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Original Article

Calcium channel γ_6 subunits are unique modulators of low voltage-activated (Cav3.1) calcium current

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Abstract

The calcium channel gamma (γ) subunit family consists of eight members whose functions include modulation of high voltage-activated (HVA) calcium currents in skeletal muscle and neurons, and regulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propanoic acid (AMPA) receptor targeting. Cardiac myocytes express at least three γ subunits, γ_4, γ_6 and γ_7, whose function(s) in the heart are unknown. Here we compare the effects of the previously uncharacterized γ_6 subunit with that of γ_4 and γ_7 on a low voltage-activated calcium channel (Cav3.1) that is expressed in cardiac myoctyes. Co-expression of both the long and short γ_6 subunit isoforms, γ_{6L} and γ_{6S}, with Cav3.1 in HEK-293 cells significantly decreases current density by 49% and 69%, respectively. Two other γ subunits expressed in cardiac myocytes, γ_4 and γ_7, have no significant effect on Cav3.1 current. Neither γ_{6L}, γ_{6S}, γ_4 nor γ_7 significantly affect the voltage dependency of activation or inactivation or the kinetics of Cav3.1 current. Transient expression of γ_{6L} in an immortalized atrial cell line (HL-1) significantly reduces the endogenous low voltage-activated current in these cells by 63%. Green fluorescent protein tagged γ_{6L} is localized primarily in HEK-293 cell surface membranes where it is evenly distributed. Expression of γ_{6L} does not affect the level of Cav3.1 mRNA or the amount of total Cav3.1 protein in transfected HEK-293 cells. These results demonstrate that the γ_6 subunit has a unique ability to inhibit Cav3.1 dependent calcium current that is not shared with the γ_4 and γ_7 isoforms and is thus a potential regulator of cardiac low voltage-activated calcium current.

Keywords: T-type calcium current; Low voltage-activated calcium channel; γ Subunit; HEK-293 cells; HL-1 cells

1. Introduction

Calcium channels are multimeric proteins consisting of a pore forming α_1 subunit and some combination of auxiliary β, $\alpha_2\delta$ and γ subunits [1]. Multiple genes and gene splice variants encode members of each subunit family. The extensive biophysical and pharmacological diversity of native calcium currents can be attributed to variation in their molecular identities [1–5]. While the main characteristics of calcium channels are determined by the identity of the α_1 subunit, the auxiliary β and $\alpha_2\delta$ subunits are thought to regulate channel insertion into the surface membrane and to modify α_1 subunit gating particularly for members of the Cav1 and Cav2 α_1 subunit gene families [6–8]. The role of the γ subunits in modifying calcium channel function is still controversial. A number of studies indicate that γ subunits modulate calcium current in skeletal muscle and neurons [9]. However, other evidence demonstrates that some, but not all, γ subunits act as regulators of neuronal alpha-amino-3-hydroxy-5-methylisoxazole-4-propanoic acid (AMPA) receptor trafficking and may not regulate calcium current in those cells [10,11].

The calcium channel γ subunit family consists of eight genes that are predicted to produce proteins with four transmembrane segments and intracellular N- and C-terminals (reviewed in [12], see also [13–21]). Only three of these genes (γ_4, γ_6 and γ_7) are robustly expressed in the rat heart

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Abbreviations: AMPA; alpha-amino-3-hydroxy-5-methylisoxazole-4-propanoic acid; CFP; cyan fluorescent protein; GFP; green fluorescent protein; PDZ, PSD-95/DLG/ZO-1.

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[19]. Interestingly, these three isoforms have distinct structural features suggesting that they may have different functional roles in cardiac cells. The γ_4 subunit contains a unique consensus PSD-95/DLG/ZO-1 (PDZ)-binding domain sequence (T-T-P-V) as the last four amino acids at the C-terminus. The γ_4 isoform has been shown to regulate surface membrane localization of AMPA receptors in neurons [11]. The γ_6 subunit does not contain this motif and has a C-terminal region that is significantly shorter than that of γ_4. Moreover, the γ_6 subunit is uniquely expressed in the heart as two isoforms and has an elongated N-terminus that is highly conserved across species [19]. The long isoform of γ_6 contains four transmembrane segments while the short isoform is missing a 46 amino acid sequence that includes the second and third transmembrane domains. Finally, the γ_7 subunit lacks both the PDZ-binding motif of γ_4 and the extensive N-terminal cytoplasmic region seen in γ_6. The significance of the sequence differences seen in γ_4, γ_6 and γ_7, if any, are unknown as is the potential role of these subunits in regulating cardiac myocyte function.

The first identified γ subunit, γ_1, was purified from rat skeletal muscle and found to form a complex with the calcium channel α_{1S} (Cav1.1), β_{1a}, and $\alpha_2\delta_1$ subunits ([13,22–25], see also review in [26]). This combination of subunits produces the high voltage-activated (HVA) L-type calcium current that is the dominant calcium current in adult skeletal muscle. This biochemical interaction was recently shown to be isoform specific since the γ_2 subunit does not incorporate into the skeletal muscle HVA channel [27]. Two groups have successfully silenced the γ_1 subunit gene in mice and both describe similar effects on the HVA calcium current in skeletal muscle cells [28,29]. The most notable phenotype in the γ_1 knockout animals is a significant increase in the density of the HVA calcium current in skeletal muscle and a shift in its steady-state inactivation curve to positive potentials. These observations indicate that the γ_1 subunit has an inhibitory effect on the Cav1.1 calcium current.

Interactions of γ subunits with low voltage-activated (Cav3) channels have not been studied extensively. However, the limited functional studies do indicate that γ subunits have inhibitory effects on the Cav3 channels similar to those described for Cav1 and Cav2 [20]. Extensive investigation of γ subunit interactions with Cav3 channels is needed to provide a complete understanding of the functional significance of the γ subunits in relation to the low voltage-activated calcium current.

As an initial step in understanding the functions of calcium channel γ subunits expressed in cardiac myocytes, we have compared the effects of γ_4, γ_{6L}, γ_{6S} and γ_7 on low voltage-activated calcium currents produced by the expression of the α_1 subunit Cav3.1 (α_{1G}). Cav3.1 is expressed in rat cardiac myocytes and presumably contributes to the generation of the native cardiac LVA calcium current [30]. Our analysis indicates that the γ_6 isoforms have a unique negative effect on Cav3.1 LVA current density that is not seen with either γ_4 or γ_7.

2. Materials and methods

2.1. Cell culture

HEK-293 cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 4 mM L-glutamine, 4.5 g/l glucose and 1.5 g/l Na-bicarbonate (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 100 U/ml:100 µg/ml:250 ng/ml of penicillin/streptomycin/amphotericin B (Life Technologies). HEK-293 cells stably transfected with Cav3.1 were a gift of Dr. L. Cribbs and Dr. E. Perez-Reyes and were maintained in media as above with the addition of 400 µg/ml G418 (Sigma). Cells were stored in a 5% CO_2 incubator at 37 °C and passed every 7 days at a 1:5 dilution. Medium was changed every 3 days.

HL-1 cells were a gift from Dr. William C. Claycomb and were maintained as previously reported [31]. Briefly, HL-1 cells were grown in HL-1 medium containing Claycomb medium (JRH Biosciences) supplemented with 10% fetal bovine serum (JRH Biosciences), 0.1 mM norepinephrine (Sigma), 2 mM L-glutamine (Sigma), and 100 U/ml:100 µg/ml:250 ng/ml of penicillin/streptomycin/amphotericin B (Life Technologies) in a humidified 5% CO_2 incubator at 37 °C. Cells were passed when confluent at a 1:3 dilution into flasks or plates pre-coated with a 1:80 dilution of fibronectin and 0.02% gelatin (Sigma). Cells from passages 86–108 were used for this study.

