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BRIEF COMMUNICATION

γ_1-Dependent Down-regulation of Recombinant Voltage-gated Ca^{2+} Channels

Alejandro Sandoval · Jyothi Arikkath · Eduardo Monjaraz · Kevin P. Campbell · Ricardo Felix

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Abstract (1) Voltage-gated Ca^{2+} (Ca_V) channels are multi-subunit membrane complexes that allow depolarization-induced Ca^{2+} influx into cells. The skeletal muscle L-type Ca_V channels consist of an ion-conducting $Ca_V1.1$ subunit and auxiliary $\alpha_2\delta$–1, β_1 and γ_1 subunits. This complex serves both as a Ca_V channel and as a voltage sensor for excitation–contraction coupling. (2) Though much is known about the mechanisms by which the $\alpha_2\delta$–1 and β_1 subunits regulate Ca_V channel function, there is far less information on the γ_1 subunit. Previously, we characterized the interaction of γ_1 with the other components of the skeletal Ca_V channel complex, and showed that heterologous expression of this auxiliary subunit decreases Ca^{2+} current density in myotubes from γ_1 null mice. (3) In the current report, using Western blotting we show that the expression of the $Ca_V1.1$ protein is significantly lower when it is heterologously co-expressed with γ_1. Consistent with this, patch-clamp recordings showed that transient transfection of γ_1 drastically inhibited macroscopic currents through recombinant N-type ($Ca_V2.2/\alpha_2\delta$–$1/\beta_3$) channels expressed in HEK-293 cells. (4) These findings provide evidence that co-expression of the auxiliary γ_1 subunit results in a decreased expression of the ion-conducting subunit, which may help to explain the reduction in Ca^{2+} current density following γ_1 transfection.

Keywords Ca^{2+} channels · γ_1 subunit · HEK-293 cells · Patch-clamp

A. Sandoval · R. Felix (✉)
Department of Cell Biology, Center for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav-IPN), Avenida IPN 2508, Colonia Zacatenco, Mexico City, DF CP 07300, Mexico
e-mail: rfelix@fisio.cinvestav.mx

A. Sandoval
School of Medicine FES Iztacala, National Autonomous University of Mexico, Tlalnepantla, Mexico

J. Arikkath · K. P. Campbell
Howard Hughes Medical Institute (HHMI); Departments of Molecular Physiology and Biophysics, Internal Medicine, and Neurology, University of Iowa Carver, College of Medicine, Iowa, USA

E. Monjaraz
Laboratory of Neuroendocrinology, Institute of Physiology, University of Puebla, Puebla, Mexico

Introduction

Excitation–contraction (EC) coupling is a process that links skeletal muscle cell membrane depolarization to the generation of a Ca^{2+} signal. This task is performed by an assembly of two Ca^{2+} channels that function as integral parts of one molecular signaling device. During EC coupling, the activation of a voltage-gated Ca^{2+} (Ca_V) channel (the dihydropyridine receptor, DHP receptor) located in the cell membrane promotes a conformational change that opens an intracellular Ca^{2+} channel (the ryanodine receptor type-1, RyR1), present in the membrane of the sarcoplasmic reticulum leading to an increase in cytosolic Ca^{2+} (Dulhunty 2006).

At the molecular level, the skeletal muscle DHP receptor is a hetero-multimeric complex formed by four different proteins: one pore-forming $Ca_V1.1$ subunit and auxiliary $\alpha_2\delta$–1, β_1, and γ_1 subunits (Felix et al. 2005; Flucher et al. 2005). The $\alpha_2\delta$–1 and β_1 subunits modulate the voltage-dependent kinetics of the channel complex and/or the trafficking of the $Ca_V1.1$ subunit to the cell membrane (Kang and Campbell 2003; Flucher et al. 2005; Melzer et al. 2006). However, there is only limited information on the regulatory actions of γ_1. This auxiliary subunit is a 32 kDa polypeptide that exhibits four putative membrane-spanning helices with N and C termini located on the cytoplasmic side. Although γ_1 expression seems to be restricted to skeletal muscle (Kang and Campbell 2003), apparently it is not indispensable for EC coupling (Melzer et al. 2006). Nevertheless, γ_1 may alter the functional properties of Ca_V channels and play an important modulatory role in skeletal muscle.

