Calcium channel β-subunit binds to a conserved motif in the I–II cytoplasmic linker of the α₁-subunit

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THE β-subunit is an integral component of purified voltage-sensitive Ca^{2+} channels[1-3]. Modulation of Ca^{2+} channel activity by the β-subunit, which includes significant increases in transmembrane current and/or changes in kinetics, is observed on coexpression of six α₁-subunit genes with four β-subunit genes in all α₁–β combinations tested[4-12]. Recent reports suggest that this regulation is not due to targeting of the α₁-subunit to the plasma membrane but is probably a result of a conformational change induced by the β-subunit[11,13]. Here we report that the β-subunit binds to the cytoplasmic linker between repeats I and II of the dihydropyridine-sensitive α₁-subunits from skeletal (α₁S) and cardiac muscles (α₁C₋ₐ), and also with the more distantly related neuronal α₁A and ω-conotoxin GVIA-sensitive α₁B-subunits. Sequence analysis of the β-subunit binding site identifies a conserved motif (QQ-E--L-GY--WI---E) positioned 24 amino acids from the IS6 transmembrane domain in each α₁-subunit. Mutations within this motif

reduce the stimulation of peak currents by the β-subunit and alter inactivation kinetics and voltage-dependence of activation. Conservation of the β-subunit binding motif in these functionally distinct calcium channels suggests a critical role for the I–II cytoplasmic linker of the α₁-subunit in channel modulation by the β-subunit.

An ^{35}S-labelled *in vitro*-translated rat β₁ᵦ-subunit[14] protein probe (Fig. 1a) was used to demonstrate an interaction between the β- and α₁S-subunits of the dihydropyridine receptor (DHPR) from skeletal muscle. A single prominent radioactive band corresponding to a relative molecular mass of 170,000 (M_r 170K) was detected in purified DHPR[15] which colocalized with immuno-stained α₁S-subunit (Fig. 1b). On prolonged exposure, β-subunit interaction with the DHPR α₁S-subunit was also detected in triads and T-tubule membranes. Incubations with ^{35}S-labelled sham *in vitro* translates showed no interaction (not shown). In addition, the *in vitro*-translated β₁ₐ-subunit of DHPR and the β₃-subunit of the ω-conotoxin GVIA receptor (CgTxR)[3] identified the same 170K protein, suggesting a conserved site for α₁-subunit interaction among β-subunits from different genes (not shown).

To identify the β-subunit interaction site on the α₁S-subunit, we screened an epitope library of the rabbit DHPR α₁S-subunit[16] with the ^{35}S-labelled β₁ᵦ-subunit probe. Figure 2a shows an autoradiogram of a single purified positive clone amplified and probed with the translated β₁ᵦ-subunit. Similar results were obtained with a β₃-probe (not shown), whereas an overlay of this α₁S-positive clone with a ^{35}S-labelled α₂ᵦ-probe showed no interaction (Fig. 2a). Seven positive plaques were purified to homogeneity and DNA sequencing showed that all clones were in the appropriate reading frame. The epitopes ranged in size from 50 to 67 amino acids and all shared a 45-amino-acid overlap that extends from amino acid 341 to 385 of the α₁S-subunit. Analysis of the transmembrane topology of the α₁S-subunit maps this β-subunit interaction site to the putative cytoplasmic linker between repeats I and II and is consistent with the predicted cytoplasmic location of the β-subunit[1,2] (Fig. 2d).

Because coexpression of β-subunits with various α₁-subunits greatly enhances peak inward currents and alters kinetic and voltage-dependent properties[4,12], we addressed whether this interaction site is conserved in distantly related calcium channel α₁-subunits. We constructed epitope libraries of the complemen-

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tary DNA encoding the rabbit cardiac dihydropyridine-sensitive α_{1C-a}-subunit[5] and the rat neuronal ω-conotoxin GVIA-sensitive α_{1B}-subunit[17]. Although these subunits exhibit the characteristic transmembrane topology of voltage-sensitive calcium channels, they are encoded by different genes and share only 66 and 34% identity, respectively, with the DHPR α_{1S}. Positive clones were again detected with ^{35}S-labelled β_{1b}-subunit probe and found to map to the same location on the I–II cytoplasmic linker of each

α_1-subunit. The smallest of two overlapping clones from the α_{1C-a} λgt11 expression library encoded an 84-amino-acid epitope extending from amino acids 434 to 517 of this subunit. Similarly, the smallest of two epitopes cloned from a screen of the α_{1B} library yielded a 56-amino-acid domain representing amino acids 378 to 434. To extend these observations to the α_{1A}-subunit, which shares 33% identity with DHPR α_{1S}, we constructed a glutathione-S-transferase (GST) fusion protein expressing