2.2. Sub-cloning of vectors for transfection in functional and localization studies

For studies on the electrophysiological effects of independently transcribed γ subunits in fluorescence-labeled cells, the coding regions of rat γ_4, γ_{6L}, γ_{6S}, and γ_7 subunit cDNAs [19] were subcloned into pCR II vectors (InVitrogen) by TA cloning. The accession numbers of these previously described genes are as follows: rat γ_4 (AF361341), rat γ_{6L} (AF361343), rat γ_{6S} (AF361344), and rat γ_7 (AF361345). From pCR II vectors, rat γ_4, γ_{6L}, γ_{6S} and γ_7 subunit cDNAs were then transferred to an expression vector (adCGI, or "adenovirus-CMV-GFP-IRES"), a gift of Dr. Jeffrey R. Holt containing a CMV-driven promoter, the coding region of green fluorescent protein (GFP) and an internal ribosomal entry site (IRES). Each target γ subunit cDNA was linked at an EcoRI site immediately after the IRES for independent expression of GFP and specific γ subunit proteins, insuring that cells expressing GFP also expressed γ subunits and that the secondary and/or tertiary structure of the γ subunits was not affected as might be the case with the creation of a GFP-γ fusion protein. For analyzing the sub-cellular localization of γ subunit proteins, expression vectors encoding fusion proteins of GFP and specific γ subunits were made. The coding regions of all γ subunit cDNAs were linked in frame downstream of the GFP cDNA in a pEGFP-C1 vector (BD Clontech). A commercially available vector (pECFP-Mem, Clontech) encoding a modified cyan fluorescent protein (CFP) protein fused to the N-terminal 20 amino acids of the neuromodulin (GAP-43) protein was used as a membrane marker.

2.3. Transfection

Untransfected HEK-293 cells, HEK-293 cell lines stably transfected with Cav3.1, and HL-1 cells were transiently transfected with vectors containing γ subunit, GFP, and modified CFP cDNAs using Lipofectamine 2000 reagent (InVitrogen) as per the manufacturer's recommendations. HEK-293 cells were grown to 70–80% confluence before transfection. HL-1 cells were grown to 50–75% confluence before transfection.

2.4. Electrophysiology

Whole-cell calcium currents were recorded using an Axopatch-1D patch clamp system and Clampex 8.0 software. Pipettes were fabricated from boroscillicate glass and had typical resistances of 2–4 Mω. The pipette solution contained (in mM): 130 NaCl, 10 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 3 Tris–ATP, and either 0.3 Li_2GTP or 0.3 Tris–GTP, adjusted to pH 7.4 with NaOH and 280 mOsm with sucrose. The bath solution contained (in mM): 137 NaCl (for HEK-293 experiments) or 120 TEACl (for HL-1 experiments), 1 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 2 $CaCl_2$ (for HEK-293 experiments) or 5 $CaCl_2$ (for HL-1 experiments), 10 glucose (for HL-1 experiments only), 10 HEPES, and 30 µM TTX (for HEK-293 experiments only), adjusted to pH 7.4 with NaOH (for HEK-293 experiments) or TEAOH (for HL-1 experiments) and 280 mOsm with sucrose. NaCl was used as the major salt in the pipette solution order to eliminate any outward K^+ currents. TTX was added to the bath solution to block Na^+ currents, as a small inward current with kinetics resembling those of previously described Na^+ currents was observed in untransfected parental HEK-293 control cells.

Recordings from HEK-293 cells were made 48–96 h post-transfection, at which time expression, as measured by GFP fluorescence, peaked. HL-1 cells were used for recording 24–36 h post-transfection. Current densities in HEK-293/Cav3.1 cell experiments were normalized to control for variation in current expression in the different frozen stocks of stable cell lines used. The current densities of different frozen stocks used for HEK-239/Cav3.1 experiments ranged from 49.1 to 58.5 pA/pF and were not significantly different from each other. HL-1 experiments were performed using a single stock of cells. All HEK-293 data are from current records in which pipette and whole-cell capacitance were ~100% compensated and series resistance was compensated to >80%. Whole-cell capacitance and series resistance were not compensated for HL-1 recordings as currents were typically <100 pA. Series resistance values were typically <10 MΩ, and no records were used in which the voltage error (as defined by $V_{er} = I_{max} \times R_a$) was greater than 5 mV. The holding potential of all protocols used for HEK-293 cells was –100 mV except for steady-state inactivation protocols where the holding potential was –120 mV. The holding potential for protocols used for HL-1 cells was –90 mV. Current–voltage relationships for all HEK-293 and HL-1 cells were measured using 50 ms test depolarizations. Peak current amplitudes for activation curves were determined by fitting tail currents, elicited by stepping membrane voltage from varying test potentials to –60 mV, with the Boltzman equation. Test potential steps for activation protocols were of varied durations, corresponding to the time to peak of the elicited current. Inactivation curves were determined by fitting peak amplitudes of currents, observed in a test pulse to –20 mV, following 500 ms steps to varying potentials, with the Boltzman equation. Time to peak was determined using the current–voltage protocol described above. Inactivation time constants were determined by fitting the decay phase of currents to an exponential equation and deactivation time constants were determined by fitting the decay phase of tail currents to an exponential equation. Tail currents for Cav3.1 were found to be best fit by a double exponential. Currents were recorded at 40 kHz and on-line filtered at either 5 or 10 kHz. In some cases currents were off-line filtered at 1 kHz to further reduce noise. Current/voltage relationships and tail currents were recorded from HEK cells using an on-line P/-4 subtraction procedure. Calcium currents recorded from HL-1 cells were off-line leak subtracted. The average membrane capacitance of untransfected HEK-293 cells in pilot experiments was 12.5 ± 1.4 pF (n = 8). The capacitance of HL-1 cells ranged from 5.6 to 14.1 pF, with averages of 9.9 ± 0.9 and 8.8 ± 1.4 pF (mean ± S.E.M.) for GFP-transfected and GFP-γ_6 transfected cells (n = 9 for each).

2.5. Statistical analysis

All data are reported as mean ± S.E.M. and tested for significance by one-way ANOVA. A value of $P < 0.05$ was considered significant and when significant P-values were encountered groups were compared using the Tukey post-hoc test.

2.6. RT-PCR

Total RNA from cultured HL-1 cells and ventricular tissue of adult C57B2 mice was used for first strand cDNA synthesis using Superscript II reverse transcriptase (InVitrogen) in the presence of gene-specific primers or oligo-dT. PCR was carried out by cycling 35–39 times at 94 °C (1 min), 56 or 60 °C (30–60 s), and 72 °C (1 min), followed by a 10-min extension step at 72 °C. A ribosomal RNA gene, PHE-7, was used as a housekeeping gene. All PCR products were subcloned and their identities confirmed by sequencing or restriction enzyme digest. For quantitative RT-PCR, the absolute abundance of Cav3.1 mRNA in HEK-293 cells was measured as described [30]. For all RT-PCR experiments a negative RT reaction was used as a control. See Table 1 for primer sets employed in RT-PCR.