The relatively small number of γ_1 functional assays have suggested mild changes in current amplitude, current kinetics, and/or voltage dependence of Ca_V channels (Kang and Campbell 2003; Flucher et al. 2005; Melzer et al. 2006). Though it has been difficult to reach a definite conclusion regarding the actions of γ_1 as the results of the initial functional assays were conflicting, more recent studies on γ_1 null mice have shown that this auxiliary subunit exerts important inhibitory effects on the activity of Ca_V channels in skeletal muscle (Freise et al. 2000; Ahern et al. 2001; Held et al. 2002; Arikkath et al. 2003).

Even though the available biochemical and electrophysiological evidence support the idea that γ_1 is a fundamental component of the skeletal Ca^{2+} channel complex, not much information is available regarding the mechanisms of its inhibitory effects on channel activity. In the current report, we have addressed this issue by examining the change in the levels of the $Ca_V1.1$ protein after γ_1 co-expression using a heterologous expression system. Furthermore, we investigated the inhibitory actions of γ_1 on macroscopic currents through recombinant channels. Our data suggest that decreased levels of the pore-forming protein may give rise to reduced Ca_V channel functional expression after γ_1 transient transfection.

Materials and Methods

Transient Transfection and Immunoprecipitation

The full-length rabbit $Ca_V1.1$ (GeneBank M23919) cDNA was fused in frame to the first 11 amino acids of the phage T7 gene 10 protein in the mammalian expression vector pSG5 (Stratagene) using *Age*I and *Not*I cloning sites (Ahern et al. 2001). The γ_1EGFP construct was generated by inserting the rabbit γ_1 subunit (GenBank M32231) cDNA into the pEGFP-N1 vector (Clontech) in-frame with the enhanced green fluorescent protein (EGFP) (Arikkath et al. 2003). The $\gamma_1$sspnEGFP and the γ_2/γ_1 chimeras were generated as previously described (Arikkath et al. 2003). Other clones used in this study were: the α1BpKCRH2 construct

containing the cDNA clone coding the rabbit brain $Ca_V2.2$ (GenBank D14157) subunit; the rat brain $\alpha_2\delta$–1 (GenBank M86621), the rat brain β_3 (GenBank M88751); mouse brain γ_2 subunit (GenBank NM_007583); and human skeletal muscle sarcospan (GenBank AF016028) (Sandoval et al. 2007).

For Western blotting, Ca_V channel subunits were expressed in tsA-201 cells (a subclone of the human embryonic kidney (HEK)-293 cell line) using transient transfection based on calcium phosphate precipitation, as described previously (Felix et al. 1997). Two days after transfection, cells were lysed in buffer containing 50 mM Tris, 1% digitonin, 0.5 M NaCl, plus protease inhibitors, and the solubilized material was isolated by centrifugation. Aliquots of the cell lysate were immunoblotted to examine the expression of the proteins as described elsewhere (Arikkath et al. 2003).

Electrophysiology

For functional assays, HEK-293 cells were grown in standard culture conditions and transfected using the Lipofectamine Plus reagent as described elsewhere (Sandoval et al. 2007). Ba^{2+} currents through heterologously expressed $Ca_V2.2$ channels were recorded as described previously (Sandoval et al. 2007) using the whole-cell configuration of the patch-clamp technique. Briefly, macroscopic currents were recorded with an Axopatch 200B amplifier (Molecular Devices) and filtered at 2 kHz. All experiments were performed at room temperature ($\sim$22°C) and a holding potential (HP) of –80 mV. Currents were digitized at 5.71 kHz, using a DigiData 1320A interface (Molecular Devices) and analyzed using the pCLAMP (Molecular Devices) and SigmaPlot (Systat Software) software. Linear capacitative currents were minimized via the capacitative transient cancellation feature of the amplifier. The remaining linear components were subtracted using a P/4 protocol. Membrane capacitance was determined and used to normalize currents. The bath recording solution contained the following (in mM): 10 $BaCl_2$, 125 TEA-Cl, 10 HEPES, and 15 glucose, pH 7.3. The internal solution consisted of the following (in mM): 110 CsCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, 4 Na-ATP, and 0.1 Na-GTP, pH 7.3. Whole-cell K^+ currents were recorded as previously described (Avila et al. 2004).

