Calcium channel \( \beta \)-subunit binds to a conserved motif in the I–II cytoplasmic linker of the \( \alpha_1 \)-subunit

Marlon Pragnell*, Michel De Waard†, Yasuo Mori‡, Tsutomu Tanabe‡, Terry P. Snutch§ & Kevin P. Campbell*)

* Howard Hughes Medical Institute, Department of Physiology and Biophysics and Program in Neuroscience, University of Iowa College of Medicine, 400 EMRB, Iowa City, Iowa 52242, USA
† Department of Chemical Medicine, Kyoto University, Kyoto 606, Japan
‡ Howard Hughes Medical Institute, Department of Cellular and Molecular Physiology, Yale University, New Haven, Connecticut 06536, USA
§ Biotechnology Laboratory and Departments of Zoology and Neuroscience, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

The \( \beta \)-subunit is an integral component of purified voltage-sensitive Ca\(^{2+} \) channels\(^1,2 \). Modulation of Ca\(^{2+} \) channel activity by the \( \beta \)-subunit, which includes significant increases in transmembrane current and/or changes in kinetics, is observed on cocexpression of six \( \alpha_1 \)-subunit genes with four \( \beta \)-subunit genes in all \( \alpha_{1-\beta} \) combinations tested\(^3,4 \). Recent reports suggest that this regulation is not due to targeting of the \( \alpha_1 \)-subunit to the plasma membrane but is probably a result of a conformational change induced by the \( \beta \)-subunit\(^1,12 \). Here we report that the \( \beta \)-subunit binds to the cytoplasmic linker between repeats I and II of the dihydropyridine-sensitive \( \alpha_1 \)-subunits from skeletal (\( \alpha_{1s} \)) and cardiac muscles (\( \alpha_{1c} \)), and also with the more distantly related neuronal \( \alpha_{1n} \) and \( \alpha_{1p} \)-conotoxin GV1A-sensitive \( \alpha_{1w} \)-subunits. Sequence analysis of the \( \beta \)-subunit binding site identifies a conserved motif (QQ-E-L-GY-WI-E) positioned 24 amino acids from the S6 transmembrane domain in each \( \alpha_1 \)-subunit. Mutations within this motif reduce the stimulation of peak currents by the \( \beta \)-subunit and alter inactivation kinetics and voltage-dependence of activation. Conservation of the \( \beta \)-subunit binding motif in these functionally distinct calcium channels suggests a critical role for the I–II cytoplasmic linker of the \( \alpha_1 \)-subunit in channel modulation by the \( \beta \)-subunit.

An \( ^{35} \)S-labelled \textit{in vitro} translated rat \( \beta_{1s} \)-subunit\(^6 \) protein probe (Fig. 1a) was used to demonstrate an interaction between the \( \beta \) and \( \alpha_{1s} \)-subunits of the dihydropyridine receptor (DHPR) from skeletal muscle. A single prominent radioactive band corresponding to a relative molecular mass of 170,000 (M, 170K) was detected in purified DHPR\(^5 \) which colocalized with immunostained \( \alpha_{1s} \)-subunit (Fig. 1b). On prolonged exposure, \( \beta \)-subunit interaction with the DHPR \( \alpha_{1s} \)-subunit was also detected in triads and T-tubule membranes. Incubations with \( ^{35} \)S-labelled sham \textit{in vitro} translated showed no interaction (not shown). In addition, the \textit{in vitro} translated \( \beta_{1s} \)-subunit of DHPR and the \( \beta \)-subunit of the \( \omega \)-conotoxin GV1A receptor (CgTxR\(^5 \)) identified the same 170K protein, suggesting a conserved site for \( \alpha_1 \)-subunit interaction among \( \beta \)-subunits from different genes (not shown).

