Absence of the γ Subunit of the Skeletal Muscle Dihydropyridine Receptor Increases L-type Ca\(^{2+}\) Currents and Alters Channel Inactivation Properties

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In skeletal muscle the oligomeric α\(_{1S}\), α/δ-1 or α/δ-2, β1, and γ L-type Ca\(^{2+}\) channel or dihydropyridine receptor functions as a voltage sensor for excitation contraction coupling and is responsible for the L-type Ca\(^{2+}\) current. The γ subunit, which is tightly associated with this Ca\(^{2+}\) channel, is a membrane-spanning protein exclusively expressed in skeletal muscle. Previously, heterologous expression studies revealed that γ1 might modulate Ca\(^{2+}\) currents expressed by the pore subunit found in heart, α\(_{1C}\), shifting steady state inactivation, and increasing current amplitude. To determine the role of γ1 assembled with the skeletal subunit composition in vivo, we used gene targeting to establish a mouse model, in which γ1 expression is eliminated. Comparing litter-matched mice with control mice, we found that, in contrast to heterologous expression studies, the loss of γ1 significantly increased the amplitude of peak dihydropyridine-sensitive I\(_{Ca}\) in isolated myotubes. Whereas the activation kinetics of the current remained unchanged, inactivation of the current was slowed in γ1-deficient myotubes and, correspondingly, steady state inactivation of I\(_{Ca}\) was shifted to more positive membrane potentials. These results indicate that γ1 decreases the amount of Ca\(^{2+}\) entry during stimulation of skeletal muscle.

High voltage activated Ca\(^{2+}\) channels are oligomeric protein complexes composed of the ion conducting α\(_1\) protein that is tightly associated with the auxiliary subunits α/δ, β, and γ. Each of the subunits is encoded by a separate gene, selected from seven α\(_1\), three α/δ, four β, and two γ genes, some of which exist as splice variants. Various α\(_1\) proteins define different types of Ca\(^{2+}\) channels, which differ in current properties, pharmacology, and G-protein-dependent modulation. In skeletal muscle, the high voltage activated L-type Ca\(^{2+}\) channel or dihydropyridine receptor functions as voltage sensor for excitation contraction coupling and is responsible for the L-type Ca\(^{2+}\) current present in this tissue (1, 2). The purified skeletal muscle L-type Ca\(^{2+}\) channel is a heterooligomeric complex of the α\(_{1C}\), β1, α/δ-1, or α/δ-2 and γ subunits (3–5). The γ subunit is a membrane-spanning protein (6, 7) encoded by a single-copy gene consisting of four translated exons (8, 9). A similar organization has been described for the gene of a novel γ subunit, γ2 or stargazin (10), which is the target of the stargazer mutation in mice. Whereas γ2 mRNA is expressed in adult mouse brain but not in other mouse tissues like heart, kidney, or skeletal muscle (10), the γ1 subunit is exclusively expressed in skeletal muscle (6, 7, 9, 11).

Up to now, functional data on the γ1 subunit were only obtained in expression systems. Coexpression of γ1 with α\(_{1S}\), β1, and α/δ-1 in Xenopus oocytes (12) and L-cells (13) did not reveal significant effects on Ca\(^{2+}\) currents. Due to low expression of the α\(_{1S}\) skeletal muscle Ca\(^{2+}\) channel subunit (12, 14, 15), the role of γ1 has been studied by heterologous coexpression with the cardiac α\(_{1C}\) subunit in Xenopus oocytes (16, 17) and human embryonic kidney (293) cells (11, 18). In these studies, coexpression of the γ1 subunit shifted the steady state inactivation of α\(_{1C}\)-induced I\(_{Ca}\) to more negative membrane potentials, accelerated current activation and inactivation, and, in one study (17), increased peak currents. Considering the discrepancies obtained through coexpression of γ1 with either α\(_{1C}\) or α\(_{1S}\) and the considerable structural and functional diversity of α\(_{1S}\) and α\(_{1C}\) (1, 19), it does not appear feasible to simply transfer the results obtained with α\(_{1C}\) to the “correct” subunit α\(_{1S}\).