Results and Discussion

In a previous study, we characterized domains mediating the interaction of the γ_1 subunit with other components of the skeletal muscle DHP-receptor, and reported that expression of this ancillary protein decreases Ca^{2+} conductance in γ_1 null myotubes (Arikkath et al. 2003). More recently, we have reported that the co-expression of the γ_2 and γ_3 subunits reduces significantly whole-cell currents through recombinant neuronal N-type Ca^{2+} channels heterologously expressed in HEK-293 cells through a dual mechanism that involves alterations in the function and the translation of the channels (Sandoval et al. 2007). Considering these findings, we investigated the possibility that the co-expression of γ_1 subunit reduces current density by affecting the expression of the channels at the level of the protein.

To test this hypothesis, Western blot experiments using specific antibodies were performed. In agreement with our previous studies (Arikkath et al. 2003), this analysis revealed that the ion-conducting $Ca_V1.1$ protein of the skeletal muscle DHP-receptor was expressed in the lysates of tsA-201 cells transiently transfected with the $Ca_V1.1$ subunit cDNA construct (Fig. 1A). Likewise, the use of a specific green fluorescent protein (GFP) antibody allowed us

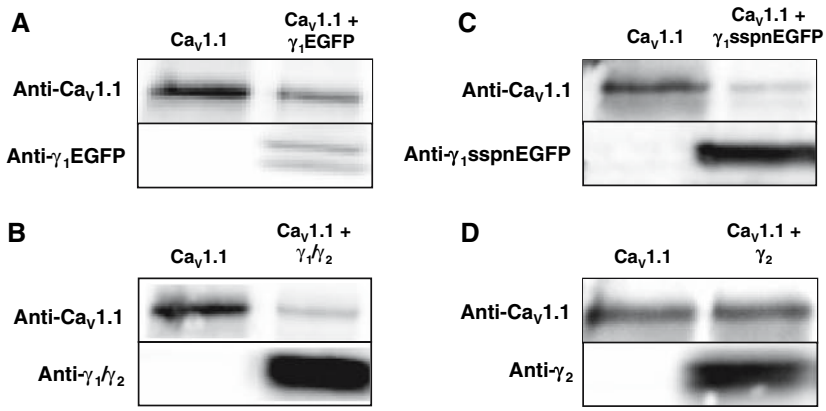

Fig. 1 Heterologously expressed γ_1 constructs interact with the Ca$_V$1.1 subunit of the skeletal muscle DHP receptor and contribute to determine its levels of expression. Aliquots of the lysate material obtained from tsA-201 cells transiently transfected with the indicated constructs were analyzed by western blot for the presence of the Ca$_V$1.1 protein and the γ_1EGFP (**A**) subunit, or the γ_1/γ_2 (**B**), γ_1/sspnEGFP chimerical proteins (**C**), and the neuronal γ_2 subunit (**D**)

to identify the expression of the γ_1 protein in the lysates of cells transfected with the γ_1EGFP construct. Interestingly, the amount of Ca$_V$1.1 protein expressed seemed to depend on the presence of the γ_1 subunit. Immunodetection revealed that the Ca$_V$1.1 protein band intensity decreased significantly when γ_1 was present.