FIG. 1 β-Subunit interaction with the α_1-subunit of the dihydropyridine-sensitive calcium channel from skeletal muscle. a, Autoradiogram of SDS–polyacrylamide gel of in vitro-translated ^{35}S-labelled probe. The β_{1b}-subunit migrates differently from the β_{1a} of DHPR because the primary structure of the β_{1b} splice variant predicts it to have an M_r 7.81K larger than β_{1a}[14]. b, CB, Coomassie blue-stained SDS–polyacrylamide gel of rabbit skeletal muscle crude membranes (Mic), triads (Tr), T-tubule system (TS) and purified dihydropyridine receptor (DHPR). α_1 mAb, Corresponding immunoblot stained with a mix of monoclonal antibodies (mAbs IIF7, IIC12 and IIID5; ref. 15) to α_{1S} of DHPR. $[^{35}S]\beta$, Autoradiogram of an identical nitrocellulose transfer incubated with in vitro-translated $[^{35}S]\beta_{1b}$ probe. Arrowhead indicates the 170K protein, α_{1S}.
METHODS. The $[^{35}S]\beta_{1b}$-subunit[14] probe was synthesized by coupled in vitro transcription and translation in the TNT system (Promega). To enhance probe yield, a 50-nucleotide alfalfa mosaic viral consensus initiation site was engineered into the cDNA. Translation was in the presence of a cocktail containing protease inhibitors pepstatin A, chymostatin, aprotinin, antipain and leupeptin (Boehringer Mannheim) at 0.1 µg ml^{-1} and calf liver tRNA (Sigma) at 40 µg ml^{-1} to minimize proteolysis and reduce background translation. Rabbit skeletal muscle crude membranes, triads, T-tubule system and purified dihydropyridine receptor[15] were electrophoretically separated on 3–12% SDS polyacrylamide gels in the presence of 1% 2-mercaptoethanol and transferred
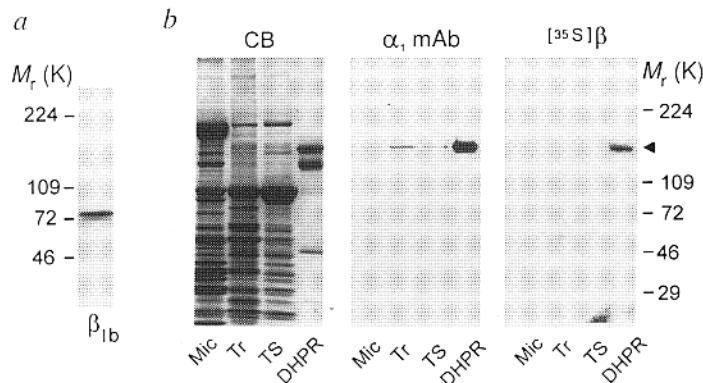
to nitrocellulose. These blots were blocked with 5% non-fat dry milk in 150 mM NaCl, 50 mM sodium phosphate (PBS), followed by an overlay buffer of 5% bovine serum albumin (BSA) and 0.5% non-fat dry milk in PBS. The translation reaction was added at 1 µl ml^{-1} of overlay buffer and incubated with gentle mixing for 12 h at 4 °C. The transfers were washed 1 h with 5% BSA in PBS at room temperature, air-dried and exposed to film (X-OMAT AR, Kodak).
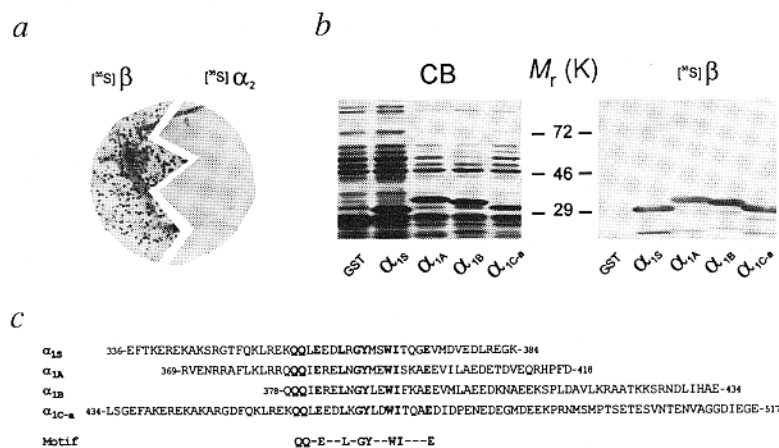