Table 1
RT-PCR primers. Primers used for RT-PCR to detect α_1 and γ subunits present in HL-1 cells and for quantitation of Cav3.1 mRNA in HEK-293 cells

Cav3.1 (HL-1)	Fwd: 5′ AAATGGTGGTGAAGATGG 3′
	Rev. 5′ GACGAAGAAACAGAGCAG 3′
Cav3.1 (Quant.)	Fwd: 5′ GAGGACTGATGACCCCAAC 3′
	Rev. 5′ ATGATCCGGTGACACAGG 3′
Cav3.2	Fwd: 5′ TCCCCCGTCTACTTCGTCACCTTC 3′
	Rev. 5′ GCGAGAGCATCCTGGACACAGATAC 3′
Cav1.2	Fwd: 5′ GACCATCGGGAACATCGTAAT 3′
	Rev. 5′ GGAAGAGGCGGAAGAAAGTGA 3′
γ_2	Fwd: 5′ ACC AGC AAG AAG AAC GAG 3′
	Rev. 5′ AGA CAC GAA GAA GAT GCC 3′
γ_3	Fwd: 5′ CAA GTG ACA ATG AAA CCA G 3′
	Rev. 5′ AGA GAC GAA GAA GAT GCC 3′
γ_4	Fwd: 5′ TGA CGA AGA CAA AAA GAA C 3′
	Rev. 5′ TAA GGA GAG GAG GAA GAG G 3′
γ_5	Fwd: 5′ CAT AAT CCT CCC ACA GAA C 3′
	Rev. 5′ TAC ACA GAC ATC ACC CC 3′
γ_6	Fwd: 5′ AGC TGC CAG GAG AAG CAA AC 3′
	Rev. 5′ CTC TGC ACC TTT ACT GAG CAC 3′
γ_7	Fwd: 5′ TGG TGA CGG AAA ACA CGG AG 3′
	Rev. 5′ AGA AAA GAG GAA GCA GCG AAG 3′
γ_8	Fwd: 5′ GGA GTC ATT GAA ACG CTG 3′
	Rev. 5′ TCA TCA CCT GCT GTG AG 3′
PHE-7	Fwd: 5′ CTG CGA AAG GCA AGG AGG AA 3′
	Rev. 5′ TGG CTC CAC GAT CCT CAG CA 3′

2.7. Fluorescent image detection

Expression vectors encoding GFP, GFP-γ_{6L} fusion protein, or modified CFP were transfected using Lipofectamine 2000 reagent (InVitrogen) into HEK-293 cells plated on glass coverslips according to the manufacturers instructions. The modified CFP contained the N-terminal 20 amino acids of neuromodulin (GAP 43, a post-translational membrane localization signal). Following transfection, cells were maintained in a 5% CO_2 incubator at 37 °C for 48–72 h. Cells were then immersed in 4% paraformaldehyde, and washed three times with PBS before being mounted onto microscope slides using a ProLong Antifade kit (Molecular Probes). Cells were viewed on a Zeiss laser scanning confocal microscope with an oil-immersed 63× objective. GFP and CFP were visualized using excitation wavelengths of 488 and 418 nm, respectively. Images were obtained by *z*-sections with a step size of ~0.5 µm and analyzed using LSM 510 software (Carl Zeiss).

2.8. Western blot

Untransfected HEK-293 or HEK-293/Cav3.1 stably transfected cells were grown in 10-cm tissue culture dishes and transiently transfected when approximately 70% confluent. Twenty micrograms of each plasmid expression vector was transfected per 10-cm dish using Lipofectamine 2000 reagent (InVitrogen) as per the manufacturer's recommendations. Cells were harvested 72 h post-transfection and processed for immunoblot analysis according to Costagliola et al. [32] with some modification. Briefly, the cells were scraped from the plates in HBSS and centrifuged for 5 min at 60 × *g*. All subsequent manipulations were done at 4 °C. The cell pellets were resuspended in 1.0 ml of homogenization buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris pH 7.5, 10% glycerol) supplemented with protease inhibitors (50 µg/ml leupeptin, 50 µg/ml aprotinin, 2.5 µg/ml pepstatin, 0.4 µM PMSF, 5 mM NaF and 8 µg/ml calpeptin). Samples were processed for 3 min using a motor driven homogenizer with a Teflon pestle. Cell lysates were centrifuged for 10 min at 600 × *g* to remove nuclei and debris. The resulting supernatant was centrifuged again for 5 min at 15,000 × *g* to obtain the P2 fraction (mitochondrial and lysosomal fraction). The remaining supernatant was centrifuged for 45 min at 125,000 × *g* to obtain the P3 fraction (microsomal/plasmalemmal). Membrane fractions were resuspended in 400 µl of solubilization buffer (100 mM $(NH_4)_2SO_4$, 20 mM Tris pH 7.5, 10% glycerol, 1% *N*-dodecyl-maltoside and protease inhibitors listed above). Protein concentration of cell extracts was determined using BCA (Pierce, Rockford, IL). Protein samples (25 µg) were separated by SDS-PAGE on a 6% or 10% acrylamide gel containing 0.4% SDS to enhance resolution of membrane proteins. Samples were electrophoretically transferred to PVDF membrane and subjected to immunoblot analysis according to standard protocols. Primary antibody for Cav3.1 was obtained from Alomone Laboratories (Jerusalem, Israel) and used at a concentration of 0.4 µg/ml. Monoclonal antibody GFP β2, which cross-reacts with CFP (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 0.2 µg/ml. All incubations were done at room temperature in 1% milk/0.2% Tween 20 in TBS. The secondary antibodies, HRP conjugated goat anti-rabbit IgG and HRP conjugated goat anti-mouse IgG, for Cav3.1 and GFPβ2 antibodies, respectively (Zymed, South San Francisco, CA), were diluted 1:20,000 in incubation solution. Protein products were visualized by ECL-Plus (Amersham/Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions and analyzed by phosphorimager detection.

3. Results

3.1. Effects of γ subunits on current through heterologously expressed LVA calcium channels

Representative current traces elicited from a HEK-293/Cav3.1 cell show the typical voltage dependency and kinetics of Cav3.1 calcium current (Fig. 1A, Table 2). Averaged, normalized current–voltage relationships for the HEK-293/Cav3.1 cell line transiently transfected with either the blank adCGI vector, adCGI-γ_{6L} or adCGI-γ_{6S} show that co-expression of the γ_6 subunits causes a significant decrease in peak calcium current. Co-expression of both γ_{6L} and γ_{6S} significantly reduced peak current density through Cav3.1 without significantly affecting the voltage at which peak current occurred. Peak current density decreased to 51% ± 17% of control value ($n = 10$) in γ_{6L} transfected cells ($P < 0.05$, Fig. 1B, C). A similar effect was seen with γ_{6S}, where mean peak current density decreased 69% (to 31% ± 13% of control value, $n = 5$, $P < 0.01$). In contrast, neither γ_4 nor γ_7 had a statistically significant effect on mean peak current density (mean percentages of control value were 93% ± 7%, $n = 10$, $P > 0.05$, and 99% ± 8%, $n = 11$, $P > 0.05$, for γ_4 and γ_7, respectively). Doubling the amount of γ_4 DNA transfected into HEK-293/Cav3.1 cells resulted in neither a significant reduction nor enhancement of peak current (128% ± 19% of control value, Fig. 1C) suggesting that the failure of γ_4 to reduce peak calcium current was not due to limited availability of this subunit. While there appears to be a slight hyperpolarizing shift in V-peak with γ_{6L} co-expression it is within the limits of voltage error and is not statistically significant (see Table 2).