To corroborate this observation, we next examined the effects of a chimerical γ_1–γ_2 subunit on the level of Ca$_V$1.1 protein expression. Previous biochemical studies have shown that this chimerical protein is capable to interact with the Ca$_V$1.1 subunit (Arikkath et al. 2003). In agreement with the result found for the wild-type γ_1, heterologous expression of the γ_1–γ_2 subunit resulted in a significant reduction of the intensity of the Ca$_V$1.1 protein band compared with that of cells transfected with Ca$_V$1.1 alone (Fig. 1B). Qualitatively similar results were obtained when a second chimerical subunit (γ_1-sspnEGFP) was employed in the transfection assays (Fig. 1C). It is worth noting that the γ_1-sspnEGFP chimera has the first extracellular loop of the γ_1 subunit replaced by an extracellular loop of the unrelated four-transmembrane protein sarcospan and EGFP at the carboxyl terminus, and it has been demonstrated that it can also associate with the Ca$_V$1.1 subunit (Arikkath et al. 2003). Conversely, co-expression of the neuronal γ_2 subunit did not affect apparently Ca$_V$1.1 protein band intensity (Fig. 1D). Taken together, these results (i) corroborate that the first two transmembrane domains and/or the intracellular N terminal loop in γ_1 contain the determinants that allow interaction with the Ca$_V$1.1 subunit and (ii) support a specific role for the γ_1 subunit in determining the level of expression of the Ca$_V$1.1 subunit.

The impact of the γ_1-induced decrease in Ca$_V$1.1 protein levels on the functional properties of the macroscopic Ca^{2+} currents was next investigated using patch-clamp recording. Given that the Ca$_V$1.1 subunit resists functional expression in non-muscle cells (Fig. 2A), the cardiac or neuronal ion-conducting subunits have been frequently used for investigating the effects of γ_1 on Ca^{2+} current properties (Kang and Campbell 2003; Flucher et al. 2005; Melzer et al. 2006). Hence, we used HEK-293 cells transiently transfected with neuronal Ca$_V$2.2, β_3, and $\alpha_2\delta$–1 subunits (Sandoval et al. 2007) to enable recording of the currents. Interestingly, our functional results matched up well with the Western blot data.