FIG. 2 Identification of the β-subunit interaction site on the α_1-subunit by epitope cloning. a, Autoradiogram of an amplified single positive plaque isolated by screening a skeletal muscle DHPR α_{1S} subunit epitope library with an in vitro-translated $[^{35}S]\beta_{1b}$ subunit probe. The nitrocellulose lift was cut and each half was incubated with either the $[^{35}S]\beta_{1b}$ subunit or a $[^{35}S]\alpha_{2b}$ subunit probe. b, Coomassie blue-stained SDS–polyacrylamide gel of glutathione-S-transferase (GST) control and α_{1S}, α_{1A}, α_{1B} and α_{1C-a} epitopes expressed as GST fusion proteins in total E. coli lysate (left), and autoradiogram of corresponding overlay with $[^{35}S]\beta_{1b}$ subunit on these nitrocellulose-immobilized fusion proteins (right). c, Alignment of amino-acid sequences of these fusion protein epitopes identifying a conserved α_1–β subunit interaction motif. The first and last amino acids of each epitope are numbered according to their location in the primary structure, as deduced from the full-length cDNA. d, Schematic showing the β-subunit interaction site on the I–II cytoplasmic linker of the α_1-subunit.
METHODS. α_1-Subunit epitope libraries in the λgt11 vector were made by digesting the α_1-subunit cDNA in a plasmid vector with DNAse I in the presence of 10 mM MnCl$_2$. Following the addition of EcoRI linkers, the randomly digested fragments were size-selected to <500 bp in Nusieve agarose gel (FMC Bioproducts). These fragments were then purified, digested with EcoRI and ligated into λgt11. The β_{1b}-subunit

probe was used to screen 2×10^4 clones of each α_1-subunit epitope library. Inserts were amplified from pure phage positives by polymerase chain reaction (PCR) using primers directed to λgt11 phage arms. These were directly subcloned into a T-vector[23] (made from Bluescript SK-plasmid) for sequencing, or digested with EcoRI and ligated into pGEX1 vector for GST fusion protein production. A fusion protein epitope of the α_{1A} subunit was constructed by amplifying base pairs 1,387–1,553 with the following primers: 5'-AGGGAATTCAAAGGGATGTCTCTG-3' and 5'-AAGGATCCGAGCGGGTGGAGAAC-3'. This PCR product was then digested with BamHI and EcoRI and subcloned into the pGEX2T vector. All inserts were sequenced in both directions by the dideoxy chain termination method using Sequenase II (US Biochemicals). Each recombinant pGEX molecule was introduced into E. coli DH5α cells. Overnight cultures of the pGEX-epitope constructs were diluted 1:10, incubated for 1 h and induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside. 75 µl of each culture was dissolved in SDS sample buffer and proteins separated electrophoretically on 3–12% SDS–polyacrylamide gels and transferred to nitrocellulose. Overlays were done as described in the legend to Fig. 1 in 5% BSA and 0.5% non-fat dry milk in PBS. Peptide sequence homology searches were done at the National Center for Biotechnology Information using the BLAST[18] network service.

amino acids 369 to 418 of the rabbit neuronal α_{1A}-subunit[12], which spans the region common to all cloned epitopes. As shown in Fig. 2b, the β_{1b}-subunit binds to the epitopes in all four α_1-subunits when expressed as GST fusion proteins. All epitopes mapped to the I–II cytoplasmic linker, although a comparison of this linker sequence among the four α_1-subunits shows only 19% overall identity.

Sequence comparison of the epitopes (Fig. 2c) suggests the presence of an interaction motif on each α_1-subunit that can be minimally described by QQ-E--L-GY--WI---E. A BLAST[18] search with this motif identified sequence records representing all voltage-sensitive calcium channel α_1-subunits so far reported. Although the lengths of the cytoplasmic linkers separating repeats I and II among these four α_1-subunits vary in size from 99 to 129 amino acids, the conserved motif is always positioned 24 amino acids downstream from the S6 transmembrane element of repeat I. Splice variations in the I–II cytoplasmic linker encoded by the α_{1C} gene have been reported which are close to the motif and hence may be important in differential β-subunit regulation of these channels. These include a 33-amino-acid exon that replaces the IS6 transmembrane segment and a 25-amino-acid insertion positioned 19 residues downstream of the motif in the α_{1C} cloned from lung[19]. Three additional splice variations of this region in α_{1C} have been reported at a location immediately downstream of the latter insertion[20].