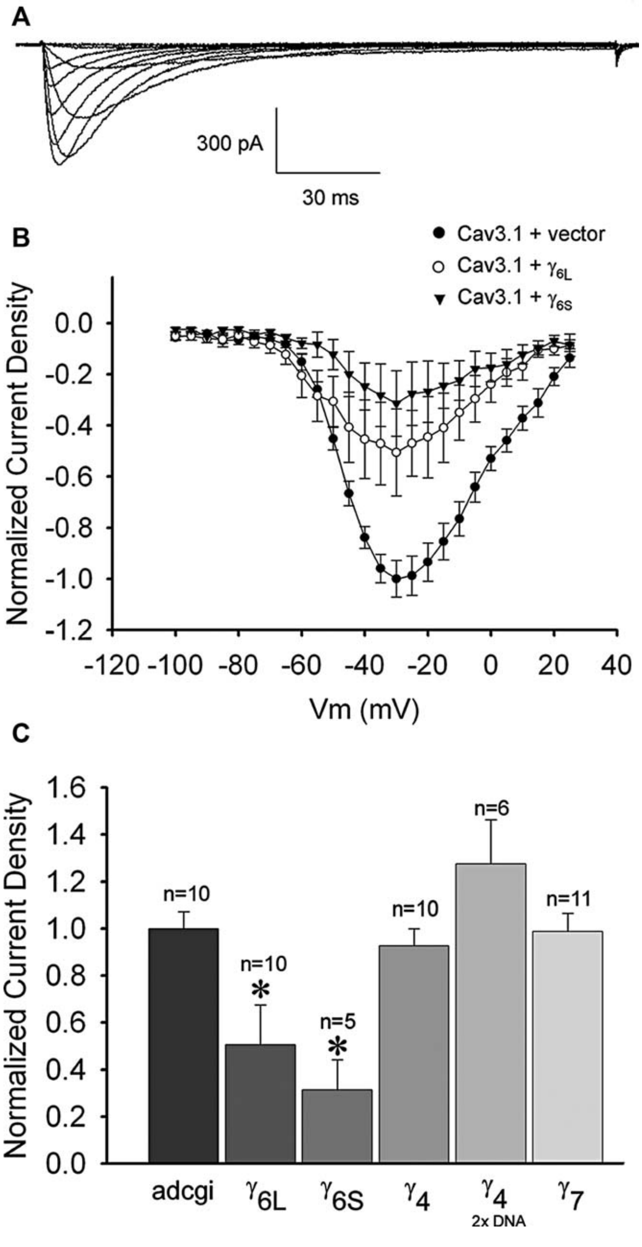

Fig. 1. Only the γ_6 subunit decreases Cav3.1 dependent LVA currents in HEK-293 cells. A: Representative whole-cell current traces from an individual HEK-293 cell stably transfected with Cav3.1. Traces were elicited by stepping membrane voltage from a holding potential of –100 to +10 mV, in 10 mV increments, for 250 ms. B: Average normalized current–voltage relationships from HEK-293/Cav3.1 transiently transfected with a bicistronic vector (adCGI) expressing either GFP, GFP plus γ_{6L}, or GFP plus γ_{6S}. C: Average normalized peak current density of HEK-293/Cav3.1 transiently transfected with either GFP, GFP plus γ_{6L}, GFP plus γ_{6S}, GFP plus γ_4, GFP plus γ_4 using a twofold increase in DNA concentration or GFP plus γ_7. Only the γ_6 isoforms significantly decrease LVA current density in these cells.

In addition to decreasing peak current density it has been reported that γ subunits alter the voltage dependency of inactivation and the time course of inactivation of HVA currents [28,33,34]. However, analysis of the LVA currents recorded from HEK-293/Cav3.1 cells demonstrate that co-expression of the γ_4, γ_{6L}, γ_{6S}, and γ_7 subunits did not alter current kinetics or voltage dependency (Fig. 2, Table 2). Averaged data for $V_{\max}$, the voltage at which peak current occurred, the $V_{0.5}$ and k for the voltage dependencies of activation and inactivation, the time to peak current at –20 mV, the time constant of inactivation at –20 mV, and the fast time constant of deactivation at –100 mV show no statistically significant differences (Table 2). The slow component of the time constant for deactivation accounted for less than 10% of the deactivation curve, did not show a consistent voltage dependency, and is not reported. Similarly there are no significant differences in the voltage dependency of inactivation nor the voltage dependency of the time constant of deactivation for HEK293/Cav3.1 cells expressing either adCGI vector only, γ_4, γ_{6L}, γ_{6S}, or γ_7.

Thus the γ_6 subunit isoforms have a unique ability, as compared to γ_4 and γ_7, to decrease Cav3.1 dependent LVA current density in stably transfected HEK-293 cells without modifying the biophysical properties of the remaining current.

3.2. *Effects of γ_{6L} on endogenous LVA current in a cardiac cell line*

To study the effects of γ subunits on an endogenous LVA current we used HL-1 cells, a cell line derived from immortalized mouse atrial myocytes. Analysis of the α_1 subunit mRNAs in HL-1 cells by RT-PCR (Fig. 3A) shows that HL-1 cells express both Cav3.1 and Cav3.2. Consistent with the observation that this sub-line of HL-1 cells did not express measurable HVA current, no Cav1.2 mRNA was detected (data not shown). We also used RT-PCR to determine whether murine HL-1 cells express endogenous γ subunits. Only the γ_7 subunit mRNA was detected in HL-1 cells (Fig. 3B). Results were normalized to the expression of PHE-7, a constitutively active ribosomal housekeeping gene. As a positive control we used mRNA from mouse brain and ventricle, tissues known to express Cav3.1 and Cav3.2 as well as γ_2–γ_8. No products were amplified from HL-1 cells

Table 2
Effects of γ subunits on the biophysical properties of Cav3.1 dependent currents in HEK-293 cells. There are no significant differences in the voltage at peak current, voltage dependency, or kinetic parameters between cells transiently transfected with vector as compared to cells transfected with any of the γ subunits studied

V peak (mV)	Cav3.1 + adcgi	Cav3.1 + g6l	Cav3.1 + g6s	Cav3.1 + g4	Cav3.1 + g7
	–29.5 ± 0.9 (*n* = 10)	–31.1 ± 3.1 (*n* = 9)	–27.0 ± 2.5 (*n* = 5)	–30.6 ± 1.5 (*n* = 9)	–24.1 ± 0.9 (*n* = 11)
Activation					
V 0.5 (mV)	–32.3 ± 1.5 (*n* = 9)	–36.3 ± 6.2 (*n* = 4)	–36.3 ± 6.2 (*n* = 4)	–37.3 ± 0.9 (*n* = 8)	–28.1 ± 1.2 (*n* = 11)
k	13.3 ± 0.3 (*n* = 9)	12.7 ± 1.5 (*n* = 4)	12.7 ± 1.5 (*n* = 4)	11.8 ± 0.5 (*n* = 8)	11.1 ± 0.3 (*n* = 11)
Time to peak @ –20 mV (ms)	5.7 ± 0.4 (*n* = 10)	6.0 ± 0.8 (*n* = 5)	6.4 ± 0.5 (*n* = 3)	5.2 ± 0.5 (*n* = 9)	7.0 ± 0.4 (*n* = 11)
Inactivation					
V 0.5 (mV)	–71.9 ± 1.4 (*n* = 8)	–78.5 ± 2.6 (*n* = 6)	–72.0 ± 5.9 (*n* = 3)	–79.2 ± 1.5 (*n* = 7)	–68.6 ± 1.4 (*n* = 10)
k	5.4 ± 0.1 (*n* = 8)	5.4 ± 0.3 (*n* = 6)	5.9 ± 0.7 (*n* = 3)	6.1 ± 0.2 (*n* = 7)	6.0 ± 0.1 (*n* = 10)
Tau @ –20 mV (ms)	19.8 ± 1.3 (*n* = 8)	18.6 ± 1.1 (*n* = 6)	22.9 ± 0.7 (*n* = 2)	15.8 ± 0.7 (*n* = 7)	17.2 ± 0.5 (*n* = 10)
Deactivation tau @ –100 mV (ms)					
Fast	1.19 ± 0.19 (*n* = 9)	1.54 ± 0.48 (*n* = 4)	1.20 ± 0.06 (*n* = 3)	1.84 ± 0.28 (*n* = 8)	1.30 ± 0.10 (*n* = 11)

