Excitation–Contraction Coupling

Calcium current modulation by the γ1 subunit depends on alternative splicing of CaV1.1

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The skeletal muscle voltage-gated calcium channel (CaV1.1) primarily functions as a voltage sensor for excitation–contraction coupling. Conversely, its ion-conducting function is modulated by multiple mechanisms within the pore-forming α1S subunit and the auxiliary αδ-1 and γ1 subunits. In particular, developmentally regulated alternative splicing of exon 29, which inserts 19 amino acids in the extracellular IVS3-S4 loop of CaV1.1a, greatly reduces the current density and shifts the voltage dependence of activation to positive potentials outside the physiological range. We generated new HEK293 cell lines stably expressing γ1 subunit to exert its inhibitory action on CaV1.1 calcium currents.

Introduction

Excitation–contraction (EC) coupling in skeletal muscle is initiated by action potentials that activate the voltage-gated calcium channel CaV1.1 located in the transverse tubules (T-tubules). In adult skeletal muscle, CaV1.1 functions as a voltage sensor that triggers the opening of the calcium release channel, the ryanodine receptor (RYR1), in the SR via protein–protein interactions, thus initiating muscle contraction (Rios and Brum, 1987; Schneider and Chandler, 1973). Because of the conformational coupling between CaV1.1 and RYR1, CaV1.1 currents are dispensable for skeletal muscle EC coupling (Armstrong et al., 1972; Dayal et al., 2017). Accordingly, in mammals, CaV1.1 channels activate only upon strong, non-physiological membrane depolarization and conduct small and slowly activating currents (Tanabe et al., 1988). This is strikingly different in the embryonic splice variant (CaV1.1e), which lacks 19 amino acids in the extracellular loop connecting segments S3 and S4 in the fourth homologous repeat, owing to alternative splicing excluding exon 29 (Tuluc et al., 2009). The embryonic CaV1.1e isoform activates upon physiological membrane depolarization and conducts currents that are substantially larger in amplitude than those of the adult CaV1.1a isoform.

CaV1.1 is a multiprotein complex consisting of a pore-forming α1 subunit and several auxiliary proteins: the intracellular β1a, the glycosylphosphatidylinositol-anchored extracellular αδ-1, and the transmembrane γ1 subunits (Curtis and Catterall, 1984; Zamponi et al., 2015). While the β1a subunit was shown to be essential for the functional expression of CaV1.1 and for EC coupling (Gregg et al., 1996; Schredelseker et al., 2005), αδ-1 and γ1 are dispensable for functional expression of CaV1.1 in muscle cells but displayed an inhibitory effect on CaV1.1 currents (Freise et al., 2000; Obermair et al., 2005; Held et al., 2002; Ursu et al., 2001; Arkkath et al., 2003; Tuluc et al., 2009; Ahern et al.,

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using a knockout or knockdown approach since CaV1.1 expresses
cannot activate to more negative voltages and increased CaV1.1
current density by the IVS3-S4 loop of CaV1.1a (Wu et al., 2016; Wu et al., 2015), we
changed (Obermair et al., 2005; Ahern et al., 2001; Ursu et al.,
2004).
All the cited studies were performed in skeletal muscle cells
using a knockout or knockdown approach since CaV1.1 expresses
poorly in mammalian non-muscle cells. Whereas coexpression of
the auxiliary subunits $\beta$ and $\alpha_\delta$ is sufficient to support
functional expression of all other high voltage activated calcium
channels (Singer et al., 1991; Lacerda et al., 1991; Zamponi et al.,
2015), CaV1.1 coexpression with these subunits does not yield
functional currents in heterologous cell systems. Only recently, it
was demonstrated that the skeletal muscle–specific adaptor
protein STAC3 is essential for membrane expression and robust
currents of CaV1.1 in heterologous cells (Polster et al., 2015; Wu et al., 2018).
In the present study, we generated two HEK cell lines stably
expressing the three subunits (STAC3, $\beta_3$, and $\alpha_\delta$) necessary
to support functional membrane expression of CaV1.1. These cell
lines provide a unique tool for analysis of wild type and mutant
CaV1.1 channel currents and pharmacology in non-muscle cells.
Interestingly, in contrast to what had been reported in myo-
tubes, our current analysis of the adult and embryonic CaV1.1
splice variants in the STAC3-HEK cell lines revealed no differ-
ce in their current densities, but still displayed the typical
differences in voltage dependence of activation. Because coex-
pression of $\gamma_1$ inhibits gating of CaV1.1a calcium currents in
skeletal myotubes and tsA201 cells (Polster et al., 2016; Freise et al., 2000; Ahern et al., 2001),
and because the recently resolved CaV1.1 structure revealed an interaction of $\gamma_1$ subunit with
the IVS3-S4 loop of CaV1.1a (Wu et al., 2016; Wu et al., 2015), we
hypothesized that regulation of the gating properties of CaV1.1
channels by the $\gamma_1$ subunit occurs in a splice variant–dependent
manner. Indeed, we found that coexpressed $\gamma_1$ subunits selec-
tively reduce the current density of the adult CaV1.1a isoform,
and not that of the embryonic CaV1.1e isoform. In contrast, $\gamma_1$
similarly shifted the voltage dependence of steady-state in-
activation to more negative voltages and increased CaV1.1
membrane expression of both isoforms. Molecular modeling
predicted several ionic interactions between the $\gamma_1$ subunit and
the IVS3-S4 linker of CaV1.1a. However, site-directed muta-
genesis of the putative ion-pair partners did not abolish $\gamma_1$
dependent inhibition of the CaV1.1a currents, suggesting an
allosteric effect of exon 29 that is important for modulation of
current density by the $\gamma_1$ subunit.

**Materials and methods**

**Generation of stable cell lines**

Two HEK293 cell lines stably expressing mouse STAC3 were
generated using the Flp-In T-Rex system (Invitrogen). Host cells,
already expressing human $\alpha_\delta-1$ and $\beta_3$ subunits and containing a
flippase recognition target (FRT) site, allowed the integration
of STAC3 into the genome in a Flp recombinase–dependent
manner. Briefly, the coding sequence of mouse STAC3 (Q8BZ71)
was cloned into the pTO-HA-strepIII C GW FRT vector (con-
taining an FRT site and a hygromycin resistance gene). To
generate the cell line constitutively expressing STAC3 (HEK-
STAC3), STAC3 expression was under the control of a CMV
promoter. To generate the inducible STAC3 expression cell line
(HEK-TetOn-STAC3), STAC3 expression was under the control
of a CMV promoter with a tetracycline operator (TetOn) ele-
ment. HEK293 host cells were transfected using the calcium
phosphate method with either plasmid or a Flp recombinase-
expressing vector (pOG44). Subsequently cells were selected with
hygromycin B (50 $\mu$g/ml; cat. #CP12.2; Lactan/Roth) and
selection agents for the other subunits (see below), and single-
positive cell clones were propagated and characterized. The
electrophysiological experiments for the characterization of the
cell lines were carried out using the TetOn-STAC3 cell line (Figs.
3, 4, 6, and S1).

Although the cell lines contain the $\beta_3$ isoform, rather than the
skeletal muscle–specific $\beta_3a$, no drawbacks are expected when
expressing a non-muscle $\beta$ subunit in non-muscle cells. Accord-
ingly, the cell lines expressing $\beta_3$ efficiently supported robust
CaV1.1 currents (Fig. 2). Also, because we compared differences due
to splicing or $\gamma_1$ coexpression (mostly involving the transmem-
brane or extracellular part of the channel), the type of the intra-
cellular $\beta$ subunit is not expected to affect our current analysis.