To perturb the β-subunit binding in the protein overlay assay and thereby identify candidate residues that contribute to the structural integrity of the motif, we made the following non-conservative mutations in the GST fusion protein (FP) epitope
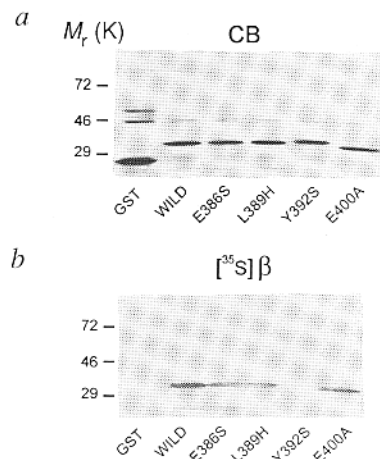
of the α_{1A}-subunit: glutamate at position 4 of the motif to serine (E386S$_{FP}$), leucine at position 7 to histidine (L389H$_{FP}$), tyrosine at position 10 to serine (Y392S$_{FP}$) or glutamate at position 18 to alanine (E400A$_{FP}$). The ability of the mutants to interact with
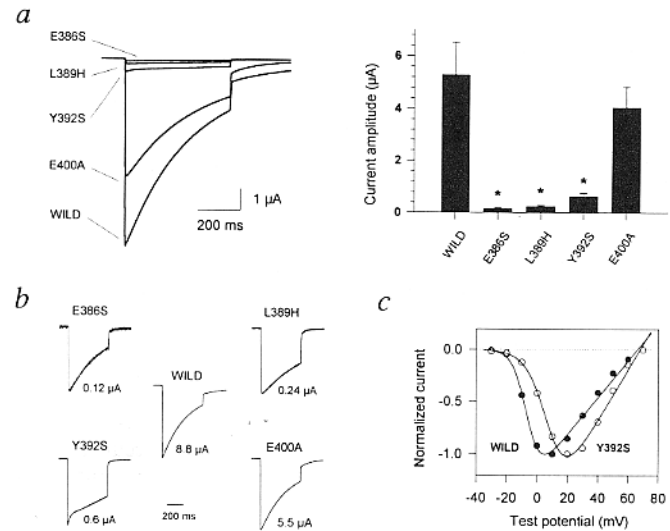
FIG. 4 Mutagenesis of the β-subunit interaction site on the α_{1A} subunit alters the effects of α_1–β subunit coexpression. a, Barium currents of α_{1A} (WILD) or mutated α_{1A}-subunits (E386S, L389H, Y392S and E400A) coexpressed with α_{2b}- and β_{1b}-subunits in Xenopus oocytes. Left panel shows superimposed representative traces of Ba^{2+} currents evoked from a holding potential of -90 mV to a $+10$ mV test potential (TP). Right panel shows average peak currents obtained ($n = 7$ to 9 oocytes). Statistically significant reductions ($P < 0.05$, t-test) from wild-type α_{1A} expression levels are denoted by asterisks. Error bars are s.e.m. b, Same representative current traces as in a, illustrating changes in inactivation kinetics. c, Normalized average current–voltage relationship from α_{1A} (WILD) or mutated α_{1A}-subunit (Y392S) to show shift in peak currents induced by the Y392S point mutation. Smooth curves were generated assuming an activation curve of a Boltzmann type $I_{Ba} = [g(TP-E)]/[1 + \exp(-(TP-V_{1/2})/k)]$ with g, the maximum normalized conductance ($g_{WILD} = 0.018$ and $g_{Y392S} = 0.024$), E, the reversal potential ($E_{WILD} = 66$ mV and $E_{Y392S} = 68$ mV), and $V_{1/2}$, the potential of half-activation ($V_{1/2,WILD} = -6.4$ mV and $V_{1/2,Y392S} = 7.6$ mV) and k, the range of potential for an e-fold change around $V_{1/2}$ ($k_{WILD} = 4.6$ mV and $k_{Y392S} = 6.7$ mV).
METHODS. To mutate the full-length α_{1A}-subunit, pSPCBI-2 (ref. 12), base pairs 1,282–1,745 were amplified by PCR and subcloned into a T-vector made from Bluescript SK$^-$. Mutagenic primers and a selection primer, 5'-CATGGTCAATCATGCTTATCGATAC-3', which eliminates the HindIII site on the recombinant molecule, were used. The 1,416–1,723 BsmI fragment encoding the motif in α_{1A} was then replaced with mutated BsmI fragments from the T-vector constructs. To facilitate cloning, the third BsmI site in the 3' non-coding region was deleted by digestion at two flanking BamHI sites, followed by recircularization of the plasmid. This BamHI fragment was restored after subcloning the mutated BsmI fragments. The mutated regions were sequenced in both directions on an Applied Biosystems Inc. Automated Sequencer. Complementary RNAs were transcribed in vitro using SP6 RNA polymerase with the rabbit brain α_{1A} plasmid or α_{1A} mutants E386S, L389H, Y392S and E400A, and T7 RNA polymerase with rat brain α_{2b} and β_{1b} plasmids. Wild-type or mutant α_{1A}-subunits were co-injected with α_{2b} and β_{1b} into stage V or VI Xenopus oocytes at concentrations: 0.6 µg ml^{-1} α_{1A} or mutants, 0.4 µg ml^{-1} α_{2b} and 0.2 µg ml^{-1} β_{1b}; about 50 nl was injected per cell. Ba^{2+} currents were recorded by a standard two-microelectrode voltage-clamp using a Dagan amplifier (TEV-200). Voltage and current electrodes (0.5–2 MΩ tip resistance) were filled with 3 M KCl. The bath solution was (in mM): Ba(OH)$_2$, 40; NaOH, 50; KCl, 2; niflumic acid, 1; EGTA, 0.1; HEPES, 5; pH 7.4, adjusted with methanesulphonic acid. Records were filtered at 0.2 to 0.5 kHz and sampled at 1–2 kHz. Leak and capacitance currents were subtracted off-line by a P/4 protocol. Ba^{2+} current through endogenous channels was less than 10 nA (no injection) and average Ba^{2+} current was 40 nA upon injection of β_{1b} and α_{2b} cRNAs.