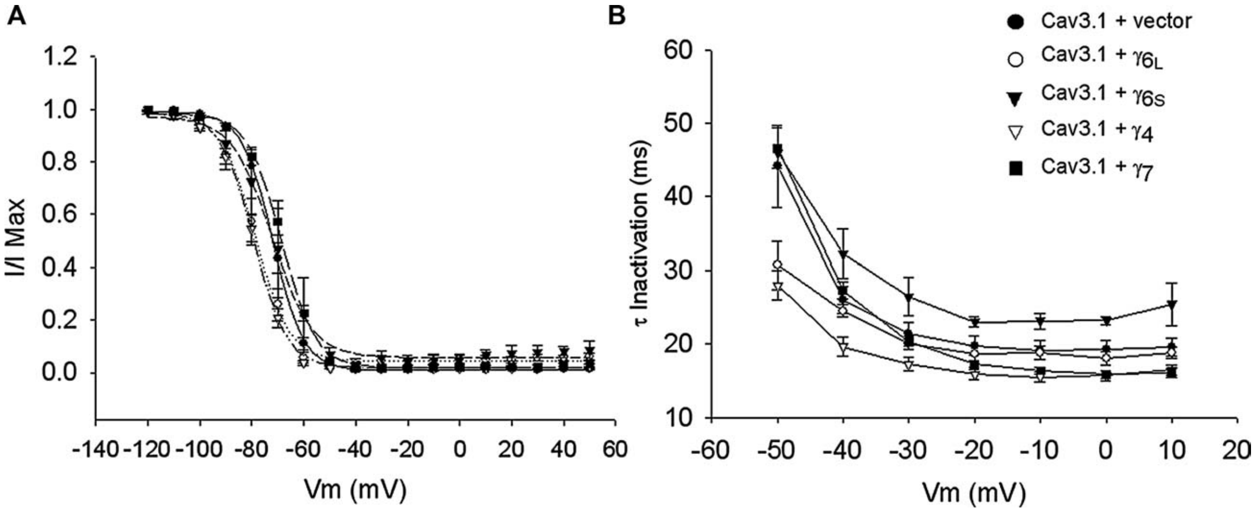

Fig. 2. Effects of γ subunits on inactivation properties of Cav3.1 expressed in HEK-293 cells. A and B: Average inactivation curves and average voltage dependence of the time constant of inactivation, respectively, from HEK-293/Cav3.1 and transiently transfected with GFP only, GFP plus γ_{6L}, GFP plus γ_{6S}, GFP plus γ_4, and GFP plus γ_7. None of the γ subunits significantly affected the inactivation properties of Cav3.1 dependent LVA current in HEK-293 cells.

using primer sets for murine γ_2, γ_3, γ_4, γ_5 or γ_8 (data not shown).

As with the HEK-293/Cav3.1 cells, HL-1 cells transfected with γ_6 but not γ_4 showed a significant decrease in LVA calcium current. Representative LVA current traces, averaged *I–V* relationships and averaged peak current densities for untransfected HL-1 cells and HL-1 cells transfected either with adCGI vector only, γ_{6L}, or γ_4 are shown in Fig. 4. The inhibitory effects of γ_{6L} on average LVA current density was even more pronounced in HL-1 cells than in HEK-293/Cav3.1 cells. LVA current density in HL-1 cells was reduced by 63% from control value ($P < 0.01$) following transfection with γ_{6L}. Current density of untransfected HL-1 cells was 9.59 ± 0.98 pA/pF, $n = 13$, HL-1 + vector was 9.18 ± 1.79 pA/pF, $n = 9$, HL-1 + γ_{6L} was 3.37 ± 0.62 pA/pF, $n = 9$, and HL-1 + γ_4 was 9.14 ± 1.34 pA/pF, $n = 10$. Current levels in HL-1 cells transiently transfected with γ subunit mRNA were too low to allow detailed analysis of voltage dependency and current kinetics.

3.3. Cellular localization of γ_{6L} in single HEK/Cav3.1 cells

Both GFP tagged γ_{6L} and plasma membrane specific CFP-neuromodulin were transiently transfected into HEK-293/Cav3.1 cells and visualized for cellular location by confocal microscopy (Fig. 5). Panel 5A is a bright field image of a HEK-293 cell expressing both GFP-γ_{6L} and CFP-neuromodulin. Using separate filter sets, the expression of GFP-γ_{6L} and CFP-neuromodulin was imaged independently (5B, C) showing that both the γ fusion protein and the plasma membrane marker are closely associated with the cell surface. This observation is supported by the overlay of panels 5B and C, which shows co-localization of the two proteins (5D). Thus, GFP-γ_{6L} and the CFP-neuromodulin exhibited similar distributions in transfected cells confirming that γ_{6L} is localized predominantly in or near the plasma membrane. Panels 5E and F show composite and cross-sectional images, respectively, of an individual HEK-293 cell expressing GFP alone demonstrating that GFP is uniformly expressed

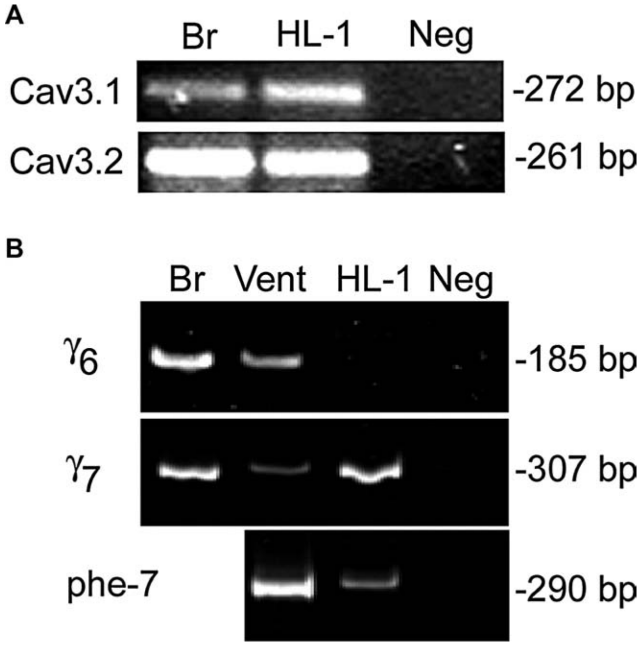

Fig. 3. Detection of α_1 and γ subunits mRNAs in HL-1 cells by RT-PCR. A: Cav3.1 (α_1G) and Cav3.2 (α_1H) are both expressed in HL-1 cells. B: HL-1 cells express only the γ_7 subunit and not the γ_6 subunit. Additionally, no expression of γ_4 was detected in HL-1 cells (data not shown). Whole rat brain, which is known to express Cav3.1 and Cav3.2 as well as γ_6 and γ_7, was used as a positive control, and a ribosomal RNA gene, PHE-7, was used as a housekeeping gene. No RT was used as a negative control.

throughout the cell. Panels 5G and H show composite and cross-sectional images, respectively, of an individual HEK-293 cell expressing GFP-γ_{6L}, demonstrating its localization at or near the plasma membrane. Panels 5I and J show composite and cross-sectional images, respectively, of an individual HEK-293 cell expressing GFP-γ_7. γ_7 is distributed throughout the cell in small distinct regions in contrast to GFP-γ_{6L} which is evenly distributed at the plasma membrane. The localization of GFP, GFP-γ_{6L} and GFP-γ_7 was also observed in HEK-293 cells not stably transfected with Cav3.1 and found to be the same as for HEK-293/Cav3.1 cells (data not shown).

3.4. Western blot analysis of GFP-tagged γ subunits in cytosolic and membrane fractions

To confirm that γ subunits are membrane associated, GFP-fusion constructs encoding either GFP-γ_{6L}, GFP-γ_7 or GFP-γ_4 were transfected into HEK-293/Cav3.1 cells. Proteins were detected using a monoclonal antibody to GFP. The anti-GFP antibody detected appropriately sized fusion proteins for GFP-γ_{6L}, GFP-γ_7 and GFP-γ_4 predominantly in cell membrane fractions (Fig. 6). The adCGI vector itself produces only the 30 kDa vectorial GFP protein that is detected mostly in the cytosolic fraction. No products were visualized in the extracts from HEK-293/Cav3.1 untransfected parent cells. Similar results were also seen when these constructs were transfected in HEK-293 parental cells not stably transfected with Cav3.1 (data not shown).