**Cells culture and transfection**

Cells were cultured in DMEM (cat. #41966052; Invitrogen)
supplemented with 10% FBS (F9665; Sigma-Aldrich), 2 mM
L-glutamine (25030-032; Invitrogen), and 10 U/ml penicillin-
streptomycin (15140122; Invitrogen) and were maintained at
37°C in a humidified incubator with 5% CO$_2$. For maintenance
of the stable cell lines, selection agents for each subunit were ap-
plied regularly (STAC3, 50 $\mu$g/ml hygromycin B; $\beta_3$, 500 $\mu$g/ml
geneticin [10131035; Gibco]; and $\alpha_\delta-1$, 15 $\mu$g/ml blasticidin S
[A113903; Gibco]).

For electrophysiological experiments, cells were plated on
35-mm dishes coated with poly-L-lysine (cat. #P2636; Sigma-
Aldrich) and simultaneously transfected with 2 $\mu$g of DNA using
Fugene HD (cat. #E2312; Promega), according to the manu-
facturer’s instructions. For the TetOn cell line, STAC3 expres-
sion was induced using 1 $\mu$g/ml doxycycline (DOX) upon transfection
(cat. #3072; Sigma-Aldrich), and cells were kept at 5% CO$_2$ and 30°C.
Cells were used for patch-clamp experiments 24–48 h after trans-
fection/induction.

**Plasmids**

Cloning procedures for GFP-CaV1.1a and GFP-CaV1.1e were pre-
viously described (Grabner et al., 1998; Tuluc et al., 2009).
Mouse $\gamma_1$ was cloned from genomic cDNA from mouse soleus
muscle. Primer sequences were selected according to GenBank
NM-007582. Briefly, the cDNA of $\gamma_1$ was amplified by PCR with a
forward primer introducing a KpnI site upstream the starting
codon (5’-ATATGGTACCATGTCACAGACCAAAACAGCGAAG-3’)
and the reverse primer introducing a Sall site after the stop
codon (5’-ATATGTCGACGCTAGTCTCTGGCTACCGGTCCATG

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The 13-residue bungarotoxin (BTX) binding site (BBS) was inserted in the IISS-S6 loop of CaV1.1a or CaV1.1e at residue 593 by overlap extension PCR. Briefly, the cDNA sequence of CaV1.1 was amplified with overlapping primers in separate PCR reactions using GFP-CaV1.1a or GFP-CaV1.1e as template. Primers used for the first fragment were forward, 5′-TACATGAGGTTGACATCGCAG-3′, and reverse, 5′-GTAGGGCTACCCCGGAGCTCTGATATTCCCATGTG TGGCCTGGTCTTGGTCAGGAAATCC-3′. Primers used for the second fragment were forward, 5′-TGGAGACGTCCCTGGGAAGCC ACCCTTGAGTCAGTGTCCAGAGACAG-3′, and reverse, 5′-GACACGGACTGGACACGC-3′. The two separate PCR products were then used as template for a final PCR reaction with flanking primers to connect the nucleotide sequences. The resulting PCR fragment was EcoRI/XhoI digested and inserted into EcoRI/XhoI-digested GFP-CaV1.1a or GFP-CaV1.1e, yielding pcDNA3-GFP-CaV1.1a-BBS or pcDNA3-GFP-CaV1.1e-BBS.

The R160A mutation was introduced by overlap extension PCR. Briefly, the cDNA sequence of γ1 was amplified with overlapping primers mutating R160 into an alanine in separate PCR reactions using pcDNA3-γ1 as template. Primers used for the first fragment were forward, 5′-ATAATGTCGATCTGACACA GACCACAAACAGGAAAG-3′, and reverse, 5′-CACCAGCATGGCGC CATGAACCTCCAGCAGGACATGAG-3′. Primers used for the second fragment were forward, 5′-GAGTCATCGGGCGACTGCG GTGAAAGCTATGATTGAC-3′, and reverse, 5′-ATAATGCACGACT GTAGTCTGCTTGGCCAGCTGAC-3′. Two separate PCR products were then used as template for a final PCR reaction with flanking primers to connect the nucleotide sequences. The resulting PCR fragment was Kpnl/Sall digested and inserted into the Kpnl/XhoI-digested pcDNA3 vector, yielding pcDNA3-γ1-R160A.

The K102A and E103A mutations were introduced by PCR. Briefly, the cDNA sequence of γ1 (nt 288–672) was amplified by PCR with a forward primer introducing the K102A and the E103A mutations downstream of the EcoRI site and the reverse primer introducing an Apal site after the stop codon. Primers used were forward, 5′-TGAATTCCACCAAGCCGGGTACAG CATCTCAGCGACGGCCATT-3′, and reverse, 5′-GAAATAGGG CCCCCCTCTGACGCT-3′. After EcoRI/Apal digestion, the PCR fragment obtained was inserted into the EcoRI/Apal-digested pcDNA3-γ1 vector, yielding pcDNA3-γ1-K102A-E103A. To combine the three mutations, we introduced the K102A and E103A mutations as described above, but using γ1-R160A as template for the PCR, yielding γ1-R160A-K102A-E103A (γ1-RKE AAA). Sequence integrity of all newly generated constructs was confirmed by sequencing (MWG Biotech).

RT-PCR
RNA was isolated from the three HEK293 cell lines after 48 h in culture using the RNeasy Protect Mini Kit (cat. #74124; Qiagen). After reverse transcription (Super-Script II reverse transcriptase, cat. #18064022; Invitrogen), the absolute number of transcripts in each sample was assessed by quantitative TaqMan PCR (Mm01159196_m1; Thermo Fisher Scientific), using a standard curve generated from known concentrations of a PCR product containing the target of the assay as described previously (Rufenach et al., 2020).

Western blotting
Proteins isolated from the three HEK cell lines were prepared as previously described (Campiglio and Flucher, 2017). Briefly, cells plated in 100-mm dishes were trypsinized after 48 h in culture. Cells were lysed in radioimmunoprecipitation assay buffer with a pestle and left on ice for 30 min. The lysates were then centrifuged for 10 min. The protein concentration was determined using a BCA assay (cat. #23250; Pierce). 20 µg of protein samples were loaded on a NuPage gel (4–12% polyacrylamide, cat. #NP0321; Invitrogen) and separated by SDS-PAGE at 160 V. The protein samples were then transferred to a PVDF membrane at 25 V and 100 mA for 3 h at 4°C with a semidry blotting system (Roht). The membrane was then cut and incubated with rabbit anti-STAC3 (1:2,000; cat. #20392-1; Proteintech; RRID:AB_10693618) or mouse anti-GAPDH (1:100,000; cat. sc-32233, Santa Cruz Biotechnology; RRID:AB_626769) antibodies overnight at 4°C and then with HRP-conjugated secondary antibody (1:5,000; Pierce) for 1 h at room temperature. The chemiluminescent signal was developed with ECL Supersignal WestPico kit (cat. #34579; Thermo Fisher Scientific) and detected with ImageQuant LAS 4000.