To determine directly the role of the γ1 subunit in L-type channel activity in skeletal muscle in vivo, we therefore used gene targeting to inactivate the γ1 gene. In the absence of the γ1 subunit, peak Ca\(^{2+}\) current amplitudes in skeletal muscle myotubes were increased and the steady state inactivation was shifted to more positive potentials. These results demonstrate that a specific modulatory function of the skeletal muscle L-type Ca\(^{2+}\) channel can be assigned to the γ1 subunit, which contributes to tissue-specific differences between high voltage activated L-type Ca\(^{2+}\) channels.

**Experimental Procedures**

**Gene Targeting and Generation of γ1 Mutant Mice**—γ1 DNA was isolated from a 129 SvJ murine genomic library (Genome Systems, St. Louis, MO), and the structure of the murine γ1 gene has been characterized (9). The replacement type targeting vector was constructed as follows. The multiple cloning site of the pBluescript-SK vector (Stratagene) was replaced by the multiple cloning sites 5′-CCG CCA ATC GAT TTG GAT CCA ACT CGA GAT TGG CCG TCG TGG TGC GGG CCC AGC CGG GGA CGG CCG 3′ (plasmid pCON1) and 5′-CGC GCA AGC GGC CGC AAG TGG CTA CCT CTA CCT GAT ACC TGA AGC ACC TCG GAG GAG GTC CGG 3′ (plasmid pCON2). To obtain the vector pKO3, the 5.1-kb BamHI/NcoI fragment, representing the 5′ non-translated region of the γ1 gene and nucleotides 1–73 encoding the

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1 The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); S, siemen(s); F, farad(s); DMEM, Dulbecco’s modified Eagle’s medium; ES, embryonic stem.
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initial 24 amino acid residues of \(\gamma 1\), and the 1.8-kb \(SalI/Notl\) fragment from \(pGEM7\) (Kijj) (20) comprising the neomycin resistance gene, were subcloned in \(pCON1\). The 3.75-kb \(SalI/AscI\) fragment containing intron sequence 3′ of exon 1 of the \(\gamma 1\) gene and the herpes simplex thymidine kinase cassette from \(pNTK\) (21) were subcloned in \(pCON2\) to yield \(pCON\); \(pCON\) was linearized with EcoRI and treated with Klenow. Defective virus vector phage lambda vector DNA (BRL) was cotransfected with \(pCON\); the phage DNA was linearized by restriction endonuclease \(SalI\); 5.9-kb \(NotI/SalI\) fragment from \(pKO\) into the \(pKO\) vector 3′ of the neomycin resistance gene. About 1.1 × 10\(^{10}\) R1 embryonic stem (ES) cells (22) were electroporated with \(CiaI\)-linearized pKO\(_Y\) (25 µg; setting of 800 V and 3 µF, Bio-Rad Gene Pulser) and plated on irradiated G418-resistant embryonic feeder cells (23). Recombinant clones were selected with G418 (0.3 mg/ml) and 1-2-day 2-fuguransol-disulfuryl-3,5-dioiouracil (0.2 µM), and 11 out of 334 double-resistant colonies showed correct homologous recombination with the targeting vector as confirmed by screening with both the 5′- and 3′-probes. Germline chimeras were obtained by injecting mutant ES cells of the clone 212 into C57Bl/6 blastocysts and transferring the blastocysts into the uteri of pseudopregnant recipients. Chimeric mice were mated with C57Bl/6 females to test germline transmission or with 129/SvJ females to obtain inbred lines carrying the mutated \(\gamma 1\) gene.

**Genotyping of ES Cells and Mice—**For Southern blot analysis genomic DNA from ES cells or mouse tails (24) were digested with EcoRV or \(SalI\), respectively, electrophoresed on a 0.6% agarose gel, and transferred to a nylon membrane (Hybond-N). Hybridizations were performed with \(9\)− and \(3\)-probes. Germ-line chimeras were obtained that transmitted the mutant allele to their progeny. These heterozygous F1 offspring were intercrossed to produce a stable homozygous \(\gamma 1\)−/− litter size was determined using the BCA method (Pierce).