To identify the \( \beta \)-subunit interaction site on the \( \alpha_{1s} \)-subunit, we screened an epitope library of the rabbit DHPR \( \alpha_{1s} \)-subunit\(^6 \) with the \( ^{35} \)S-labelled \( \beta_{1s} \)-subunit probe. Figure 2a shows an autoradiogram of a single purified positive clone amplified and probed with the translated \( \beta_{1s} \)-subunit. Similar results were obtained with a \( \beta_1 \)-probe (not shown), whereas an overlay of this \( \alpha_{1s} \)-positive clone with a \( ^{35} \)S-labelled \( \delta_{2o} \)-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 epitope 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 \( \alpha_{1s} \)-subunit. Analysis of the transmembrane topology of the \( \alpha_{1s} \)-subunit maps this \( \beta \)-subunit interaction site to the putative cytoplasmic linker between repeats I and II and is consistent with the predicted cytoplasmic location of the \( \beta \)-subunit\(^1,12 \) (Fig. 2d).

Because coexpression of \( \beta \)-subunits with various \( \alpha_1 \)-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 \( \alpha_1 \)-subunits. We constructed epitope libraries of the complemen-
FIG. 1. β-Subunit interaction with the α1 subunit of the dihydro- 
pyridine-sensitive calcium channel from skeletal muscle. a, Autoradi 
ogram of SDS–polyacrylamide gel of in vitro translated 35S-labelled probe. The β1subunit migrates differently from the β12, DHPR  
because the primary structure of the β12 splice variant predicts it to  
have an M, 78,717 larger than β12. b, CB, Coomassie blue 
stained SDS–polyacrylamide gel of rabbit skeletal muscle crude  
membranes (MIC), tris (Tr), T-tube (T) system (S) and purified di 
hydropyridine receptor (DHPR). α1 mAb, Corresponding immuno 
blot stained with a mix of monoclonal antibodies (mAbs) IIF7, IIC12 and  
IIIDS; ref. 15) to α1 of DHPR. [35S]β, Autoradiogram of an identi 
fical nitrocellulose transfer incubated with in vitro translated [35S]βpro 
bote. Arrowhead indicates the 170k protein, α13. 

METHODS. The [35S]β subunit (β) probe was synthesized by coupled  
into vitro transcription and translation in the TNT system (Promega). To  
enhance probe yield, a 50-nucleotide dUTP oligo viral cDNA initiation  
site was engineered into the cDNA. Translation was in  
the presence of a cocktail containing protease inhibitors pepstatin A, 
chymotrypsin, aprotinin and leupeptin (Boehringer Mannheim) at  
0.1 μg/ml and calf liver RNA (Sigma) at 40 μg/ml to minimize  
proteolysis and reduce background translation. Rabbit skeletal muscle  
crude membranes, tris, T-tube system and purified dihydropyridine  
receptor12 were electrophoretically separated on 3–12% SDS polya 

crylamide gels in the presence of 1% 2-mercaptoethanol and transferred  

FIG. 2. Identification of the β-subunit interaction site on the α1 subunit  
by epitope cloning. a, Autoradiogram of an amplified single positive  
plaque isolated by screening a skeletal muscle DHPR α1 subunit epi 

tope library with an in vitro translated [35S]α1 subunit probe. The nitro 
cellulose lift was cut and each half was incubated with either the  
[35S]β12 subunit or [35S]α12 subunit probe. b, Coomassie blue 

stained SDS–polyacrylamide gel of glutathione-S-transferase (GST) control and  
α1, α1, α, and α1α epitopes expressed as GST fusion proteins  
in total E. coli lysate (left), and autoradiogram of corresponding overlay 
with [35S]β12 subunit on these nitrocellulose-immobilized fusion pro 

teins (right). c, Alignment of amino-acid sequences of these fusion pro 

tein epitopes identifying a conserved α1β 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 
gthen cDNA. d, Schematic showing the β-subunit interaction site on the  
I- and cytoplasmic linker of the α1 subunit. 