FIG. 3 Mutation of the motif in the α_{1A} epitope perturbs the β-subunit interaction in the overlay assay. a, Coomassie blue-stained SDS–poly-acrylamide gel of GST, α_{1A} epitope (WILD) and mutants (E386S, L389H, Y392S and E400A) expressed as GST fusion proteins in total E. coli lysate, and b, autoradiogram of corresponding overlay with [^{35}S]β_{1b}-subunit probe on these nitrocellulose-immobilized GST fusion proteins.
METHODS. Site-directed mutagenesis was performed on the fusion protein containing the α_{1A} epitope using the Transformer site-directed mutagenesis system (Clontech). This method works by simultaneously annealing two oligonucleotide primers to one strand of a denatured double-stranded plasmid. One primer introduces the desired mutation and the second mutates a restriction site unique to the plasmid for the purpose of selection. To generate fusion protein mutants the following mutagenic primers were used: 5'-GCGGCAGCAGCAGATATCACG-CGAGCTCAACGGG-3' (E386S), 5'-GCAGATTGAACGCGAGCACAAC-GGGTACATGGAG-3' (L389H), 5'-CGAGCTCAACGGGTCCATGGAGTGG-ATCTCAAAAGC-3' (Y392S) and 5'-GGATCTCAAAAGCAGCTGA-GGTGATCCTCGCAGAGG-3' (E400A). The selection primer 5'-GACATCC-CTTGGATATCATCGTGACTG-3' was used to mutate EcoRI to EcoRV on the recombinant PGEX2T molecule. All mutations were verified by sequence analysis. Overlays were done as described in Fig. 1 legend. Autoradiographs were scanned with a Molecular Dynamics computing densitometer.

the β-subunit was determined by the overlay assay described above. The β-subunit interaction was reduced with the E386S$_{FP}$, L389H$_{FP}$ and Y392S$_{FP}$ mutants compared with the wild-type epitope (Fig. 3a, b). This reduction was greatest with the Y392S$_{FP}$ mutation, which required prolonged exposure of the film for visualization. No reduction in intensity was observed with E400A$_{FP}$. Densitometric scans of autoradiogram band intensities normalized for protein concentration and averaged over three experiments measured a 27-fold reduction in β-subunit binding to Y392S$_{FP}$ compared with the wild-type epitope. Binding to mutants E386S$_{FP}$ and L389H$_{FP}$ were both reduced 1.7-fold, whereas a 1.3-fold enhancement was measured with E400A$_{FP}$.