Immunocytochemistry
The three HEK cell lines were plated on poly-L-lysine–coated coverslips and fixed in paraformaldehyde at room temperature after 2 d in culture. Fixed cells were incubated in 5% normal goat serum in PBS/BSA/Triton for 30 min. The rabbit anti-STAC3 antibody (1:2,000) was applied overnight at 4°C and detected with Alexa Fluor 594–conjugated secondary antibody. During the last washing step, cells were incubated with Hoechst dye to stain nuclei. Preparations were analyzed on an Axiosimager microscope (Carl Zeiss) using a 63×, 1.4-NA objective. Images were recorded with a cooled charge-coupled device camera (SPOT; Diagnostic Instruments) and Metamorph image processing software (Universal Imaging). Images were arranged in Adobe Photoshop CS6 (Adobe Systems), and linear adjustments were performed to correct black level and contrast. To quantify the fluorescence intensity of the STAC3 staining, 14-bit grayscale images of the red (STAC3) and blue (Hoechst) channels were acquired for each cell line. A region of interest was manually traced around each cell in the STAC3 staining image, and its intensity was recorded and background corrected using Metamorph. For each condition, between 15 and 31 cells were analyzed from each of three independent experiments.

Labeling of cell surface CaV1.1 channels with QD555
For cell-surface labeling, a 13-amino acid high-affinity BBS was inserted into CaV1.1a and CaV1.1e as described (Yang et al., 2010) and expressed in HEK-293 cells. 48 h after transfection, cells were resuspended from 35-mm dishes with ice-cold PBS containing calcium and magnesium (pH 7.4, 0.9 mM CaCl2, and 0.49 mM MgCl2), washed, and incubated with 5 µM biotinylated α-BTX (cat. #B1196; Invitrogen) in PBS/BSA in the dark for 1 h on ice. Cells were washed twice with PBS/BSA and incubated with 10 nM streptavidin-conjugated quantum dots
Microscopy

Cells were mounted in Tyrode’s physiological solution and imaged using a 63×, 1.4-NA objective Axioimager microscope (Carl Zeiss). 14-bit images were recorded with a cooled charge-coupled device camera (SPOT; Diagnostic Instruments) and Metaview image processing software (Universal Imaging). Image composites were arranged in Adobe Photoshop CS6.

Multiparameter flow cytometry

Labeled cells were counted by flow cytometry using a BD FACSVerse analyzer. For flow cytometric analyses, labeled cells were counted and analyzed using BD FACSuite v1.0.6 and BD FACS Diva v9.0 software. Cells expressing GFP were excited at 488 nm, and red signal was excited at 633 nm. Our gating strategy assured that the same cell population in terms of size and granularity was counted in each condition. In each set of experiments, untransfected or unlabeled cells, as well as single-color controls, were used to adjust threshold values, and these settings were then used when analyzing all samples.

Electrophysiology

Calcium currents in HEK cells were recorded with the whole-cell patch-clamp technique in voltage-clamp mode using an Axopatch 200A amplifier (Axon Instruments). Patch pipettes (borosilicate glass; Science Products) had resistances between 1.8 and 4.0 MΩ when filled with (in mM) 135 CsCl, 1 MgCl₂, 10 HEPES, 10 EGTA, and 4 ATP-Na₂ (pH 7.4 with CsOH). Data acquisition and command potentials were controlled by pCLAMP software (Clampex version 10.2; Axon Instruments); analysis was performed using Clampfit version 10.7 (Axon Instruments) and SigmaPlot version 12.0 (SPSS Science) software. The current–voltage dependence of activation was determined using 300- or 500-ms-long square pulses to various test potentials (holding potential –80 mV), and curves were fitted according to

\[
I = \frac{G_{\text{max}}}{1 + \exp\left[-\frac{(V-V_{1/2})}{k}\right]} + I_{\text{residual}},
\]

where \(V_{1/2}\) is the half-maximal inactivation voltage and \(k\) is the inactivation slope factor an \(I_{\text{residual}}\) is the residual fractional current.

Statistical analysis

All experimental groups were analyzed in transiently transfected cells from at least three independent cell passages/transfections. The means, SEM, and \(P\) values were calculated using Student’s \(t\) test, two-tailed, with significance criteria as follows: *; \(P < 0.05\); **; \(P < 0.01\); ***; \(P < 0.001\); and ****; \(P < 0.0001\). \(P\) values of the experiments in which more than two groups were compared to each other were calculated using ANOVA and Tukey’s or Sidak’s post-hoc test. The software used for statistical analysis was GraphPad Prism v9.

Structure modeling

The complex structures of both splice variants of the human α₁-subunit (Caₙ₁.1e and Caₙ₁.1a) and the γ₁-subunit were modeled based on the rabbit cryo-EM structure of Caₙ₁.1 in the inactivated state, with voltage sensors in the “up” conformation and a closed intracellular gate (PDB accession no. SGJV; Wu et al., 2016). Homology modeling has been performed using MOE (Molecular Operating Environment, version 2018.08; Molecular Computing Group). Additionally, ab initio Rosetta modeling was used to generate structures for loops that were not resolved in the original Caₙ₁.1 α₁-subunit and γ₁-subunit templates (Rohl et al., 2004). The structures for the putative mutants were derived from both WT splice variant models by replacing the mutated residue and carrying out a local energy minimization using MOE. The C-terminal and N-terminal parts of each domain were capped with acetylamine and N-methylamide to avoid perturbations by free charged functional groups. The structure model was embedded in a plasma membrane consisting of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and cholesterol in a 3:1 ratio, using the CHARMM-GUI Membrane Builder (Lee et al., 2019; Jo et al., 2009). Water molecules and 0.15 M KCl were included in the simulation box. Energy minimizations of Caₙ₁.1e and Caₙ₁.1a WT and mutant structures in the membrane environment were performed. The topology was generated with the LEAP tool of AmberTools18 (Case et al., 2008), using force fields for proteins (ff14SBonlysc) and lipids (Lipid14; Dickson et al., 2014). The structure models were heated from 0 to 300°K in two steps, keeping the lipids fixed, and then equilibrated over 1 ns. Molecular dynamics simulations were performed for 10 ns, with time steps of 2 fs, at 300°K and in anisotropic pressure scaling conditions. Van der Waals and short-range electrostatic interactions were cut off at 10 Å, whereas long-range electrostatics were calculated by the particle mesh Ewald method (Salomon-Ferrer et al., 2013). As extracellular loop 1 was not resolved in the cryo-EM structure, we modeled 100 loop structures with Rosetta ab initio modeling (Rohl et al., 2004). By clustering on the loops using an RMSD distance criterion of 2 Å, we obtained 10 clusters. These 10 clusters were carefully evaluated, and the two energetically most favorable cluster representatives, which formed interactions...
with the S3-S4 loop of VSD IV (exon 29), were considered for further minimizations in the membrane environment. MOE and Pymol were used to visualize the key interactions and point out differences in structure models (The PyMOL Molecular Graphics System; version 2.0, Schrödinger, LLC).

Online supplemental material
Fig. S1 shows the activation and inactivation kinetics analysis pertaining to Fig. 3 (activation) and Fig. 4 (inactivation).

Results
Generation of two HEK cell lines expressing β2, α2δ-1, and STAC3
In order to generate HEK293 cell lines that could reliably support CaV1.1 expression, we inserted STAC3 into the genome of a host cell line already available, stably expressing α2δ-1 and β2, using the Flp-In T-Rex system. We generated two cell lines: one in which the expression of STAC3 was constitutive (HEK-STAC3) and one in which the expression of STAC3 was DOX inducible (HEK-TetOn-STAC3). While the parental HEK293 cell line showed neither STAC3 mRNA nor protein expression, the selected clone of the constitutive HEK-STAC3 cell line strongly expressed STAC3 (Fig. 1). As expected, without DOX induction, the selected clone of the inducible HEK-TetOn-STAC3 cell line showed only weak basal STAC3 mRNA and protein expression. However, 24 h after the beginning of DOX induction, STAC3 expression levels were strongly increased and comparable to those of the constitutive HEK-STAC3 cell line (Fig. 1).