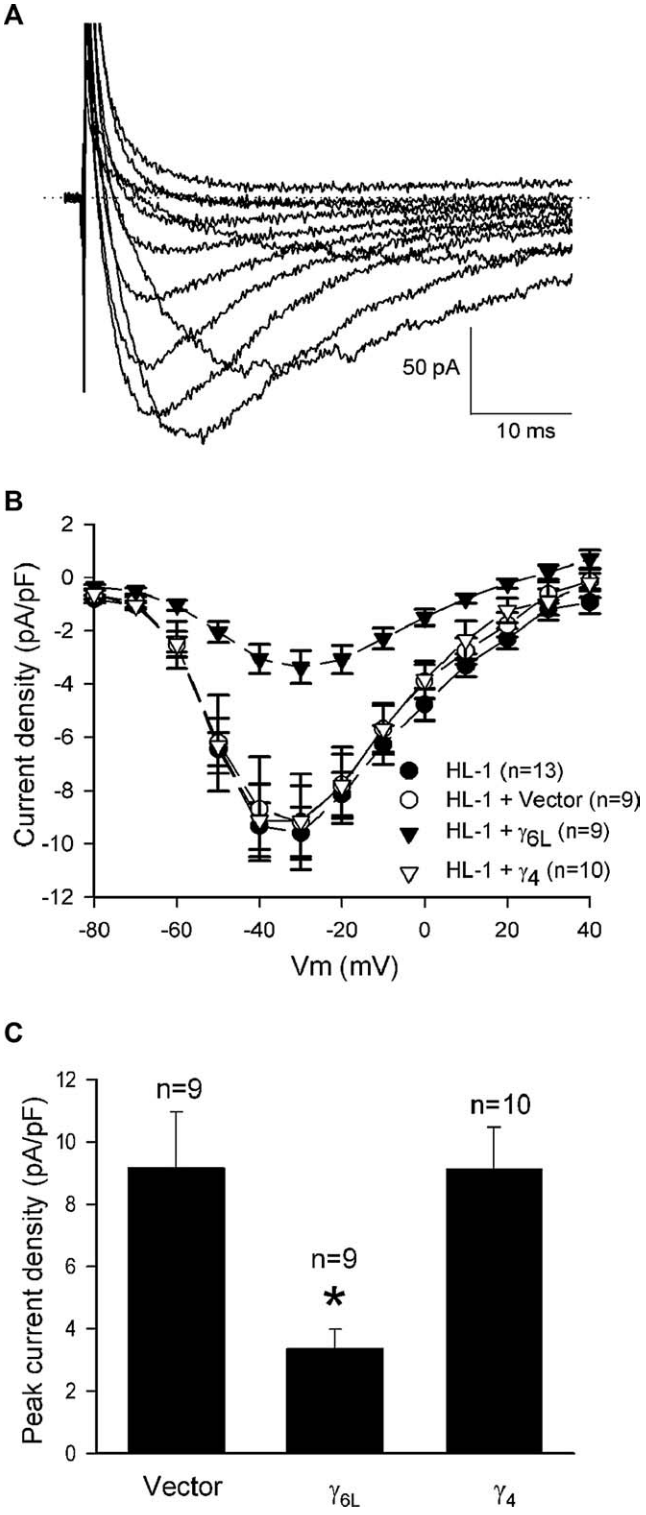

Fig. 4. γ_{6L} decreases endogenous LVA calcium current density in HL-1 cells. A: Sample whole-cell current traces from an untransfected HL-1 cell. Traces were elicited by stepping membrane potential from a holding potential of –90 to +10 mV in 10 mV increments. B and C: Average current–voltage relationships and peak current density, respectively, from untransfected HL-1 cells, from cells transfected with GFP only, and from cells transfected with GFP plus γ_{6L}. γ_{6L} significantly reduced endogenous LVA current as compared to vector control, without altering the voltage at which current peaks.

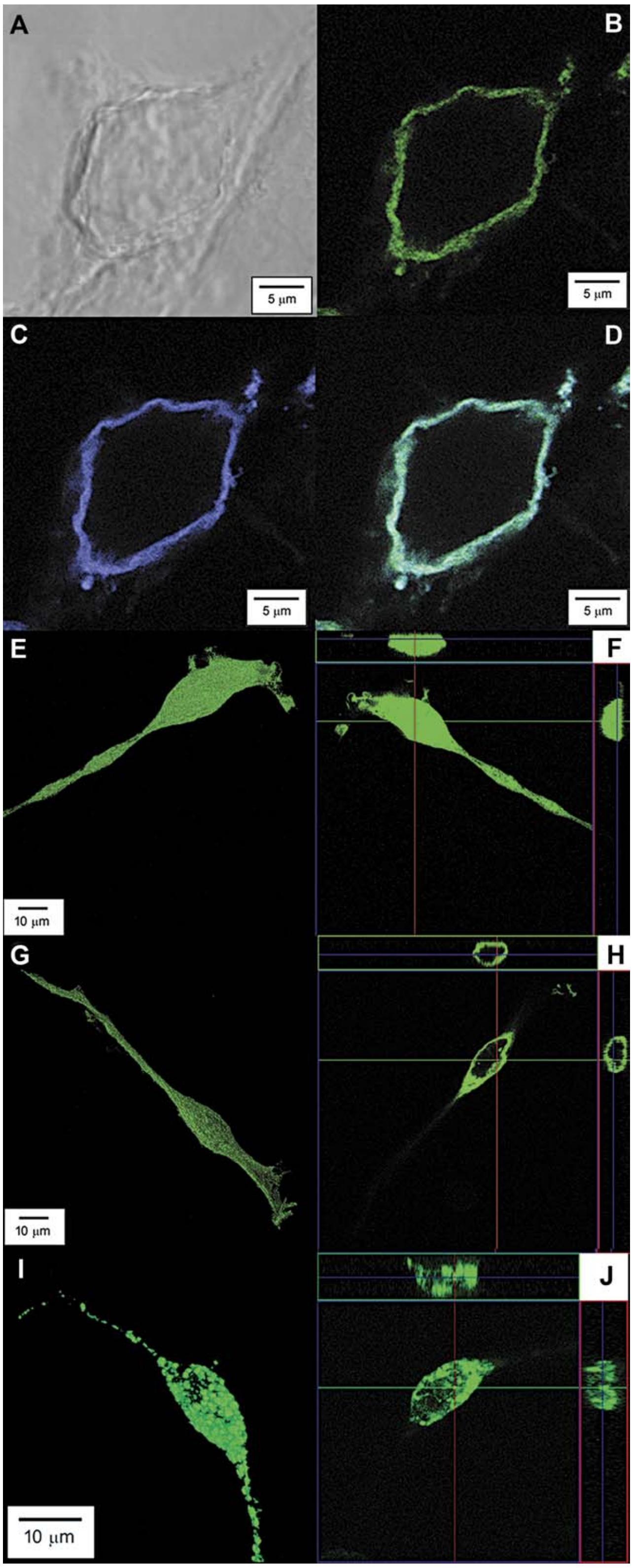

Fig. 5. γ_{6L} is localized in or near the plasma membrane. A: Bright field image of individual HEK-293/Cav3.1 cells transfected with a vector encoding a GFP-γ_{6L} fusion protein and a vector encoding a CFP protein that is targeted to cell membranes by a neuromodulin fragment. B: Same cell seen in panel A showing the distribution of the GFP-γ_{6L} fusion protein. C: Same cell seen in panel A showing the distribution of CFP–neuromodulin. D: Composite image of panels B and C showing the co-localization of GFP-γ_{6L} and CFP. Composite confocal microscopic images of representative HEK-293 cells transiently transfected with either GFP only, a GFP-γ_{6L} fusion construct, or a GFP-γ_7 fusion construct (E–J). E: Cell transfected with GFP only. G: Cell transfected with GFP-γ_{6L}. I: Cell transfected with GFP-γ_7. F, H, and J: Single horizontal cross-sectional images of cells seen in E, G, and I, respectively. GFP expressed alone was evenly distributed throughout the cell. GFP-γ_{6L} appears to be localized to the plasma membrane, where it is evenly distributed. However, GFP-γ_7 is distributed in small distinct regions throughout the cell. The localization of GFP, GFP-γ_{6L}, and GFP-γ_7 was the same in HEK-293 parental cells as in HEK-293/Cav3.1 cells (not shown).