**RESULTS**

**Targeted Disruption of the Skeletal Muscle Calcium Channel \(\gamma 1\) Subunit Gene—**One allele of the \(\gamma 1\) gene was mutated by replacing the \(3′\)-part of its first translational exon and the adjacent splice donor site (Fig. 1A) with the neomycin resistance gene (neo) in murine embryonic stem cells. Chimeric males were obtained that transmitted the mutant allele to their progeny. These heterozygous F1 offspring were intercrossed to produce F2 homozygous \(\gamma 1\)−/− mice, as confirmed by Southern blot analysis of tail DNA (Fig. 1B). The mutation was trans-
FIG. 1. Targeted disruption of the Ca\(^{2+}\) channel \(\gamma 1\) gene. \(A\), depicted are the first translated exon (filled box, exon 1) of the murine \(\gamma 1\) gene and the adjacent intron (lines) including the positions of recognition sites for relevant restriction enzymes for the wild-type allele (top), the targeting vector, and the targeted allele (bottom). A 420-bp NcoISstI (N/S) fragment, containing part of exon 1 and of the following intron was replaced by the neomycin resistance cassette (neo'). The position of the probes (closed bars) used for Southern blot analysis and expected sizes of hybridizing fragments (arrows) after SstI (St) and EcoRV (E) digests of genomic DNA are shown. Abbreviations: A, ApaI; B, BamHI; C, ClaI; E, EcoRV; N, NcoI; S, SstI; St, StI; th, thymidine kinase cassette. \(B\), identification of \(\gamma 1^+/+, \gamma 1^-/-\), and \(\gamma 1^-/-\) mice by Southern blot analysis. EcoRV fragments obtained from wild-type (10.0 kb) and targeted alleles (8.2 kb) were detected by the 5' probe. Using the 3' probe SstI fragments of 6.5 and 5.2 kb representing wild-type and mutated alleles, respectively, were detected. Absence of additional random integrations of the targeting construct was checked by hybridization with a CDNA probe representing a 634-bp fragment of the neo' cassette (data not shown). \(C\), Northern blot analysis of polyA\(^+\) RNA (9 \(\mu\)g), extracted from skeletal muscle of \(\gamma 1^+/+, \gamma 1^-/-\), and \(\gamma 1^-/-\) mice. The blot was hybridized with the nucleotide 236–672 cDNA fragment encoding the translated exons 2, 3, and 4 of the murine \(\gamma 1\); lower panel, hybridization of the same filter with a human glycerol-3-phosphate dehydrogenase CDNA probe. \(D\), immunoblot analysis of \(\gamma 1\) expression in skeletal muscle microsomes (70 \(\mu\)g) from \(\gamma 1^+/+, \gamma 1^-/-\), and \(\gamma 1^-/-\) mice. 

mitted at Mendelian ratio, suggesting normal fetal and embryonic development of homozygous mutant mice. \(\gamma 1^-/-\) mice grew and reproduced normally and were indistinguishable from their wild-type littermates. The deletion of the \(\gamma 1\) gene and the lack of expression was confirmed by Northern and Western blot analysis (Fig. 1, \(C\) and \(D\)). No transcripts could be detected in polyA\(^+\) RNA prepared from \(\gamma 1^-/-\) skeletal muscle using as probes either the mouse \(\gamma 1\) CDNA covering the translated exons 2, 3, and 4 (Fig. 1C) or the 160-bp fragment of exon 1 which has been replaced by neo' (data not shown). Using polymerase chain reactions, \(\gamma 1\) transcripts encoding the complete \(\gamma 1\) protein could be amplified from \(\gamma 1^+/+\) and \(\gamma 1^-/-\) polyA\(^+\) RNA but not from polyA\(^+\) RNA isolated from \(\gamma 1^-/-\) skeletal muscle. In addition, the DNA fragments encoding exon 2 or exons 3 and 4 could be amplified from \(\gamma 1^-/+\) and \(\gamma 1^-/-\) polyA\(^+\) RNA but not from polyA\(^+\) RNA (data not shown), indicating that the replacement of part of exon 1 and of the adjacent intron completely prevents expression of \(\gamma 1\) specific transcripts. Accordingly, the \(\gamma 1\) protein (–32 kDa) is recognized by a \(\gamma 1\) subunit-specific polyclonal antibody (9) in skeletal muscle microsomes prepared from \(\gamma 1^-/+\) mice and \(\gamma 1^-/-\) mice but not in microsomes prepared from \(\gamma 1^-/-\) mice (Fig. 1D). No transcripts of the neuronal \(\gamma 2\) subunit (10) could be detected in skeletal muscle RNA from wild-type or \(\gamma 1\)-deficient mice.