We then analyzed the ability of the cell lines to support the expression of functional CaV1.1 currents by transient transfection with the adult CaV1.1a or the embryonic CaV1.1e isoforms. The two CaV1.1 isoforms differ in the length of the linker connecting helices S3 and S4 of the fourth homologous repeat, with the embryonic isofrom skipping exon 29 and lacking 19 amino acids. Although both isoforms support skeletal muscle EC coupling, they display very different current properties when expressed in dystrogenic (CaV1.1-null) myotubes. In contrast to the adult CaV1.1a isoform, the embryonic CaV1.1e splice variant activates at more hyperpolarizing potentials and conducts calcium currents that are several-fold larger than those of CaV1.1a (Tuluc et al., 2009). Our experiments show that both the constitutive (HEK-STAC3) and the inducible (HEK-TetOn-STAC3) cell lines efficiently supported functional expression of both the adult and the embryonic CaV1.1 variants (Fig. 2, A, B, E, and F; and Table 1). More interestingly, while the two CaV1.1 splice variants displayed the expected difference in the V1/2 of activation (Fig. 2, C, D, G, and H; and Table 1), the expected smaller current density in CaV1.1a was not observed in the two STAC3-HEK cell lines (Fig. 2, A, B, E, and F; and Table 1).

We reasoned that some factor is missing in HEK cells that specifically mediates the splicing-dependent effect on the current amplitude in muscle cells. In muscle, the specific function of exon 29 is to curtail the calcium currents, and in our STAC3-HEK cells the currents were equally large, so the missing factor might be a muscle-specific protein capable of diminishing CaV1.1 currents specifically in the adult splice variant. The only CaV1.1 subunit not present in our expression system is the γ1 subunit. Moreover, the γ1 subunit acts as a negative regulator of CaV1.1 currents both in skeletal muscle and in tsA201 cells (Freise et al., 2000; Ahern et al., 2003; Andronache et al., 2007; Polster et al., 2016), and its expression is restricted to skeletal muscle (Biel et al., 1991; Jay et al., 1990). Therefore, we inferred that the γ1 subunit may be the missing factor selectively reducing the currents of CaV1.1a and not those of CaV1.1e. This notion was further supported by the fact that cryo-EM structures of CaV1.1 predicted an interaction of the γ1 subunit with the CaV1.1 IVS3-S4 region, exactly the site containing the alternatively spliced exon 29 (Wu et al., 2015, 2016).

The γ1 subunit selectively reduces the current density of CaV1.1a but not that of CaV1.1e
To test this hypothesis, we measured the calcium current density of CaV1.1a and CaV1.1e in the presence and the absence of γ1 in one of the newly established cell lines (HEK-TetOn-STAC3). As previously reported (Polster et al., 2016; Freise et al., 2000), the presence of γ1 significantly reduced CaV1.1a current amplitudes, with no significant effect on the voltage dependence of

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activation (Fig. 3, A–D; and Table 2). The activation kinetics were unaltered by coexpression of the γ1 subunit (Fig. S1, A–D; and Table 2), in agreement with what had been observed in myotubes (Freise et al., 2000) but contrary to what was previously reported in tsA201 cells (Polster et al., 2016). More importantly, in contrast to CaV1.1a, coexpression of γ1 had no effect on the current density of CaV1.1e (Fig. 3, E–H; and Table 2), suggesting that the inclusion of the 19 amino acids encoded in exon 29 is essential for suppression of the CaV1.1 current by the γ1 subunit.

Figure 2. Exclusion of exon 29 in CaV1.1e shifts the voltage dependence of activation to more negative voltages but does not affect current density in either of the two STAC3-HEK cell lines. (A–D) Current properties of CaV1.1a (blue, n = 15) compared with CaV1.1e (red, n = 15) in the HEK-STA3 cell line. (E–H) Current properties of CaV1.1a (blue, n = 19) compared with CaV1.1e (red, n = 15) in the inducible cell line HEK-TetOn-STA3 treated with DOX. (A and E) Exemplary current traces at Vmax show similar activation kinetics of the CaV1.1a and CaV1.1e variants and no difference in the peak current density (Ipeak; peak current normalized to the cell size) in both the HEK-STA3 (B) and HEK-TetOn-STA3 (F) cell lines (P = 0.94 and P = 0.56, respectively). The current–voltage relationship (C and G) and the normalized steady-state activation curves (D and H) show that exclusion of exon 29 (in CaV1.1e) results in a 20.4- and 21.1-mV left shift of activation when expressed in the HEK-STA3 and HEK-TetOn-STA3 cell line, respectively. Mean ± SEM; P values calculated with Student’s t test (see Table 1 for parameters and statistics).

The γ1 subunit shifts the steady-state inactivation to more negative potentials in both CaV1.1 isoforms

The γ1 subunit inhibits CaV1.1 currents not only by decreasing the current amplitude, but also by promoting inactivation. In fact, previous studies demonstrated that, in the presence of γ1, the voltage dependence of inactivation shifted toward more negative potentials, while the voltage dependence of activation remained unaltered (Ahern et al., 2001; Freise et al., 2000; Held et al., 2002; Ursu et al., 2004).

To determine whether this γ1 effect on CaV1.1 currents is also restricted to the adult CaV1.1a isoform, we performed a steady-state inactivation protocol comparing the current size of test pulses before and after 45-s conditioning prepulses at incrementally increasing potentials (Fig. 4 A, inset). The normalized steady-state inactivation was plotted as a function of the prepulse potential. As previously demonstrated, coexpression of the γ1 subunit resulted in a robust left shift in the voltage dependence of inactivation of the adult CaV1.1a isoform (Fig. 4 A). In the presence of γ1, the half-maximal inactivation potential was

Table 1. Current activation properties of CaV1.1a and CaV1.1e expressed in HEK-STA3 and HEK-TetOn-STA3 cells

| Property                | HEK-STA3               | HEK-TetOn-STA3          |
|-------------------------|------------------------|-------------------------|
|                         | CaV1.1a                | CaV1.1e                 | CaV1.1a                | CaV1.1e                 |
| Ipeak (pA/pF)           | −19.8 ± 2.7            | −19.5 ± 2.4             | −18.2 ± 3.1            | −21.3 ± 4.5             |
| V1/2 activation (mV)    | 24.4 ± 0.7             | 4.0 ± 1.0               | 24.1 ± 1.4             | 3.0 ± 1.5               |
| k activation (mV)       | 8.1 ± 0.4              | 7.1 ± 0.2               | 7.0 ± 0.5              | 5.3 ± 0.3               |
| Vrev (mV)               | 86.3 ± 1.2             | 75.3 ± 1.1              | 81.2 ± 1.7             | 72.8 ± 1.6              |
| Time to peak (ms)       | 173.1 ± 10.8           | 162.6 ± 12.0            | 198.3 ± 20.4           | 184.1 ± 14.3            |
| n                       | 15                     | 15                      | 19                      | 15                      |

Data are expressed as mean values ± SEM. Statistically significant: *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
shifted by 14.1 mV toward more hyperpolarizing potentials (Fig. 4 B and Table 2).

Surprisingly, these effects were recapitulated with the embryonic CaV1.1e isoform. In the presence of the γ1 subunit, the half-maximal inactivation potential was shifted to hyperpolarizing potentials by 13.7 mV (Fig. 4, C and D; and Table 2).

