troponin T or other extended parts of the Limulus troponin complex might also contribute to the strand density. By localizing Limulus troponinomyosin (and possibly parts of troponin), in both the ‘off’ and ‘on’ states, to positions on actin originally proposed in the seminal steric model and again more recently on the basis of further modelling, we offer direct structural support for the hypothesis. Earlier reconstructions of thin filaments reconstituted from vertebrate proteins either failed to resolve troponinomyosin from actin, or revealed it only in the ‘on’ state, perhaps because in the vertebrate ‘off’ state troponinomyosin is disordered (R. A. Milligan, personal communication).

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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 35S-labelled in vitro-translated rat β1-subunit protein probe (Fig. 1a) was used to demonstrate an interaction between the β- and α15-subunits of the dihydropridine receptor (DHPR) from skeletal muscle. A single prominent radioactive band corresponding to a relative molecular mass of 170,000 (M, 170K) was detected in the purified DHPR, which colocalized with immuno-stained α15-subunit (Fig. 1b). On prolonged exposure, β-subunit interaction with the DHPR α15-subunit was also detected in triads and T-tubule membranes. Incubations with 35S-labelled sham in vitro translates showed no interaction (not shown).

In the in vitro-translated β1-subunit of DHPR and the β-subunit of the α-conotoxin GVIA receptor (CgTxR) 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 α15-subunit, we screened an epitope library of the rabbit DHPR α15-subunit with the 35S-labelled β1-subunit probe. Figure 2a shows an autoradiogram of a limited purified positive clone amplified and probed with the translated β1-subunit. Similar results were obtained with a β1-probe (not shown), whereas an overlay of this α15-positive clone with a 35S-labelled α2-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 43-amino-acid overlap that extends from amino acid 341 to 385 of the α15-subunit.

Analysis of the transmembrane topology of the α15-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 (Fig. 2d).

Because coexpression of β-subunits with various α1-subunits greatly enhances peak inward currents and alters kinetic and voltage-dependent properties, we addressed whether this interaction site is conserved in distantly related calcium channel α1-subunits. We constructed epitope libraries of the complemen-
tary DNA encoding the rabbit cardiac dihydropyridine-sensitive $\alpha_{1C}$-subunit and the rat neuronal $\omega$-conotoxin GV1A-sensitive $\alpha_{1B}$-subunit. 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 $\alpha_{1S}$. Positive clones were again detected with $^{35}$S-labelled $\beta_{1S}$-subunit probe and found to map to the same location on the I–II cytoplasmic linker of each $\alpha_{1}$-subunit. The smallest of two overlapping clones from the $\alpha_{1C}$-Lgt11 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 $\alpha_{1B}$ library yielded a 56-amino-acid domain representing amino acids 378 to 434. To extend these observations to the $\alpha_{1N}$-subunit, which shares 33% identity with DHPR $\alpha_{1S}$, we constructed a glutathione-S-transferase (GST) fusion protein expressing 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 $\mu$l 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. 1.** $\beta$-Subunit interaction with the $\alpha_{1S}$-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 $\beta_{1S}$-subunit migrates differently from the $\beta_{1S}$, of DHPR because the primary structure of the $\beta_{1S}$ splice variant predicts it to have an M. 7.8 K larger than $\beta_{1S}$. b, CB, Coomassie blue-stained SDS–polyacrylamide gel of rabbit skeletal muscle crude membranes (Mic), triads (Tri), T-tubule system (TS) and purified dihydropyridine receptor (DHPR), $\alpha_{1S}$, mAb, Corresponding immunoblot stained with a mix of monoclonal antibodies (mAbs IIF7, IIC2 and IIIID5; ref. 15) to $\alpha_{1S}$ of DHPR. [H]35β, Autoradiogram of an identical nitrocellulose transfer incubated with in vitro-translated [H]35β- probe. Arrowhead indicates the 170K protein, $\alpha_{1S}$. Methods. The [H]35β- subunit probe was synthesized by coupled in vitro transcription and translation in the TNT system (Promega). To enhance probe yield, a 50-nucleotide alafafa 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 $\mu$g ml$^{-1}$ and calf liver tRNA (Sigma) at 40 $\mu$g ml$^{-1}$ to minimize proteolysis and reduce background translation. Rabbit skeletal muscle crude membranes, triads, T-tubule system and purified dihydropyridine receptor 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 $\mu$l 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 $\beta$-subunit interaction site on the $\alpha_{1S}$-subunit by epitope cloning. a, Autoradiogram of an amplified single positive plaque isolated by screening a skeletal muscle DHPR $\alpha_{1S}$ subunit epitope library with an in vitro-translated [H]35β- subunit probe. The nitrocellulose lift was cut and each half was incubated with either the [H]35β- subunit or a [H]35β- subunit probe. b, Coomassie blue-stained SDS–polyacrylamide gel of glutathione-S-transferase (GST) control and $\alpha_{1S}$, $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1C}$-epitopes expressed as GST fusion proteins in total E. coli lysate (left), and autoradiogram of corresponding overlay with [H]35β- subunit on these nitrocellulose-immobilized fusion proteins (right). c, Alignment of amino-acid sequences of these fusion protein epitopes identifying a conserved $\alpha_{1S}$-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 $\beta$-subunit interaction site on the I–II cytoplasmic linker of the $\alpha_{1S}$-subunit. METHODS. $\alpha_{1}$-Subunit epitope libraries in the Lgt11 vector were made by digesting the $\alpha_{1}$-subunit cDNA in a plasmid vector with DNase I in the presence of 10 $\mu$M MnCl$\text{2}$. Following the addition of EcoRI linkers, the randomly digested fragments were size-selected to <500 bp in Nuasea agarose gel (FMC Bioproducts). These fragments were then purified, digested with EcoRI and ligated into Lgt11. The $\beta_{1S}$-subunit probe was used to screen 2 $\times$ 10$^{5}$ clones of each $\alpha_{1}$-subunit epitope library. Inserts were amplified from pure phage positives by polymerase chain reaction (PCR) using primers directed to Lgt11 phage arms. These were directly subcloned into a T-vector (made from Bluescript SK plasmid) for sequencing, or digested with EcoRI and ligated into the pGEX1 vector for GST fusion protein production. A fusion protein epitope of the $\alpha_{1S}$-subunit was constructed by amplifying base pairs 1,387–1,553 with the following primers: 5′-AGGGAGATTCAAGGGAATGTGTGGGTGAC-3′ and 5′-AACGGATCTCGAGGCGGTTGGAACAC-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 dyeoxy chain termination method using Sequenase II (US Biochemicals). Each recombinant pGEX molecule was introduced into E. coli DHBa cells. Overnight cultures of the pGEX-epitope constructs were diluted 1:10, incubated for 1 h and induced for 2 h with 1 $\mu$m isopropyl-$\beta$-D-thiogalactopyranoside. 75 $\mu$1 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 α1A-subunit12, which spans the region common to all cloned epitopes. As shown in Fig. 2b, the β10-subunit binds to the epitopes in all four α1- 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 α1-subunits shows only 19% overall identity.