Coexpression of α_{1A} with a β-subunit dramatically increases the functional expression[12] and alters the voltage-dependent and kinetic properties of the channel in *Xenopus* oocytes (not shown). Alteration of the β-subunit binding motif on α_{1A} should therefore alter the modulatory contribution by the β-subunit. A deletion of 30 amino acids that includes the interaction motif (α_{1A}377–406) or a smaller 16-amino-acid deletion (α_{1A}377–393) of part of the motif had the result that no detectable current (<10 nA) was observed on coexpression with the β_{1b}-subunit in *Xenopus* oocytes (not shown). To determine more accurately the functional importance of the motif, the four point mutations described above were introduced into the full-length α_{1A}-subunit. Coexpression of E386S$_{\alpha1A}$, L389H$_{\alpha1A}$ or Y392S$_{\alpha1A}$ with α_{2b}- and β_{1b}-subunits resulted in significantly reduced peak currents compared with the wild-type α_{1A}-subunit (Fig. 4a). Average reductions in current expression were: E386S$_{\alpha1A}$, 36-fold; L389H$_{\alpha1A}$, 22-fold; Y392S$_{\alpha1A}$, 9-fold; E400A$_{\alpha1A}$, 1.3-fold. Although autoradiogram band intensities of β-subunit overlay were also reduced for mutations E386S, L389H and Y392S, no quantitative correlations were attempted because of the vastly different conditions of these assays. Mutant Y392S$_{\alpha1A}$ altered the inactivation kinetics of the channel (Fig. 4b). At test pulses to $+10$ mV, Ba^{2+} current inactivated with similar average time constants of (mean $\pm$ s.d.) $\tau = 350 \pm 59$ ms (wild-type α_{1A}, $n = 7$), $\tau = 290 \pm 69$ ms (E386S$_{\alpha1A}$, $n = 9$), $\tau = 434 \pm 158$ ms (L389H$_{\alpha1A}$, $n = 8$) and $\tau = 522 \pm 119$ ms (E400A$_{\alpha1A}$, $n = 7$). However, Ba^{2+} current conducted by Y392S$_{\alpha1A}$ consistently inactivated along two components, $\tau_1 = 22 \pm 4$ ms (15% of the total current) and $\tau_2 = 1,806 \pm 682$ ms (85% of the total current). In addition to this change in inactivation kinetics, this Y392S mutation also shifted the voltage-dependence of activation (Fig. 4c). All currents, independent of magnitude, peaked at a test potential of $+10$ mV, except for Y392S$_{\alpha1A}$, which peaked at $+20$ mV. More precise estimates of Y392S$_{\alpha1A}$ peak currents were obtained by a fit of the data and showed a general shift by 15 mV towards depolarized test potentials. Although the Y392S mutation greatly modified the biophysical properties of $\alpha_{1A}\alpha_{2b}\beta_{1b}$ complexes, this was not due to a complete loss of α_1-β-subunit interaction. β_{1b}-subunit still overlayed on the mutant epitope (Fig. 3b) and also, peak current amplitudes with Y392S$_{\alpha1A}\alpha_{2b}$ were enhanced from (mean $\pm$ s.e.m.) -10 ± 4 nA ($n = 4$) to -613 ± 137 nA ($n = 8$) by β_{1b}-subunit coexpression. Ba^{2+} currents obtained in the absence of the β_{1b}-subunit were, however, too small for accurate description of the current properties.

The non-conserved residues interspersed among conserved amino acids of the β-subunit interaction motif may be important for the variation in effects of β-subunits on different α_1-subunits. For example, when the α_{1E}-subunit is coexpressed with the β-subunit, shifts in voltage-dependence of activation and inactivation occur, but no appreciable stimulation of current expression is observed[9]. Sequence variations of the motif in the carp skeletal muscle α_1-subunit[21] in position 4 (E→D) and in an α_1 subunit (doe-4)[22] of torpedo electric organ at positions 7 (L→F) and 9 (G→R) may indicate differences in modulation by β-subunits in these non-mammalian channels or a structural divergence in their respective β-subunits to accommodate these substitutions. Our results show that substitutions of critical residues in the

β-subunit interaction motif on the Ca^{2+} channel α_1-subunit alter the ability of the α_1-subunit to be modulated by the β-subunit. The properties altered by these mutations correlate well with those modified by β-subunits on coexpression with α_1-subunits[4–12]. These findings suggest a critical role for the conserved interaction site on the I–II cytoplasmic linker of the α_1-subunit in voltage-sensitive calcium-channel modulation by the β-subunit. $\square$

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