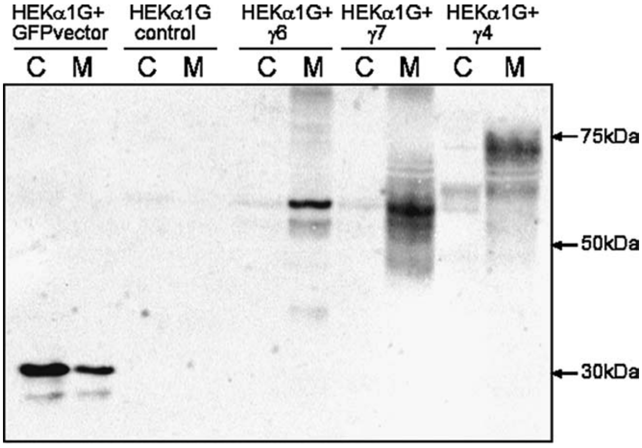

Fig. 6. Membrane localization of GFP-γ_{6L}, GFP-γ_7, and GPF-γ_4 in HEK-293/Cav3.1. Western blot of cytosolic (C) and total membrane (M) fractions of HEK-293/Cav3.1 cells transfected with GFP and cells transfected with GFP fusion constructs for either γ_{6L}, γ_7, or γ_4. Proteins were visualized using a monoclonal antibody to GFP. The GFP antibody detects appropriately sized fusion proteins for GFP-γ_{6L}, GFP-γ_7, GFP-γ_4 almost exclusively in the total membrane fraction of the cell lysate. When expressed alone, GFP protein is predominately expressed in the cytosolic fraction. No significant bands are seen in protein extracts from control cells (blank vector). Similar results were seen in HEK-293 parental cells, which were not stably transfected with Cav3.1 (data not shown).

3.5. Effects of γ subunit co-expression on Cav3.1 mRNA and protein

We investigated whether changes in either Cav3.1 mRNA or protein levels can explain the decrease in Cav3.1 current when co-expressed with γ_6. Quantitative RT-PCR was performed to monitor the amount of Cav3.1 mRNA expressed in untransfected HEK-293 cells and HEK-293/Cav3.1 cells transfected either γ_4, γ_{6L} or γ_7 (Fig. 7). Over-expression of γ_4, γ_{6L} and γ_7 had no significant effect ($P > 0.05$) on the level of Cav3.1 message as compared to transfection with adCGI vector alone in HEK-293/Cav3.1 cells. Amounts of Cav3.1 transcript ranged from $1.0 \pm 0.24 \times 10^{+5}$ molecules of Cav3.1 mRNA per µg of total cellular RNA (vector only) to $0.83 \pm 0.11 \times 10^{+5}$ (γ_4 over-expression), $1.3 \pm 0.14 \times 10^{+5}$ (γ_{6L} over-expression) and $1.3 \pm 0.12 \times 10^{+5}$ (γ_7 over-expression). No appreciable amount of Cav3.1 mRNA was detected in parental HEK-293 cells. These results demonstrate that transient transfection with γ_6 does not affect Cav3.1 gene transcription in HEK-293 cells.

To determine whether the γ subunits modulate Cav3.1 protein levels, protein samples from HEK-

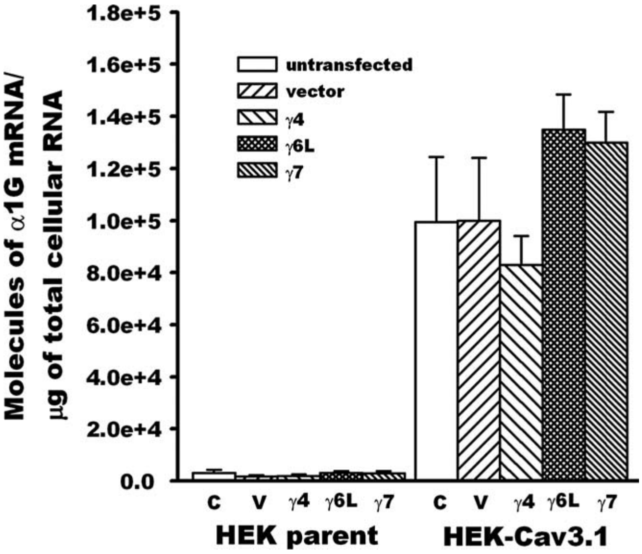

Fig. 7. Expression of γ subunits does not alter Cav3.1 mRNA levels. Equal amounts of either γ_4, γ_{6L} or γ_7 DNA were transiently transfected into parental HEK-293 cells or HEK-293/Cav3.1. Control cells were transfected with the adCGI vector (V) or underwent a mock transfection (C, no DNA). Quantitative RT-PCR was performed to monitor the amount of Cav3.1 mRNA expressed in each of these groups. Transient transfection of γ_4, γ_{6L} or γ_7 had no significant effect on Cav3.1 mRNA levels in either HEK-293 parental cells, or HEK-293/Cav3.1 when compared to vector control (V).

293/Cav3.1 cells expressing γ_4, γ_{6L} and γ_7 were analyzed using western blot analysis with an anti-Cav3.1 antibody. An exemplar blot (Fig. 8A) indicates that Cav3.1 protein is highly expressed in membrane fraction of the cell lysate and runs predominantly as a ~260 kDa band. No Cav3.1 protein was detected in parental HEK-293 control cells (data not shown). Similar results were obtained for the other γ subunits as shown in the averaged data from three independent experiments by quantifying the 260 kDa band (Fig. 8B). The relative amounts of Cav3.1 protein expressed in cytosolic and membrane fractions for mock transfection, vector, γ_{6L}, and γ_4 were normalized to cell extracts from cells transfected with adCGI vector alone. The co-expression of the γ subunits does not significantly alter Cav3.1 protein levels in either the cytoplasm; lysosomal and mitochondrial membrane fraction; or microsomal and plasma membrane fraction ($P > 0.05$). Additionally, there is no significant effect of γ subunit expression on the total amount of Cav3.1 protein expressed in all fractions combined from HEK-293/Cav3.1 cell lysates ($P > 0.05$). Most importantly γ_{6L} causes no down-regulation of Cav3.1 protein expression when transfected in Cav3.1/HEK-293 cells.

4. Discussion

In this study, we describe the functional effects of the three γ subunits that are robustly expressed in rat cardiac myocytes on current produced by a heterologously expressed LVA calcium current and on endogenous LVA current in an atrial derived cell line. Our results provide the first functional analysis of the γ_6 subunit and show that this subunit has unique inhibitory effects on the density of Cav3.1 dependent LVA currents not seen with the other γ isoforms expressed in the heart.

Our finding that γ_6 co-expression reduces LVA calcium current density is consistent with previous reports that γ subunit co-expression generally reduces current density through HVA channels [35,36]. In contrast to the effect of γ_6, neither γ_4 nor γ_7 had statistically significant effects on