Sequence comparison of the epitopes (Fig. 2c) suggests the presence of an interaction motif on each α1-subunit that can be minimally described by QQ--E--L--G--Y--W--I--E. A BLAST search with this motif identified sequence records representing all voltage-sensitive calcium channel α1-subunits so far reported. Although the lengths of the cytoplasmic linkers separating repeats I and II among these four α1-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 α1C 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 I6S transmembrane segment and a 25-amino-acid insertion positioned 19 residues downstream of the motif in the α1C cloned from lung19. Three additional splice variations of this region in α1C 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 α1Aβ1C-subunit: glutamate at position 4 of the motif to serine (E386SFP), leucine at position 7 to histidine (L389HFP), tyrosine at position 10 to serine (Y392SFP) or glutamate at position 18 to alanine (E400A FP). The ability of the mutants to interact with

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**FIG. 4** Mutagenesis of the β-subunit interaction site on the α1A subunit alters the effects of α1β subunit coexpression. a, Barium currents of α1A(WILD) or mutated α1A-subunits (E386S, L389H, Y392S and E400A) coexpressed with α2β and β2β-subunits in Xenopus oocytes. Left panel shows superimposed representative traces of Ba2+ currents evoked from 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 α1A 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 α1A(WILD) or mutated α1A-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 type f∞ = [g(−TP–E)]/(1 + exp(−(TP–V1/2))/k) and k, the normalized maximum conductance (g∞ = 0.018 and g∞392S = 0.024). E, the reversal potential (E∞ = 66 mV and E∞392S = 68 mV), and V1/2, the potential of half-activation (V1/2E∞ = −6.4 mV and V1/2E∞392S = −7.6 mV) and k, the range of potential for an e-fold change around V1/2 (k∞ = 4.6 mV and k∞392S = 6.7 mV).