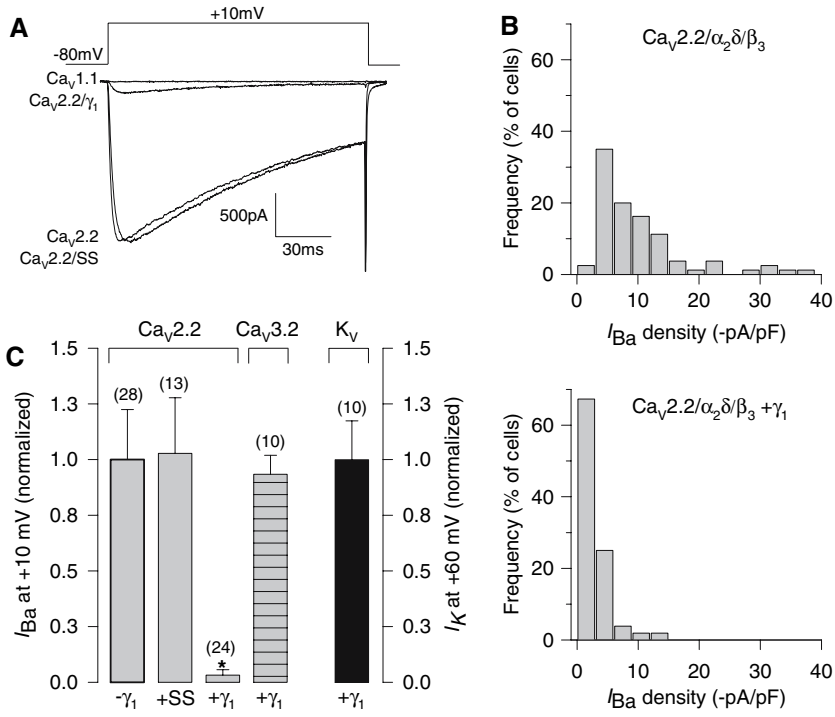

Fig. 2 γ_1 inhibits currents through $Ca_V2.2/\alpha_2\delta$–$1/\beta_3$ channels. (**A**) Representative current traces from control HEK-293 cells expressing $Ca_V1.1$ or $Ca_V2.2/\alpha_2\delta$–$1/\beta_3$ (with and without γ_1) or cells co-expressing sarcospan (SS). Currents were elicited by steps to +10 mV from a HP of –80 mV. $Ca_V1.1$ (flat trace with null channel activity) exemplify recordings in cells transfected with the $Ca_V1.1/\alpha_2\delta$–$1/\beta_1$ subunits of the skeletal DHP receptor (n = 15). (**B**) Comparison of frequency distribution of I_{Ba} density in HEK-293 cells determined as the current amplitude at +10 mV normalized by cell capacitance in control cells (upper panel) and cells co-expressing γ_1 (lower panel). (**C**) Comparison of normalized endogenous K^+ peak current density (K_V) in untransfected HEK-293 cells and peak Ba^{2+} current density in cells expressing neuronal recombinant channels ($Ca_V2.2$) or T-type channels ($Ca_V3.2$) in absence (-γ) and presence of γ_1. The number of tested cells is listed in parentheses

Representative traces of Ba^{2+} current (I_{Ba}) evoked by test pulses to +10 mV recorded from control (without the γ_1 subunit) and cells expressing γ_1 are depicted in Fig. 2A. As can be seen, co-expression of γ_1 significantly decreased I_{Ba} amplitude. In contrast, co-expression of sarcospan (Crosbie et al. 1997), an unrelated tetraspan protein, had no effect on I_{Ba} suggesting a specific functional interaction of γ_1 with the $Ca_V2.2/\beta_3/\alpha_2\delta$–1 channel complex. The inhibitory effect of the auxiliary subunit revealed by electrophysiology is better observed in Fig. 2B, which presents the frequency distribution of I_{Ba} density in control (upper panel) and γ_1 expressing cells (lower panel). As can be seen, the frequency distribution in the γ_1 transfected cells was shifted to the left, which indicates a drastic inhibitory effect in I_{Ba} density in presence of the auxiliary subunit. Likewise, γ_1 regulation was specific for $Ca_V2.2$-containing channels as γ_1 expression had no effect on endogenous K^+ currents normally expressed in the HEK-293 cells (Avila et al. 2004). Similarly, as expected from the fact that the auxiliary γ subunits seem not to associate with low voltage activated Ca^{2+} (T-type) channels (Perez-Reyes 2006), co-expression of γ_1 did not affect recombinant $Ca_V3.2$ T-current activity (Fig. 2C).

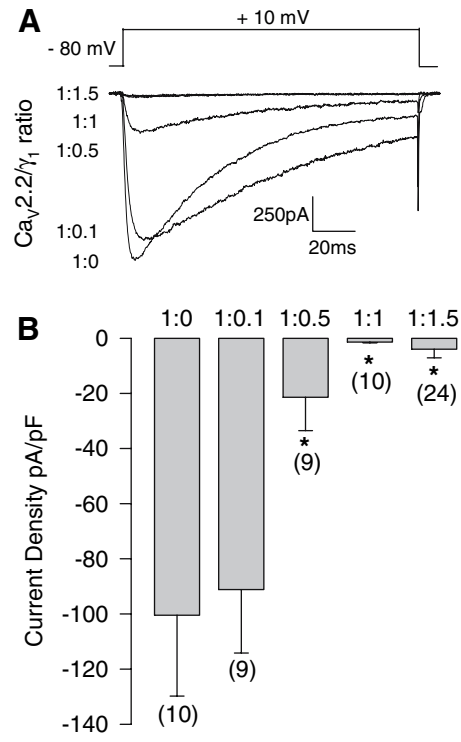

Fig. 3 The γ_1 subunit inhibits recombinant N-type Ca^{2+} channels in a concentration-dependent manner. (**A**) Representative Ba^{2+} currents recorded (upper panel) and comparison of averaged peak current density (lower panel) in HEK-293 cells expressing $Ca_V2.2/\alpha_2\delta$–1/$Ca_V\beta_3$ channels. Currents were elicited by depolarizing pulses to +10 mV from a HP of –80 mV, in the absence (1:0) and the presence of different concentrations of γ_1 cDNA in the transfection mixture. (**B**) Peak I_{Ba} density through $Ca_V2.2/\alpha_2\delta$–1/$Ca_V\beta_3$ channels in control cells (1:0) and cells co-expressing the γ_1 cDNA in molar ratios ranging from 1:0.1 to 1:1.5 in relation to the $Ca_V2.2$ subunit. The asterisks denote significant differences ($p < 0.05$) as compared with control