These results suggest that, although the γ1 subunit fails to suppress the current of the embryonic CaV1.1e splice variant by

Table 2. Current properties (activation and inactivation) of CaV1.1a and CaV1.1e in the presence and absence of γ1

| Property                          | CaV1.1a       | CaV1.1a + γ1 | P value | CaV1.1e       | CaV1.1e + γ1 | P value |
|----------------------------------|---------------|--------------|---------|---------------|--------------|---------|
| $I_{peak}$ (pA/pF)               | −18.9 ± 2.9   | −7.8 ± 1.2   | 0.012*  | −24.1 ± 4.0   | −26.1 ± 2.7  | 0.69    |
| $V_{1/2}$ activation (mV)         | 26.3 ± 1.0    | 29.4 ± 2.0   | 0.14    | 3.9 ± 1.1     | 4.5 ± 0.9    | 0.68    |
| $k$ activation (mV)               | 6.8 ± 0.2     | 8.3 ± 0.8    | 0.03*   | 5.1 ± 0.3     | 5.2 ± 0.2    | 0.70    |
| $V_{rev}$ (mV)                    | 81.4 ± 1.1    | 78.6 ± 2.8   | 0.07    | 75.7 ± 2.0    | 76.0 ± 1.3   | 0.90    |
| Time to peak (ms)                 | 150.1 ± 18.0  | 102.9 ± 15.0 | 0.08    | 104.4 ± 11.0  | 97.1 ± 13.3  | 0.67    |
| $\tau_{mono}$ activation (ms)     | 35.1 ± 3.0    | 32.9 ± 2.9   | 0.65    | 30.3 ± 5.5    | 23.7 ± 4.0   | 0.19    |
| $\tau_{slow}$ activation (ms)     | 107.4 ± 39.9  | 79.6 ± 21.2  | 0.53    | 79.6 ± 21.2   | 76.0 ± 13.3  | 0.53    |
| $A_{slow}$ activation (%)          | 18.7 ± 6.9    | 31.0 ± 11.8  | 0.41    | 31.0 ± 11.8   | 26.4 ± 4.4   | 0.23    |
| Time to peak (ms)                 | 26.4 ± 6.1    | 17.1 ± 4.4   | 0.23    | 17.1 ± 4.4    | 17.1 ± 4.4   | 0.23    |
| $\tau_{fast}$ activation (ms)     | 81.3 ± 6.9    | 69.0 ± 11.8  | 0.41    | 69.0 ± 11.8   | 69.0 ± 11.8  | 0.41    |
| $n$ (activation)                  | 19            | 10           | 12      | 12            | 13          |         |
| $V_{1/2}$ inactivation (mV)        | −16.9 ± 2.6   | −31.0 ± 5.3  | 0.04*   | −28.6 ± 1.7   | −42.3 ± 1.9  | ***     |
| $I_{residual}$ (%)                | 18.7 ± 7.4    | 14.7 ± 4.6   | 0.66    | 9.8 ± 3.1     | 3.9 ± 1.3    | 0.13    |
| $k$ inactivation (mV)             | 8.1 ± 0.4     | 7.9 ± 0.9    | 0.89    | 7.0 ± 0.9     | 7.3 ± 0.3    | 0.79    |
| $t_{mono}$ inactivation (ms)       | 2,062.4 ± 525.5 | 1,389.5 ± 251.5 | 0.32    | 2,159.8 ± 278.5 | 1,453.2 ± 230.3 | 0.15    |
| $n$ (inactivation)                | 6             | 6            | 7       | 6             | 6           |         |

Data are expressed as mean values ± SEM. Statistically significant: *, P < 0.05; ***, P < 0.001.
reducing its amplitude (Fig. 3, E–G), it still inhibits CaV1.1e currents by left-shifting the steady-state inactivation.

The γ1 subunit was also reported to accelerate the inactivation kinetics of CaV1.1 (Ahern et al., 2001; Freise et al., 2000). Accordingly, the time constant of inactivation was reduced to 67% in the presence of γ1 for both CaV1.1a and CaV1.1e, although not to a statistically significant extent (Fig. S1, E–H; and Table 2).

The γ1 subunit increases membrane expression of both CaV1.1 isoforms

CaV1.1 is the only 1 out of the 10 voltage-gated calcium channels that expresses poorly in non-muscle cells, unless the adaptor protein STAC3 is coexpressed (Polster et al., 2015). Recently it was shown that the γ1 subunit also supports robust membrane expression of CaV1.1a in tsA201 cells; although in the absence of STAC3, these channels produce only very small calcium currents (Polster et al., 2016). To examine whether the γ1 subunit supports only the membrane targeting of the adult CaV1.1a isoform or also of the embryonic CaV1.1e, we established a dual-labeling approach, originally developed by the lab of Henry Colecraft (Fang and Colecraft, 2011; Yang et al., 2010), to identify and quantify membrane-inserted CaV1.1a channels. To this end, a 13 amino acid high-affinity BBS was introduced into the extracellular IIS5–IIS6 domain of GFP-CaV1.1a and GFP-CaV1.1e. Then, the channels expressed on the cell surface of HEK cells (expressing β3 and αδ-1) were labeled by exposing unpermeabilized living cells to biotinylated BTX and subsequently to streptavidin-coated quantum dots (QD655; Fig. 5 A). Hence, the GFP fluorescence of a cell measures the total CaV1.1 expression, and the QD655 fluorescence quantifies the fraction of surface-expressed CaV1.1a channels.

In cells expressing CaV1.1a alone, we detected minimal QD655 fluorescence in the plasma membrane. By contrast, coexpression of STAC3 or γ1, individually or together, resulted in robust CaV1.1a membrane targeting (Fig. 5 B). To quantify membrane-inserted CaV1.1a channels, we used flow cytometry analysis, which measures the fluorescence signals of a multitude of individual cells (Fig. 5 D). This analysis confirmed the lack of a robust QD655 fluorescence signal in cells expressing only GFP-CaV1.1a but the presence of a strong QD655 fluorescence in cells coexpressing GFP-CaV1.1a together with STAC3, γ1, or both. In four independent experiments, cells coexpressing STAC3 on average displayed a 140% increase of surface-expressed CaV1.1a, cells coexpressing γ1 displayed an 80% increase, and cells expressing both STAC3 and γ1 subunits displayed a 180% increase compared with cells expressing CaV1.1a alone (Fig. 5 F). These results corroborate the importance of STAC3 and γ1 for CaV1.1a plasma membrane expression (Niu et al., 2018; Polster et al., 2016; Polster et al., 2015).

We then analyzed the effect of the STAC3 and γ1 subunits on membrane expression of the embryonic CaV1.1e isoform. In contrast to the adult isoform, the embryonic CaV1.1e channel showed substantial membrane staining even when expressed alone (Fig. 5, C [top] and E [left]). Nevertheless, coexpression of STAC3 and γ1, individually or together, further increased the amount of QD655 fluorescence (Fig. 5, C and E). In four independent experiments, cells coexpressing STAC3 displayed a 70% increase of surface-expressed CaV1.1e, cells coexpressing γ1 displayed a 50% increase, and the ones expressing both STAC3 and γ1 subunits displayed an 80% increase compared with cells expressing CaV1.1e alone (Fig. 5 G).

Altogether, these results demonstrate that, although the γ1 subunit fails to modulate the current amplitude of the embryonic CaV1.1e isoform, it still modulates its steady-state inactivation and surface trafficking. Moreover, the reduction of current density induced by γ1 cannot be explained by reduced channel availability at the cell surface.