METHODS. α1-Subunit epitope libraries in the λgt11 vector were made by digesting the α1-subunit cDNA in a plasmid vector with DMase I in the presence of 10 mM MnCl2. Following the addition of Ecoli linkers, the randomly digested fragments were size-selected to ~500 bp in Russove agarose gel (FMIC Bioproducts). These fragments were then  
digested with EcoRI and ligated into λgt11. The [35S]β12 subunit  
probe was used to screen 2 × 106 clones of each α1-subunit epitope  
library. Inserts were amplified from pure plasmid positivity by polymerase  
chain reaction (PCR) using primers directed to λgt11 phase arms. These  
were directed subcloned into a T-vector 3 (made from Bluescript SkI 
(plasmid) for sequencing, or digested with EcoRI and ligated into the  
pGEKX1 vector for GST fusion protein production. A fusion protein epitope  
of the α1 subunit was constructed by amplifying phase pairs 1,387–  
1,553 with the following primers: 5'-AGGGAATTCAGAAGGATGCTGCTGCG3'  
and 5'-AGGAATCCGCTGGTGAGAAC3'. This PCR product was then  
digested with BamHI and EcoRI and subcloned into the pGEK2X vector.  
All inserts were sequenced in both directions by the dye deoxy chain  
termination method using Sequenase II (US Biochemicals). Each recom 
binant pGEK molecule was introduced into E. coli DH5α cells. Overnight  
cultures of the pGEK-epitope constructs were diluted 1:10, incubated  
for 1 h and induced for 2 h with 1 μM isopropyl β-D-thiogalactopyrano 
side, 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 network service.
amino acids 369 to 418 of the rabbit neuronal a1 subunit, which spans the region common to all cloned epitopes. As shown in Fig. 2b, the β subunit binds to the epitopes in all four a1 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 a1 subunits shows only 19% overall identity.

Sequence comparison of the epitopes (Fig. 2c) suggests the presence of an interaction motif for each a1 subunit that can be minimally described by QQ–E–L–GY–W–I–E. A BLAST search with this motif identified sequence records representing all voltage-sensitive calcium channel a1 subunits so far reported. Although the lengths of the cytoplasmic linkers separating repeats I and II among these four a1 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 a1C 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 I8 transmembrane segment and a 25-amino-acid insertion positioned 19 residues downstream of the motif in the a1C cloned from lung. Three additional splice variations of this region in a1C have been reported at a location immediately downstream of the latter insertion.

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 nonconservative mutations in the GST fusion protein (FP) epitope of the a1 subunit: glutamate at position 4 of the motif to serine (E386S), leucine at position 7 to histidine (L389H), and tyrosine at position 10 to serine (Y392S) or glutamate at position 18 to alanine (E400A). The ability of the mutants to interact with

![FIG. 4 Mutagenesis of the β subunit interaction site on the a1 subunit alters the effects of a1–fl subunit coexpression. a, Barium currents of a1a (WILD) or mutated a1a subunits (E386S, L389H, Y392S, and E400A) coexpressed with a1a and β subunits in Xenopus oocytes. Left panel shows superimposed representative traces of Ba2+ currents evoked from a holding potential of −90 mV to +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 a1a 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 a1a (WILD) or mutated a1a subunits (Y392S) to show shift in peak currents induced by the Y392S point mutation. Smooth curves were generated assuming an activation curve of a Boltzmann function f(θ) = [1 + exp(−(TP – θ)/k)]/1 + exp(−(TP – θ)/k) with g, the maximum normalized conductance (gmax = 0.018 and gmax = 0.024), θ, the reversal potential (Erev = 56 mV and Erev = 63 mV), and k, the potential of half-activation (V1/2rev = −6.4 mV and V1/2rev = −7.5 mV) and k, the range of potential for an e-fold change around V1/2 (V1/2WILD = 4.6 mV and V1/2Y392S = 6.7 mV). METHODS. To mutate the full-length a1a subunit, pSCP-B2 (ref. 12), base pairs 1,282–1,745 were amplified by PCR and cloned into a Tet vector made from Bluescript SK. Mutagenic primers and a selection primer, 5′-CATGTCAATCATGTCATGACTATAC-3′, which eliminates the HindIII site on the recombinant molecule, were used. The 1,416–1,723 BsmI fragment encoding the motif in a1a was then replaced with mutated BsmI fragments from the Tet 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 a1a cDNA or a1a, mutants E386S, L389H, Y392S, and E400A, and T7 RNA polymerase with rat brain a2β and β plasmids. Wild-type or mutant a1a subunits were co-injected with a2β and β into stage V or VI Xenopus oocytes at concentrations: 0.6 μg μl−1 a1a or mutants, 0.4 μg μl−1 a2β and 0.2 μg μl−1 β; about 50 μl was injected per cell. Ba2+ currents were recorded by a standard two-microelectrode voltage-clamp using a Dagan amplifier (T4200). Voltage and current electrodes (0.5–2.0 M tip resistance) were filled with 3 M KC). The bath solution was in mM): Na2[OH]2; 40; NaCl, 50; KCl, 2; niflumic acid, 1; EGTA, 0.1; HEPES, 5; pH 7.4, adjusted with methanesulfonic 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. Ba2+ current through endogenous channels was less than 10 nA (no injection) and average Ba2+ current was 40 nA upon injection of a2β and a2β cRNAs.
the β-subunit interaction was determined by the overlay assay described above. The β-subunit interaction was reduced with the E386S Δβ, L389HΔβ and Y392SΔβ mutants compared with the wild-type epitope (Fig. 3a, b). This reduction was greatest with the Y392SΔβ mutation, which required prolonged exposure of the film for visualization. No reduction in intensity was observed with E400AΔβ. Densitometric scans of autoradiograms band intensities normalized for protein concentration and averaged over three experiments measured a 27-fold reduction in β-subunit binding to Y392SΔβ compared with the wild-type epitope. Binding to mutants E386SΔβ and L389HΔβ were both reduced 1.7-fold, whereas a 1.3-fold enhancement was measured with E400AΔβ.