Functional Characterization of Calcium Channels in \(\gamma 1\)-deficient Myotubes—To study the function of the \(\gamma 1\) subunit in skeletal muscle, primary cultures were obtained from fore- and hindlimb muscles of 1–3-day-old wild-type and \(\gamma 1^-/-\) litter-matched mice. Both \(\gamma 1^+/+\) and \(\gamma 1^-/-\) myotubes exhibited spontaneous contractions after day 4 in culture and were used for electrophysiological recordings up to 9 days. Fig. 2 shows representative recordings of Ca\(^{2+}\) channel activity at different test potentials from \(\gamma 1^+/+\) and \(\gamma 1^-/-\) myotubes of litter-matched mice. At positive test potentials, a large sustained L-type inward Ca\(^{2+}\) current was observed in \(\gamma 1^+/+\) and \(\gamma 1^-/-\) cells (Fig. 2A). In all cells, the activation threshold was around –20 mV and the peak amplitude of the current was at +20 mV (Fig. 2B). In addition to L-type currents, additional transient Ca\(^{2+}\) currents at test potential > –60 mV (present at –20 mV in Fig. 2A, right panel) were observed representing T-type Ca\(^{2+}\) channels (27). This channel type was not consistently observed in all cells but, when present, was not obviously altered by the \(\gamma 1\) subunit deletion (data not shown). The peak whole-cell current through L-type Ca\(^{2+}\) channels was found to be significantly larger in \(\gamma 1^-/-\) cells compared with \(\gamma 1^+/+\) cells, as evident from the representative examples and the averaged
current-voltage (IV) relationship (Fig. 2, A and B). Individual IV curves were fitted with a Boltzmann equation, resulting in a significantly different whole-cell conductance \( g \) of 0.45 \( \pm \) 0.07 nS/pF for \( \gamma 1 / - / - \) cells compared with 0.37 \( \pm \) 0.05 nS/pF for \( \gamma 1 / + / + \) cells (\( n = 11 \), \( p < 0.05 \)). This effect is not due to a difference of cell cultivation length, as \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) myotubes were prepared on the same day and measured in a paired fashion. Furthermore, expression of L-type Ca\(^{2+} \) currents did not vary between days 5 and 9 in \( \gamma 1 / - / - / - \) mutant mice (data not shown). To look for differences between \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) myotubes concerning selectivity, gating properties, and activation kinetics of L-type Ca\(^{2+} \) channels, we analyzed the experiments in more detail. The apparent reversal potential \( E_{\text{rev}} \) (65.7 \( \pm \) 2.2 mV for \( \gamma 1 / + / + \) and 66.3 \( \pm \) 1.8 mV for \( \gamma 1 / - / - \)) as well as the half-maximal voltage for activation \( V_{1/2} \) (8 \( \pm \) 1 mV for \( \gamma 1 / + / + \) and 7 \( \pm \) 1 mV for \( \gamma 1 / - / - \)) and the slope factor for activation \( k \) (5.8 \( \pm \) 0.4 mV for \( \gamma 1 / + / + \) and 6.2 \( \pm \) 0.3 mV for \( \gamma 1 / - / - \)) remained unchanged. Activation kinetics of the L-type Ca\(^{2+} \) current were also not altered by the elimination of the \( \gamma 1 \) subunit as shown in Fig. 2C. Depicted are averaged (\( n = 11 \) for \( \gamma 1 / + / + \) and \( n = 13 \) for \( \gamma 1 / - / - \), normalized current traces (test pulse to +20 mV) from \( \gamma 1 / - / - \)-deficient and wild-type mice fitted with a double exponential function (time constants of 67.9 and 4.2 ms for \( \gamma 1 / + / + \) and 58.1 and 5.7 ms for \( \gamma 1 / - / - \)). Together, these measurements indicate that Ca\(^{2+} \) selectivity, gating, and activation kinetics of L-type Ca\(^{2+} \) channels in skeletal muscle are not significantly influenced by the \( \gamma 1 \) subunit.