CaV1.1–γ1 ion-pair partners predicted by structure modeling are not essential for CaV1.1a-specific current reduction by γ1

Because the recent cryo-EM structure of CaV1.1 revealed that the γ1 subunit interacts with IVS3–S4 (Wu et al. 2016; Wu et al. 2015), and because we found that γ1 fails to inhibit the current amplitude of the embryonic CaV1.1e isoform (Fig. 2 E), which lacks 19 amino acids in the IVS3–S4 linker, we hypothesized that γ1 and the IVS3–S4 linker of CaV1.1a may establish an interaction responsible for the current inhibition in CaV1.1a. To identify putative interaction partners between the IVS3–S4 linker and γ1, we generated a structural model of the CaV1.1 channel based on the published cryo-EM structure (Wu et al., 2016; Fig. 6). We
used Rosetta computational modeling software (Bender et al., 2016; Rohl et al., 2004) to model the structure of the IVS3-S4 linker of CaV1.1a. The resulting structure predicts a putative interaction of residues D1223 and D1225 of the IVS3-S4 linker of CaV1.1a with residue R160 in the second extracellular loop of the γ1 subunit (Figs. 7 A and 6B). To test whether the observed inhibition of the CaV1.1a current amplitude by γ1 is dependent on this ionic interaction, we performed site-directed mutagenesis to substitute the involved residues with alanines, which deletes all interactions made by side-chain atoms beyond the β carbon (Wells, 1991). However, mutation of residue R160 of the γ1 subunit to an alanine did not diminish its ability to inhibit the current amplitude of CaV1.1a (Fig. 7, A–D; and Table 3). Also, simultaneously mutating both D1223 and D1225 of CaV1.1a did not alter the ability of γ1 to reduce the current amplitude of CaV1.1a (Fig. 7, E–H; and Table 3). Together, these results indicate
that this putative interaction between the IVS3-S4 linker of CaV1.1a and the γ1 subunit is dispensable for current amplitude inhibition by γ1.

Previously, it has been suggested that the N-terminal half of the γ1 subunit, including the first two transmembrane domains, mediates its interaction with the calcium channel and is responsible for suppressing the current amplitude of CaV1.1 (Arikkath et al., 2003). Because the analyzed R160A mutation is located outside of this region in the C-terminal half of the γ1 subunit protein, we further modeled the structure of the extensive extracellular loop located in the first half of the γ1 subunit and searched it for possible interaction sites with the IVS3-S4 linker of CaV1.1a. We identified putative ionic interactions of residues D1225 and R1229 in the IVS3-S4 linker of CaV1.1a with K102 and E103 positioned in the first extracellular domain of the γ1 subunit (Fig. 7 I, Table 3). However, mutation of K102 and E103 to alanines did not alter the ability of γ1 to inhibit the calcium channel current amplitude (Fig. 7, J–L; and Table 3). Finally, to exclude the possibility that the interaction between the IVS3-S4 linker of CaV1.1a with either one of the two extracellular loops of γ1 was sufficient to suppress the calcium channel current amplitude, we combined the R160A and K102A/E103A mutations (Fig. 7 M). However, this triple-mutant γ1 was also capable of inhibiting the current amplitude of CaV1.1a to levels similar to the wild type γ1 (Fig. 7, N–P; and Table 3). Together, these mutagenesis experiments suggest that the current-inhibiting effect of γ1 is not mediated by the direct ionic interactions between γ1 and the IVS3-S4 loop of CaV1.1a, at least not those predicted by our structure modeling.

Discussion

Whereas the role of the auxiliary α2δ and β subunits in subsellar targeting and gating modulation have been extensively studied for high voltage activated calcium channels in heterologous cells, this has not been the case for the γ1 subunit. γ1 is a specific subunit of the skeletal muscle CaV1.1 isoform and, until recently, CaV1.1 had resisted efficient functional expression in
Figure 7. The putative interactions between the IVS3-54 loop and \( \gamma_1 \) identified by structure modeling are dispensable for CaV1.1a current reduction.

(A–H) Structure modeling of CaV1.1a and \( \gamma_1 \) indicates interactions of R160 (\( \gamma_1 \)) with D1223 and D1225 (CaV1.1a). Neutralizing the putative CaV1.1a interaction partner (R160A; A) or the \( \gamma_1 \) interaction partners (D1223A and D1225A; E), did not impair current reduction by \( \gamma_1 \) (B–D and F–H).

(I–L) Structure modeling of CaV1.1a and \( \gamma_1 \) indicates further interactions of K102 and E103 (\( \gamma_1 \)) with D1225 and R1229 (CaV1.1a). Neutralizing both of these putative CaV1.1a interaction partners to alanine (K102A/E103A; I) did not abolish the ability of \( \gamma_1 \) to reduce CaV1.1a current (J and K).

(M–P) Concomitant mutation of all three \( \gamma_1 \) residues involved in these putative interactions did not abolish the current reduction by \( \gamma_1 \) (N–P). Exemplary current traces at \( V_{\text{max}} \) (B, F, J, and N); scatter plots of \( I_{\text{peak}} \) (C, G, K, and O); and current–voltage relationship (D, H, L, and P). Mean ± SEM; P values calculated with ANOVA and Tukey’s post-hoc test. *, P < 0.05; **, P < 0.01.
heterologous expression systems. Only since the discovery of STAC3 as an essential component of the CaV1.1 channel complex, permitting the reliable heterologous expression of CaV1.1, have such analyses been possible (Horstick et al., 2013; Nelson et al., 2013; Polster et al., 2015). Here, we developed and validated two HEK cell lines stably expressing STAC3 (plus $\alpha_2\delta$-1 and $\beta_3$), which proved to be a convenient and efficient heterologous expression system for CaV1.1. By coexpression of CaV1.1 and $\gamma_1$ in these cells, we found three effects of the $\gamma_1$ subunit: facilitated membrane expression of CaV1.1, a reduction of the current density, and a shift of steady-state inactivation to hyperpolarizing potentials. The effects of the $\gamma_1$ subunit on the two splice variants of CaV1.1 expressed in our new STAC3-HEK cell lines revealed a novel, isoform-dependent mechanism of channel modulation by this subunit. Although $\gamma_1$ equally supports membrane expression of Cav1.1a and Cav1.1e, if functions only as a negative regulator of the adult Cav1.1a splice variant. This differential regulation of current density is mediated by the inclusion of the alternatively spliced exon 29 in the extracellular loop connecting helices S3 and S4 in repeat IV, but it does not require the direct ionic interactions between this loop and the $\gamma_1$ subunit. Another novel finding is that in both the adult and embryonic CaV1.1 splice variants, $\gamma_1$ reduces steady-state inward current at more negative voltages by shifting the voltage dependence of steady-state inactivation, but not of activation, to more negative voltages and by promoting the time course of current inactivation.

The $\gamma_1$ subunit supports membrane expression of CaV1.1

The substantially increased surface expression induced by coexpression of $\gamma_1$ observed with extracellular BTX labeling and flow cytometry did not translate into increased current densities. This is consistent with the observation that in $\gamma_1$-null mouse muscle, in which STAC3 is endogenously expressed, the expression levels of CaV1.1 are similar to those of wild type mice (Arikkath et al., 2003). In our experiments, this is explained by

| Property         | CaV1.1a | CaV1.1a + $\gamma_1$ | P value (t test) | CaV1.1a | CaV1.1a + $\gamma_1$ | P value (t test) |
|------------------|---------|----------------------|------------------|---------|----------------------|------------------|
| $l_{\text{peak}}$ (pA/pF) | $-26.1 \pm 6.6$ | $-10.4 \pm 2.6$ | 0.04$^*$ | $-13.9 \pm 2.1$ | $-21.0 \pm 3.3$ | 0.09 |
| $V_{1/2}$ activation (mV) | 25.1 ± 1.0 | 26.9 ± 0.9 | 0.04 | 28.2 ± 0.9 | 28.2 ± 0.9 | 0.06 |
| $k$ activation (mV) | 8.7 ± 0.5 | 9.8 ± 0.5 | 0.20 | 8.7 ± 0.4 | 8.7 ± 0.4 | 0.99 |
| $V_{\text{rev}}$ (mV) | 86.7 ± 1.8 | 91.8 ± 2.0 | 0.24 | 81.2 ± 2.7 | 81.2 ± 2.7 | 0.17 |
| Time to peak (ms) | 121.5 ± 18.5 | 99.8 ± 23.1 | 0.69 | 108.5 ± 13.3 | 108.5 ± 13.3 | 0.85 |
| $n$ | 14 | 13 | | | |