Coexpression of αtΔβ with a β-subunit dramatically increases the functional expression14 and alters the voltage-dependent and kinetic properties of the channel in Xenopus oocytes (not shown). Alteration of the β-subunit binding motif on αtΔβ should therefore alter the modulatory contribution by the β-subunit. A deletion of 30 amino acids that includes the interaction motif (αtΔβ377-406) or a smaller 16-amino-acid deletion (αtΔβ377-393) of part of the motif had the result that no detectable current (<10 nA) was observed on coexpression with the β-subunit in Xenoopus 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 αtΔβ-subunit. Coexpression of E386SΔαt, L389HΔαt, or Y392SΔαt, with α2βt and β1βt-subunits resulted in significantly reduced peak currents compared with the wild-type αtΔβ-subunit (Fig. 4a). Average reductions in current expression were: E386SΔαt, 36-fold; L389HΔαt, 22-fold; Y392SΔαt, 9-fold; E400AΔαt, 1.3-fold. Although autoradiogram band intensities of β-subunit overlap were also reduced for mutations E386S, L389H and Y392S, no quantitative correlations were attempted because of the vastly different conditions of these assays. Mutant Y392SΔαt altered the inactivation kinetics of the channel (Fig. 4b). At test pulses to +10 mV, Ba2+ current inactivated with similar average time constants of (mean ± s.d.) τ = 350 ± 59 ms (wild-type αtΔβ, n = 7); τ = 290 ± 69 ms (E386SΔαt, n = 9); τ = 434 ± 158 ms (L389HΔαt, n = 8) and τ = 522 ± 119 ms (E400AΔαt, n = 7). However, Ba2+ current conducted by Y392SΔαt consistently inactivated along two components, τ1 = 22 ± 4 ms (15% of the total current) and τ2 = 1,806 ± 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 Y392SΔαt, which peaked at +20 mV. More precise estimates of Y392SΔαt 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 αtΔββt complexes, this was not due to a complete loss of αtββ interaction. βtβt-subunit still overlaid on the mutant epitope (Fig. 3b) and also, peak current amplitudes with Y392SΔαtβt were enhanced from (mean ± s.e.m.) -10 ± 4 nA (n = 4) to -613 ± 137 nA (n = 8) by βtβt-subunit coexpression. Ba2+ currents obtained in the absence of the βtβt-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 αt-subunits. For example, when the αtΔβ-subunit is coexpressed with the β-subunit, shifts in voltage-dependence of activation and inactivation occur, but no appreciable stimulation of current expression is observed15. Sequence variations of the motif in the carp skeletal muscle αt-subunit16 in position 4 (E→D) and in an αt subunit (doe-4)17 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 Ca2+ channel αt-subunit alter the ability of the αt-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 αt-subunits14. These findings suggest a critical role for the conserved interaction site on the I-F cytoplasmic linker of the αt-subunit in voltage-sensitive calcium-channel modulation by the β-subunit.

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