)l (\( \beta \)).

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 M\( \Omega \) tip resistance) were filled with 3M 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 \( I_{Ba} = \{g(TP - E)\}/\{1 + \exp[-(TP - V_{\theta 1})/k]\} \), where \( g \) = normalized conductance, \( E \) = reversal potential, and \( k \) = range of potential for an e-fold change around \( V_{\theta 1} \). The steady-state inactivation curves were also described by a Boltzmann equation: \( I_{Ba} = \{1 + \exp[(V - V_{\theta 1})/k]\}^3 \), where the current amplitude \( I_{Ba} \) has decreased to half-amplitude at \( V_{\theta 1} \) with an e-fold change over k mV. Endogenous Ba\(^{2+}\) current was less than 10 nA (n = 8). Injection of cRNAs

Figure 9. \( \beta \) 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 \( \alpha_1 \) Subunit

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IBa = \{g(TP - E)\}/\{1 + \exp[-(TP - V_{\theta 1})/k]\}, \quad I_{Ba} = \{1 + \exp[(V - V_{\theta 1})/k]\}^3
\]
coding for \(\alpha_2\) (n = 7), \(\beta_1\) (n = 10), or \(\alpha_2\) and \(\beta_1\) (n = 5) yielded maximum average inward currents of 10-60 nA.

cDNA Constructions of the Truncated \(\beta_3b\) Subunit Forms

Truncated \(\beta_3b\) subunits were amplified by polymerase chain reaction from cDNA encoding \(\beta_3b\), using forward primers that contained an Ncol restriction site encoding the translation initiation codon and reverse primers with an Xbal 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' alpha-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 \(\beta_3b\) Subunit

Site-directed mutagenesis was performed on the full-length \(\beta_3b\) using the Transformer Site-Directed Mutagenesis System (Clontech). The following mutagenic primers were used: 5'-GTCGAC-AGAGCAGCCTGGGCGGCTATGACGGTGCCT-3' (P221R), 5'-CTATACCAGTTGCGTCATAGGAGCCCTACATCC-3' (S228R), and 5'-CATCTATGCTGCTGGAGATCTCTGAGGCTATAGG-3' (P237R). The selection primer was 5'-TAGCCAGCTAGAGAAAGCCAAGA-3'. All mutations were verified by sequence analysis.

In Vitro Translation of the \(\beta_3b\) cDNA Constructions and Overaly Experiments

The wild-type and mutant [\(^{35}\)S]methionine-labeled \(\beta_3b\) 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 peptatin A (0.1 \(\mu\)g/ml), chymostatin (0.1 \(\mu\)g/ml), aprotinin (0.1 \(\mu\)g/ml), and calf liver RNA (40 \(\mu\)g/ml) to minimize proteolysis and reduce background translation. Specific incorporation of [\(^{35}\)S]methionine and total amount of protein synthesized were determined by trichloroacetic acid protein precipitation to normalize for the amount of probe used. The fusion protein epitope of the \(\alpha_{1A}\) subunit was constructed and induced as previously described (Pragnell et al., 1994). Equivalent quantities of GST control and \(\alpha_{1A}\) 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 \(\mu\)l/ml (wild-type \(\beta_3b\), 1.2 \(\mu\)l/ml (P221R), and 0.9 \(\mu\)l/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-O MAT AR, Kodak).

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