Data are expressed as mean values ± SEM. Statistically significant: *, $P < 0.05$; **, $P < 0.01$.
the observation that the effects of γ1 and STAC3 on membrane expression are not additive, and therefore γ1 does not significantly increase CaV1.1 beyond the level already achieved by STAC3. Apparently, an independent component must be limiting for membrane targeting. The effect of γ1 on membrane targeting in heterologous cells is consistent with a previous immunocytochemistry and charge movement analysis showing that in the absence of STAC3, the γ1 subunit supports robust membrane expression of CaV1.1 in tsA201 cells, while sustaining only very small currents (Polster et al., 2016). In contrast, an earlier Western blot analysis of tsA201 cells lysates reported that coexpression of γ1 reduces the levels of CaV1.1 protein expression (Sandoval et al., 2007). In sum, our results corroborate the findings that the γ1 subunit supports membrane expression of CaV1.1 in heterologous cell systems in a splice variant–independent manner, possibly by masking retention motifs on the C-terminus (Niu et al., 2018), but without adding to the calcium influx.

The γ1 subunit promotes steady-state inactivation in CaV1.1α and CaV1.1e

Functionally, the two negative actions of γ1 on CaV1.1 currents dominate. The observed decrease in current amplitude and left-shift of steady-state inactivation are in general agreement with previous studies in muscle cells (Ahern et al., 2001; Freise et al., 2000) as well as in tsA201 cells expressing CaV1.1α (Polster et al., 2016). Limiting calcium influx through CaV1.1 during muscle activity is tolerable because of the principal role of CaV1.1 as a voltage sensor in skeletal muscle EC coupling (Schneider and Chandler, 1973; Rios and Brum, 1987). At the same time, it is important to limit interference of calcium influx with other calcium signaling events, such as those regulating fiber type specificities, and to avoid adverse effects of calcium overload on mitochondrial integrity (Sultana et al., 2016). Previously, we pointed out how intrinsic mechanisms in the CaV1.1 IVS3 subunit and the actions of auxiliary subunits cooperate in limiting the calcium currents in skeletal muscle (Tuluc et al., 2009; Flucher et al., 2005). Whereas the δ γ1 subunit slows down the activation, the γ1 subunit promotes voltage-dependent inactivation at more negative voltages. This effect was equally observed in the adult and, as shown here for the first time, the embryonic splice variant. Together with the observed increase in membrane targeting, this is the first experimental evidence demonstrating that the γ1 subunit functionally interacts with the embryonic splice variant CaV1.1e. Therefore, this modulatory effect is independent of the length of the extracellular loop connecting helices IVS3 and IVS4.

The γ1 subunit reduces the current amplitude specifically in CaV1.1α

The most interesting finding of this study is the differential down-regulation of calcium currents in CaV1.1α versus CaV1.1e. The small current size is one of the hallmarks of skeletal muscle calcium currents. Our results demonstrate that the γ1 subunit is a major determinant of this reduced current density. Whereas in skeletal muscle the adult and embryonic CaV1.1 splice variants differ substantially in voltage dependence of current activation and in current size, the currents recorded in the HEK cells (stably expressing αCaV1.1, β3, and STAC3) reproduced the difference in V1/2 of activation, but not in current density. Apparently, this difference is due to the lack of one or more muscle-specific factors in the heterologous expression system. As coexpression of γ1 restored the reduced current density in CaV1.1α compared with CaV1.1e, the γ1 subunit is such a factor. Quantitatively, the difference in current density between the two splice variants was still smaller than that observed when the same constructs were expressed in dysgenic myotubes (Tuluc et al., 2016; Tuluc et al., 2009). Therefore, it is likely that other modulatory mechanisms present in the native environment of the channel in the skeletal muscle triads contribute to the full expression of this splice variant–specific difference. The γ1 subunit is one of two proteins shown to differentially modulate the current properties of the two CaV1.1 splice variants, along with RYR1 (Benedetti et al., 2018), demonstrating the importance of the native cellular environment for the accurate expression of physiological current properties. Notably, γ1 does not reduce the current density of CaV1.1α by decreasing its plasma membrane expression. As previously shown, CaV1.1e has a higher open probability than CaV1.1α in skeletal myotubes (Tuluc et al., 2009). Therefore, the most likely explanation is that γ1 decreases the channel’s maximal open probability in a splice variant–specific manner.

The sole difference in the primary structure between the embryonic and adult splice variants is the inclusion of 19 amino acids coded in exon 29 in the IVS3-S4 loop of CaV1.1α. Apparently, this difference determines the action of the γ1 subunit on current size. There are two possible mechanisms how inclusion of exon 29 can enable this functional interaction with γ1: direct interactions between the IVS3-S4 loop and γ1 or the stabilization of a conformation of the channel complex by inclusion of exon 29 that renders CaV1.1α susceptible to this particular γ1 modulation. As the first possibility is amenable to experimental testing, we examined it by identifying and mutating putative interaction sites on both channel subunits. However, none of these ion pairs seemed to be essential for the current-reducing action of γ1. This result is in agreement with previous findings showing that CaV1.1 current reduction is mediated by the first two transmembrane domains of γ1, and that the extracellular loop is dispensable for this interaction (Arikkath et al., 2003). Therefore, it is unlikely that this effect is mediated by the direct interaction of the γ1 subunit with the IVS3-S4 loop, although our experiments do not entirely rule out this possibility.

Given that the CaV1.1 structure identified the II and III transmembrane domains of γ1 as the ones involved in the interaction with the IVS3-S4 of CaV1.1 (Wu et al., 2016), and that the first two transmembrane domains of γ1 were sufficient for reconstituting the current reduction (Arikkath et al., 2003), we can deduce that an interaction between the second transmembrane domain of γ1 and the IVS4 of CaV1.1 is the most likely scenario for mediating the current-reducing effect. We therefore conclude that insertion of exon 29 into the IVS3-S4 loop alters the conformation of the channel in a way that enables it to respond to the inclusion of γ1 with a reduced current density (Fig. 8 A). Notably, the left-shifted activation in CaV1.1e
compared with CaV1.1a is observed with or without γ1, and the left-shifted inactivation is observed with or without exon 29, whereas the decreased current amplitude requires their cooperation. Evidently, the interdependence of the analyzed gating properties on the IVS3-S4 loop and the γ1 subunit is highly specific. Each of the partners independently exerts its specific action on the voltage dependence of activation and inactivation (Fig. 8 B).

Uncoupling of the effects of the γ1 subunit on current size and inactivation

The finding that the current amplitude of the embryonic variant CaV1.1e is not modulated by the γ1 subunit, unlike the adult isoform, is surprising. In fact, in a previous study in γ1 knockout mice, it was reported that the difference in current density between wild type and knockout mice is age dependent, as it was detected only in mice <4 wk of age, but not in older animals (Held et al., 2002). However, this observation cannot be explained by the differential current regulation of CaV1.1a and CaV1.1e reported here. There is no evidence that primary cultures derived from muscles at different times after birth express different ratios of CaV1.1e and CaV1.1a. Moreover, if there were such differences, muscles of mice at ≥4 wk would be expected to express predominantly the adult isoform and thus be more susceptible to modulation by γ1 than younger muscles, not the opposite (Tang et al., 2012; Sultana et al., 2016; Tuluc et al., 2009).