The increased L-type Ca\(^{2+} \) current amplitude can in principle be explained in one of three ways. 1) The single-channel conductance is increased. 2) The open probability is increased. 3) The number of functional Ca\(^{2+} \) channels in the plasma membrane is increased. Because the open probability of skeletal muscle L-type Ca\(^{2+} \) channels is rather low and open times can be extremely short, we used the calcium channel agonist (-)-BayK 8644 to increase open times of the channels. (-)-BayK 8644 (at 1 \( \mu \)M) did increase the peak current amplitude of L-type Ca\(^{2+} \) currents at +20 mV by 64% in both \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) myotubes (\( \gamma 1 / + / + \): from -13.4 \( \pm \) 1.6 to -22.9 \( \pm \) 2.3 pA/pF, \( n = 9 \); \( \gamma 1 / - / - \): from -22.1 \( \pm \) 2.1 to -39.6 \( \pm \) 4.7 pA/pF, \( n = 9 \)), indicating a comparable sensitivity of native and \( \gamma 1 / - / - \)-deficient skeletal calcium channels toward dihydropyridines. The effect of (-)-BayK 8644 was already saturated at this concentration, as higher concentrations (between 3 and 5.6 \( \mu \)M) did not increase the peak current any further in either \( \gamma 1 / + / + \) or \( \gamma 1 / - / - \) myotubes (\( n = 22 \)). Thus, the relative difference of current amplitude of L-type channels between \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) myotubes was not changed by application of (-)-BayK 8644. Therefore, we were able to compare single-channel properties in the presence of (-)-BayK 8644. To measure single-channel conductances, a voltage ramp protocol as shown in Fig. 3A was used. Typical examples of current responses from \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) myotubes with no single-channel activity and with channel activity are overlaid in Fig. 3B. Subtracting blank traces from a trace with channel activity, we could fit a line to the current traces shown in Fig. 3C to get the single-channel conductance of each experiment. On average, there was no significant difference between recordings for \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) cells (\( \gamma 1 / + / + \); 13.0 \( \pm \) 0.8 pS, \( n = 4 \); \( \gamma 1 / - / - \); 12.3 \( \pm \) 0.3 pS, \( n = 3 \)). These values are very similar to the ones (14.5 pS) from a previous study measured under slightly different conditions (30). Next, we tested whether a difference in the amount of \( \alpha_{1S} \) protein in \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) skeletal muscle might be responsible for the difference in current densities by binding studies using the dihydropyridine isradipine as a ligand. However, the densities of isradipine binding sites were not significantly different in \( \gamma 1 / + / + \) and \( \gamma 1 / - / - \) muscles.
with γ1+/+ cells (Fig. 4B). This finding is summarized in Fig. 4C, in which normalized steady state inactivation is plotted as a function of the prepulse potential. The inactivation curve of Ca²⁺ currents from γ1−/− cells is shifted to more positive potentials and is also steeper than that from γ1+/+ cells. This behavior is also reflected in the slope factor \( k \) and half maximal-inactivation potential \( V_{1/2} \), which were found to be significantly different for γ1-deficient and wild-type cells (\( k = 5.7 \) mV, \( V_{1/2} = 0.9 \) mV for γ1+/+ cells; \( k = 10.4 \) mV, \( V_{1/2} = -7.7 \) mV for γ1+/+ cells; \( n = 10 \); \( p < 0.05 \)).