In order to confirm that current inhibition by the γ_1 subunit was not simply a non-specific effect of over-expression, we next transfected HEK-293 cells using different concentrations of cDNAs. For these experiments, we fixed the cDNA concentration for $Ca_V2.2$, β_3 and $\alpha_2\delta$–1 (subunit transfection (1:1:1 molar ratio) and varied the γ_1 concentration (Fig. 3A). Our results show that upon co-expression of the γ_1 cDNA in molar ratios ranging from 1:0.1 to 1:1.5 (with respect to the other channel subunits), the amplitude of I_{Ba} through $Ca_V2.2/\beta_3/\alpha_2\delta$–1 channels decreased significantly as the relative molar ratio of γ_1 increased (Fig. 3B). These data suggested a γ_1-specific inhibitory effect. Current regulation was observed at virtually all voltages tested (data not shown).

We have previously demonstrated that the first half of the γ_1 subunit, including the first two transmembrane domains, mediates the interaction of the protein with the L-type Ca^{2+} channel (Arikkath et al. 2003). To assess whether this region of the protein allowed interaction with the N-type Ca^{2+} channel, the functional effects of the chimeric protein γ_1–γ_2 was examined by patch-clamp experiments. In agreement with the biochemical studies, co-expression of the γ_1–γ_2 chimera significantly reduce the current density (Fig. 4A and B). This result demonstrates that the first half of γ_1 can also mediate subunit interaction of neuronal Ca_V channels.

Lastly, we tested the role of other Ca_V channel regulatory subunits on γ_1 inhibition. To evaluate this, HEK-293 cells were co-transfected with $Ca_V2.2/\alpha_2\delta$–1 channels (in the absence of the β subunit) and cDNA encoding γ_1. As shown by representative current traces (Fig. 4C), the presence of the γ_1 subunit resulted in a significant reduction of current amplitude. The comparison of mean current density at +10 mV recorded in cells expressing $Ca_V2.2/\alpha_2\delta$–1 channels and cells co-expressing γ_1 resulted in current densities of ~31% (Fig. 4D). Similar suppression effects were observed when the HEK-293 cells expressing $Ca_V2.2/\beta_3$ channels