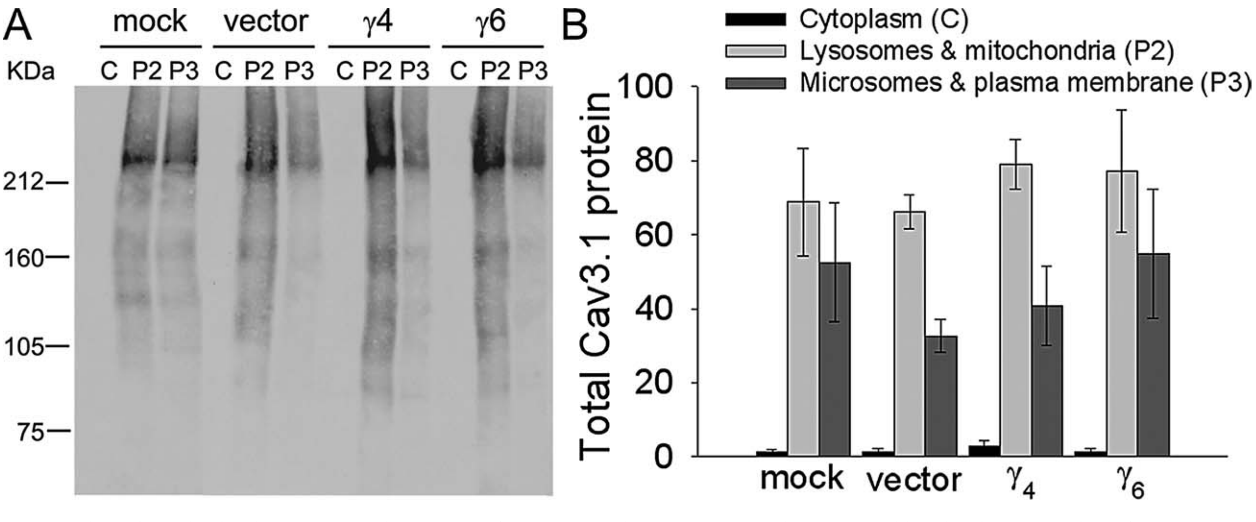

Fig. 8. Expression of γ subunits does not alter the amount of Cav3.1 protein. A: Representative Western blot showing expression of Cav3.1 protein in HEK-293/Cav3.1 cells and HEK-293/Cav3.1 cells transiently transfected with either blank vector, γ_4 or with γ_{6L}. Cell lysates were separated into cytoplasmic (C), lysosomal and mitochondrial (P2), and microsomal and plasma membrane (P3) fractions. Proteins were visualized using a polyclonal antibody for Cav3.1. The Cav3.1 protein was detected as a major band at approximately 260 kDa with the majority of protein in the membrane fractions. No Cav3.1 protein was detected in HEK-293 parental control cells not stably transfected with Cav3.1 (data not shown). B: The bar graph shows the relative amount of Cav3.1 protein expressed in each fraction normalized to percent of vector control (V) in HEK-293/Cav3.1 cells. The data represent three separate experiments. No statistically significant effect of either vector, or the γ_4 or γ_6 subunits on Cav3.1 protein expression was detected in any of the cell fractions individually, or in total cell Cav3.1 protein.

Cav3.1 dependent calcium current. These findings are in agreement with a previous report showing that γ_4 had no effect on current density through an LVA channel, Cav3.3 (α_{1I}), expressed in HEK-293 cells [20]. However, a profound effect of human γ_7 on the expression of Cav2.2 dependent HVA current has been previously reported [36]. Thus, it is possible that the functional effects of specific γ subunits are dependent on the α_1 subunit with which they interact. Our data clearly show that both of the γ_6 isoforms have a much more robust effect on LVA current density than either γ_4 or γ_7 under the same experimental conditions supporting the conclusion that the effects of γ_6 are distinct from those of other cardiac γ subunits with regards to current arising from Cav3.1 α_1 subunits. This difference suggests that there may be unique interactions between specific α_1 and γ subunits or other associated proteins.

In addition to altering calcium channel function, γ subunits containing C-terminal PDZ-binding domains have been shown to be important regulators of ligand gated ion channel trafficking [11,37]. The γ_2, γ_3, γ_4, and γ_8 subunits mediate surface membrane localization of AMPA receptors in neurons and make up a family of transmembrane AMPA receptor regulatory proteins, TARPs [11]. Neither the γ_1 nor the γ_5 subunit regulated AMPA receptor localization demonstrating that these subunits are likely to have different cellular functions. Although not specifically tested in that study, the γ_6 and γ_7 subunits, which are expressed in cardiac muscle, may not be members of the TARP family. They lack the consensus PDZ-binding motif of the TARP proteins and are closely related to γ_1 and γ_5 phylogenetically [19]. It is interesting that several of the γ subunits found to be involved in AMPA receptor trafficking have previously been shown to modulate calcium current suggesting that individual γ subunits may have pleiotropic effects in the same cells [10,21,36].

Our results show that over-expression of γ_{6L} significantly reduces endogenous LVA current in the HL-1 cell line. The similarity of the γ_6 effects on current in both HEK-293 and HL-1 cells, an atrial cell line, suggests that γ_6 effects are independent of cell type and that our HEK-293 studies are physiologically relevant to native cardiac tissue. Cardiac LVA current density changes dramatically during postnatal growth and in response to hormonal changes and pathophysiological insults [38–40] although the mechanisms by which LVA current expression in the heart is regulated are not well understood. Our results suggest that repression of LVA calcium current density by the γ_6 subunit could play a role in the regulation of this current.

A number of different mechanisms that might explain the effect of the γ_6 subunits on LVA calcium current can be ruled out by our data. Since robust effects of γ_6 are seen in HEK-293/Cav3.1 cells where non-coding (regulatory) genomic sequence does not flank the heterologously expressed gene it is unlikely that the γ subunits act as regulators of transcription. We have confirmed this idea by showing that γ_6 caused no change in the levels of Cav3.1 mRNA in Cav3.1/HEK293 cells. This result is consistent with the demonstration that native LVA current in thalamocortical relay neurons of γ_2–/– mice is significantly increased without concurrent changes in the mRNA levels of any Cav3 α_1 subunits [41]. Western analysis indicates that there is no change in the amount of Cav3.1 protein in either the lysosomal/mitochondria or the microsomal/plasmalemmal fractions of homogenized cells transiently transfected with γ_{6L} even though LVA calcium current is decreased significantly. Thus, it appears that in HEK/Cav3.1 cells the γ_{6L} dependent decrease in LVA calcium current density is not the result of a decrease in total Cav3.1 protein.

What mechanisms might then account for the decrease in Cav3.1 current that results from co-expression with γ_6? It is also possible that the γ_6 subunit uniformly alters the biophysical properties of all channels thus decreasing current density. Freise et al. [28] suggested that γ subunits decrease the open probability of channels to account for the increase in Cav1.1 (α_1S) dependent calcium current in skeletal muscle cells from γ_1 knock out mice, where the amount of Cav1.1 protein was biochemically shown to be unchanged. More recently Lacinova and Klugbauer [42] have shown that the $\alpha_2\delta_{2a}$ and γ_5 subunits modestly alter the gating currents of Cav3.1 channels thus altering current kinetics. Since we did not see any changes in the activation and kinetic properties of the LVA current our data do not provide any experimental evidence to support this mechanism although we cannot rule it out completely without more detailed biophysical analysis. Thus, it remains to be determined if an alteration of channel gating can explain the decrease in Cav3.1 current by γ_6. Another possibility is that the γ_6 subunit causes a decrease in the number of channels normally available for activation. In this case the remaining current would have a smaller peak amplitude but unaltered kinetics consistent with our data. A decrease in channel availability could entail a change in trafficking of channels to the surface membrane [43]. It also could be produced if a subgroup of channels in the membrane become electrically silent under the influence of γ_6. These possible mechanisms are currently being investigated.

The region(s) of the γ_6 subunit involved in the interaction with α_1 subunits are unclear. Arikkath et al. [27] used a chimeric strategy to show that the N-terminal half of γ_1, including transmembrane segments 1 and 2, is necessary for its interaction with Cav1.1. Since γ_1 is highly homologous to γ_6 and we have shown that both γ_{6L} and γ_{6S} have the same functional effects on Cav3.1, it is likely that transmembrane regions 2 and 3 as well as the sequence linking these two regions is not responsible for the interaction of either γ_1 or γ_6 with α_1 subunits. These results suggest that an important α_1 interaction site for γ_1 and γ_6 may lie within the N-terminal region up to and including the first transmembrane segment. However, these results do not preclude the existence of other α_1–γ interaction sites.

In summary, these studies provide the first comparative functional analysis of the calcium channel γ subunits that are expressed in cardiac muscle and show that the γ_6 subunit is unique in its ability to decrease LVA calcium current density.

This functional specificity makes the γ_6 subunit a potential target for interventions seeking to modify LVA calcium current in the heart.

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