**DISCUSSION**

We used gene targeting to generate mice that lack the γ1 subunit of the multisubunit skeletal muscle L-type Ca²⁺ channel. In γ1-deficient myotubes, (i) the peak amplitudes of L-type Ca²⁺ currents are increased, (ii) the time-dependent inactivation of \( I_{Ca} \) is decelerated, and (iii) the steady state inactivation curve is shifted to more positive potentials. As a result, Ca²⁺ influx through L-type Ca²⁺ channels is increased in γ1-deficient muscle myotubes.

The changes in inactivation properties of \( I_{Ca} \) due to the inactivation of the γ1 gene corresponds to previous results obtained after coexpression of γ1 with the cardiac L-type Ca²⁺ channel complex \( \alpha_{1C}/\beta\) (11, 16, 17, 18). In these studies, the major effects of γ1 were to accelerate current inactivation and to shift the steady state inactivation curve to more negative potentials. The latter effect may be due to the addition of negative charges into the vicinity of the external mouth of the channel. The γ1 subunit is membrane-spanning protein (6, 7), and the extracellular loop between the predicted membrane-spanning segments 1 and 2 is especially rich in negative charges. This loop may be close to the voltage sensing parts of the native α₃B in skeletal muscle but also of the recombinant cardiac α₁C. Interestingly, this negatively charged extracellular loop region is almost completely conserved among the γ1 proteins of various species (9) and corresponds to a similarly charged region of the neuronal γ2 protein (10). Accordingly, in neurons of mutant mice that exhibit a mutation in the γ2 gene, enhanced Ca²⁺ entry through voltage-activated Ca²⁺ channels due to reduced steady state inactivation of Ca²⁺ currents has been implicated (10, 31).

In contrast to the modulatory role of γ1 on current inactivation, the observed increase of peak Ca²⁺ current amplitude in γ1-deficient myotubes has not been anticipated from the results of previous expression studies. In those studies, γ1 did not have a consistent effect on the amplitude of \( I_{Ca} \) through recombinant cardiac L-type Ca²⁺ channels (11, 16, 18) or even increased the measured currents (17). Obviously, on native skeletal muscle Ca²⁺ channel activity, γ1 subunits act as a brake and Ca²⁺ channel activity increases in their absence and leads to enhanced Ca²⁺ influx in response to depolarization. This increased Ca²⁺ entry could increase the store of Ca²⁺ for release from the sarcoplasmic reticulum or slow the Ca²⁺ release process by changing the sensitivity of the ryanodine receptor toward activation (32, 33).

In skeletal muscle, single depolarizations simultaneously activate only a small fraction (~5%) of the L-type Ca²⁺ channels (34), and during single twitches, contraction is not dependent on extracellular Ca²⁺ (35). In contrast, repetitive depolarizations at high frequency substantially increase Ca²⁺ influx through skeletal muscle L-type channels (36–38) and, subsequently, contractile force of muscle fibers may be increased. This Ca²⁺ channel potentiation has been shown to be due to phosphorylation by Ca²⁺/CaM-dependent protein kinase at positive membrane potentials and results in a negative shift in the voltage dependence of channel activation and slowing of channel deactivation (39). The γ1 subunit, which dominates the inactivation process of \( I_{Ca} \), making it faster and more sensitive to voltage, might fine-tune this process.

In summary, deletion of the γ1 subunit of the skeletal muscle L-type Ca²⁺ channel specifically alters channel activity. γ1−/− mice are viable, and their phenotype differ from the phenotype seen in mice with mutations in the α₁B (40, 41) or β subunit (42) of the same multisubunit channel, muscular dysgenic (mdg) and β1−/−. The slow voltage activated L-type Ca²⁺ current is absent or 10–20-fold decreased in mdg mice (43) and β1−/− mice (44), respectively, and homozygous mutants lack excitation-contraction coupling (40, 42). The availability, now, of a viable mutant that lacks the γ1 subunit allows to study the functional impact of γ1 on Ca²⁺ channel activity and excitation-contraction coupling in parallel in embryonic skeletal muscle cells, in isolated muscle fibers and in the adult animal.

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