More importantly, unlike the age-dependent reduction in current amplitude, in muscles of γ1 knockout mice, the shift in the steady-state inactivation was found to be age independent (Held et al., 2002), suggesting that these two functional effects of the γ1 subunit are not coupled with each other. Here, we observed a similar lack of coupling of the two γ1 effects in CaV1.1e, which is subject to the shift in steady-state inactivation but not to the reduction in current density in cells coexpressing γ1. Together, these data strongly suggest that the two γ1 functional effects are independent of each other and possibly mediated by different domains.

Figure 8. Model of differential γ1 modulation on CaV1.1a and CaV1.1e currents and its consequences for retrograde coupling. (A) In both CaV1.1 splice variants, the γ1 subunit limits calcium currents by shifting the voltage dependence of inactivation to more hyperpolarizing potentials and rendering inactivation more complete. Inclusion of exon 29 in the extracellular IVS3-S4 loop stabilizes a conformation of the CaV1.1a channel complex, which enables the γ1 subunit to reduce the current amplitude. (B) The IVS3-S4 loop including exon 29 and the γ1 subunit require each other for reducing the current amplitude. In contrast, this cooperation is not required to shift the voltage dependence of activation and inactivation, which occurs in a splice variant–dependent manner. (C) In skeletal muscle cells, the negative regulation of calcium currents by the γ1 subunit is a prerequisite of retrograde current amplification by the RYR1 in CaV1.1a (red arrow from RYR1 to γ1; Grabner et al., 1999; Nakai et al., 1996). Without exon 29 in embryonic CaV1.1e, no γ1-dependent reduction of current amplitude and no RYR1-dependent relief of this inhibition occurs (Benedetti et al., 2015). The red loop in CaV1.1a indicates inclusion of exon 29.
The role of the γ1 subunit in orthograde and retrograde coupling of CaV1.1 and RYR1

The γ1 subunit was previously demonstrated to be dispensable for EC coupling, i.e., the orthograde coupling between CaV1.1 and the RYR1. In fact, in γ1-null myotubes, neither the amplitude nor the voltage dependence of the calcium transients was affected (Ahern et al., 2001; Freise et al., 2000). Likewise, calcium release was unaffected in γ1-null myotubes, and twitch and tetanic force development of adult γ1-null mice was very similar in both fast and slow muscles (Ursu et al., 2001). However, long-lasting potassium-induced contractures were significantly larger, and the shift of the steady-state inactivation in CaV1.1 currents was shown to translate into a similar shift in the inactivation curve of calcium release of adult skeletal muscle fibers (Ursu et al., 2004). Our finding that γ1 equals shifts the voltage dependence of inactivation of CaV1.1a and CaV1.1e indicates that the corresponding shift in the inactivation curve of calcium release also may be present.

In skeletal muscle, not only does CaV1.1 activate RYR1, but CaV1.1a calcium currents are also augmented by an interaction of its cytoplasmic II–III loop with the RYR1 (Grabner et al., 1999), a phenomenon termed retrograde coupling. Previously we demonstrated that this function is specific for the adult CaV1.1a splice variant (Benedetti et al., 2015). The currents of CaV1.1e are not reduced when the connection with RYR1 is severed. The dependence of the current augmentation by retrograde coupling on inclusion of exon 29 in the IVS3–S4 loop of CaV1.1 mirrors the importance of exon 29 for the current reduction by γ1. Based on the results of the earlier study, we had proposed a mechanistic model according to which retrograde coupling partially relieves the inhibition of CaV1.1 currents by an unknown, exon 29–dependent factor. Our current study suggests that the γ1 subunit may be this inhibitory factor. According to this hypothetical model, in the simultaneous presence of exon 29 and the γ1 subunit, the currents of CaV1.1a are reduced, and this effect is partially counteracted by the interaction with RYR1. If either exon 29 or the γ1 subunit is missing, this inhibition is absent and there is nothing to be relieved by retrograde coupling (Fig. 8 C).

Conclusions

This analysis of the actions of the γ1 subunit on the two splice variants of CaV1.1 in heterologous cells revealed multiple functions of γ1 in membrane targeting and functional modulation of the skeletal muscle calcium channel. Interestingly, some of the γ1 effects are general for both splice variants, while another is specific for the adult CaV1.1a. Inclusion of exon 29 in CaV1.1a appears to allosterically render the channel susceptible to the reduction of its currents by γ1, as well as to the simultaneous relief of this block by RYR1. Newly generated mammalian cell systems proved highly valuable for this type of coexpression study of CaV1.1, but at the same time highlight the multitude of factors involved in shaping the physiological current properties in the native environment of skeletal muscle.

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**Figure S1.** \( \gamma_1 \) Does not affect activation kinetics but shows a trend of accelerated inactivation kinetics. (A–D) Time constants of activation of Ca\(_{v1.1a}\) (blue, \( n = 19 \)), Ca\(_{v1.1a} + \gamma_1\) (dark blue, \( n = 9 \)), Ca\(_{v1.1e}\) (red, \( n = 12 \)), and Ca\(_{v1.1e} + \gamma_1\) (dark red, \( n = 13 \)) of a monoexponential and biexponential fit (Ca\(_{v1.1e}\)) on the rising phase of the inward calcium current during a 500-ms depolarization to \( V_{\text{max}} \). (A) Example traces of 500-ms depolarization to \( V_{\text{max}} \) in Ca\(_{v1.1a}\) (left) and Ca\(_{v1.1e}\) (right), normalized to the peak current. (B and C) No differences were found between the time constant of activation of Ca\(_{v1.1a}\) or Ca\(_{v1.1e}\) with or without \( \gamma_1 \) coexpression when fitted monoexponentially (B) or between the fast or slow time constant of Ca\(_{v1.1e}\) (\( n = 6 \)) and Ca\(_{v1.1e} + \gamma_1\) (\( n = 7 \)) of the recordings that could be fitted biexponentially (C). Ca\(_{v1.1a}\) and Ca\(_{v1.1a} + \gamma_1\) could be fitted only monoexponentially. (D) The current contribution of the fast component was bigger than that of the slow component in both Ca\(_{v1.1e}\) (slow:fast \( \approx 80:20 \)) and Ca\(_{v1.1e} + \gamma_1\) (slow:fast \( \approx 70:30 \)), but the ratios were similar (P = 0.41). (E and F) Time constant of inactivation of Ca\(_{v1.1a}\) (blue, \( n = 6 \)), Ca\(_{v1.1a} + \gamma_1\) (dark blue, \( n = 5 \)), Ca\(_{v1.1e}\) (red, \( n = 6 \)), and Ca\(_{v1.1e} + \gamma_1\) (dark red, \( n = 5 \)) of a monoexponential fit on the decay phase of the inward calcium current during a 45-s depolarization to \( V_{\text{max}} \). (E) Example traces of 45-s depolarization to \( V_{\text{max}} \) in Ca\(_{v1.1a}\) and Ca\(_{v1.1e}\), normalized to the peak current. (F) The time constant of inactivation was accelerated by 33% (not significant) when Ca\(_{v1.1a}\) (P = 0.32) or Ca\(_{v1.1e}\) (P = 0.15) was coexpressed with \( \gamma_2 \). Mean ± SEM; P values were calculated with Student’s t test.