METHODS. To mutate the full-length α1A β-subunit, pSPCB-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′-CATGTTCACTGCTATCAGTAC-3′, which eliminates the HindIII site on the recombinant molecule, were used. The 1,416–1,723 BsmI fragment encoding the motif in α1A 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 BsmI 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 α1A plasmid or α1A mutants E386S, L389H, Y392S and E400A, and T7 RNA polymerase with rat brain α2β and β1β plasmids. Wild-type or mutant α1A subunits were co-injected with α2β and β1β into stage V or VI Xenopus oocytes at concentrations: 0.6 µg µl−1 α1A or mutants, 0.4 µg µl−1 α2β and 0.2 µg µl−1 β1β; about 50 nl was injected per cell. Ba2+ 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 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 β10β and α2β cRNAs.
the β-subunit was determined by the overlay assay described above. The β-subunit interaction was reduced with the E386SβPP, L389HβP, and Y392SβP mutants compared with the wild-type epitope (Fig. 3a, b). This reduction was greatest with the Y392SβP mutation, which required prolonged exposure of the film for visualization. No reduction in intensity was observed with E400AβP. 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 Y392SβP compared with the wild-type epitope. Binding to mutants E386SβP and L389HβP were both reduced 1.7-fold, whereas a 1.3-fold enhancement was measured with E400AβP.

Coexpression of α1A with a β-subunit dramatically increases the functional expression12 and alters the voltage-dependent and kinetic properties of the channel in Xenopus oocytes (not shown). Alteration of the β-subunit binding motif on α1A should therefore alter the modulatory contribution by the β-subunit. A deletion of 30 amino acids that includes the interaction motif (a1A377-406) or a smaller 16-amino-acid deletion (a1A377-393) of part of the motif had the result that no detectable current (<10 nA) was observed on coexpression with the β3α2 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 a1Aβ4 subunit. Coexpression of E386Sα1A, L389Hα1A or Y392Sα1A with α1Sβ and β1Hβ-subunits resulted in significantly reduced peak currents compared with the wild-type α1A-subunit (Fig. 4a). Average reductions in current expression were: E386Sα1A, 36-fold; L389Hα1A, 22-fold; Y392Sα1A, 9-fold; E400Aα1A, 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 Y392Sα1A 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 α1A, n = 7), τ = 290 ± 69 ms (E386Sα1A, n = 9), τ = 434 ± 158 ms (L389Hα1A, n = 8) and τ = 522 ± 119 ms (E400Aα1A, n = 7). However, Ba2+ current conducted by Y392Sα1A 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 Y392Sα1A, which peaked at +20 mV. More precise estimates of Y392Sα1A 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 α1Aβ2β3H3 complex, this was not due to a complete loss of α1β-subunit interaction. β1Hβ-subunit still overlay on the mutant epitope (Fig. 3b) and also, peak current amplitudes with Y392Sα1Aα1H were enhanced from (mean ± s.e.m.) −10 ± 4 nA (n = 4) to −613 ± 137 nA (n = 8) by β1Hβ-subunit coexpression. Ba2+ currents obtained in the absence of the β1Hβ-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 α1-subunits. For example, when the α1H-subunit is coexpressed with the β-subunit, shifts in voltage-dependence of activation and inactivation occur, but no appreciable stimulation of current expression is observed12. Sequence variations of the motif in the carp skeletal muscle α1-subunit13 in position 4 (E→D) and in an α1-subunit (doc-e4)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 Ca2+ channel α1-subunit alter the ability of the α1-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 α1-subunits12. These findings suggest a critical role for the conserved interaction site on the I-II cytoplasmic linker of the α1-subunit in voltage-sensitive calcium-channel modulation by the β-subunit.

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A 13-amino-acid motif in the cytoplasmic domain of FcγRIIB modulates B-cell receptor signalling

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The Fc receptor on B lymphocytes, FcγRIIB (β1 isofrom), helps to modulate B-cell activation triggered by the surface immunoglobulin complex1–3. Crosslinking of membrane immunoglobulin by antigen or anti-Ig F(ab')2 antibody induces a transient increase in cytosolic free Ca2+, a rise in inositol-3-phosphate, activation of protein kinase C, and enhanced protein tyrosine phosphorylation4–10. Crosslinking FcγRIIB with the surface immunoglobulin complex confers a dominant signal that prevents or aborts lymphocyte activation triggered through the ARH-1 motifs of the signal transduction subunits Ig-α and Ig-β. Here we show that FcγRIIB modulates membrane immunoglobulin-induced Ca2+ mobilization by inhibiting Ca2+ influx, without changing the pattern of tyrosine phosphorylation. A 13-amino-acid motif in the cytoplasmic domain of FcγRIIB is both necessary and sufficient for this effect. Tyrosine

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