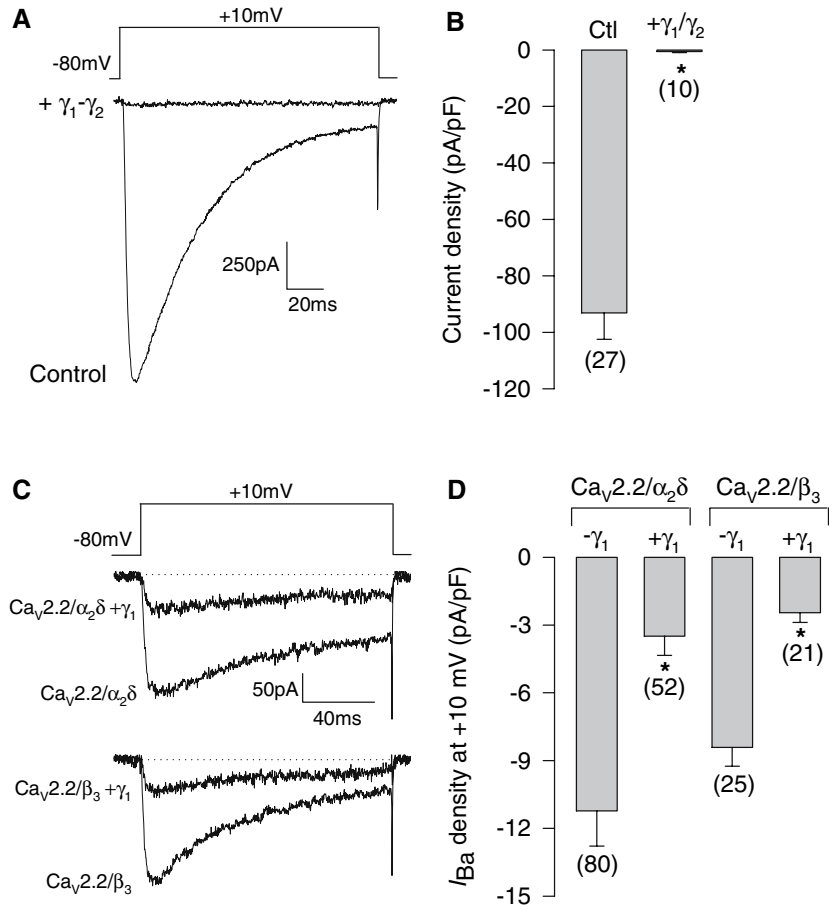

Fig. 4 The γ_1 subunit interacts with the Ca^{2+} channel complex directly via the α_1 subunit. (**A**) Representative whole-cell currents when HEK-293 cells transfected with the γ_1–γ_2 chimera were depolarized to +10 mV from a HP of –80 mV. (**B**) Mean I_{Ba} density in HEK-293 cells as in (**A**). Bars denote mean ± SE; the number of recorded cells is indicated in parentheses. *, $p < 0.05$ as compared with control. (**C**) Representative current traces from control HEK-293 cells expressing $Ca_V2.2$ channels (with or without γ_1) in absence of the β (upper panel) and $\alpha(\delta$ (lower panel) subunits. Currents were elicited by voltage steps to +10 mV from a HP of –80 mV. (**D**) Comparison of I_{Ba} density determined in HEK-293 cells co-transfected with $Ca_V2.2/\alpha_2\delta$–1 or $Ca_V2.2/\beta_3$ in absence or presence of γ_1. The number of recorded cells is given in parentheses. The asterisks denote significant differences ($p < 0.05$)

were co-transfected with γ_1 (Fig. 4C; lower panel). Comparing the mean current density at +10 mV after co-expression with γ_1 resulted in I_{Ba} density of ~33% in relation to the control (Fig. 4B). These results strongly suggest that the γ_1 subunit interacts with the Ca^{2+} channel complex directly via the α_1 subunit.

In summary, by combining biochemical and electrophysiological evidence, we were able to demonstrate that heterologously expressed γ_1 subunit is able to decrease $Ca_V1.1$ protein levels and exert inhibitory effects on neuronal recombinant channel activity. Though the mechanism of this inhibition has yet to be determined, one possibility would be that γ_1 modulates the

surface expression and/or interferes with the synthesis of new channels. Though the synthesis, assembly, and trafficking of Ca_V channels is an intricate process determined by a wide range of events, recent evidence suggest that Ca^{2+} current inhibition by neuronal γ_1 subunits may involve alterations in the functional properties of the channels as well as the activation of the unfolded protein response (UPR) to suppress channel translation (Sandoval et al. 2007).

It is worth mentioning that the UPR is a specific signaling pathway that is triggered when mammalian cells are subjected to endoplasmic reticulum (ER) stress. This signaling pathway initiates with the activation of PERK (PKR-like ER-associated kinase), which inhibits protein biosynthesis through phosphorylation of the eukaryotic translation initiation factor eIF2α (Harding et al. 2002; Marciniak and Ron 2006). Interestingly, by using genistein and mutant dominant-negative constructs of PERK, Sandoval et al. (2007) found that the γ_2-mediated suppression of $Ca_V2.2$ currents involved $Ca_V2.2$ subunit synthesis arrest. In support of this, our studies indicate that the inhibition of $Ca_V1.1$ expression and $Ca_V2.2$ currents by γ_1 may be attributable to a reduction in the level of the protein.

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