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COOH-terminal Truncated Alpha₁S Subunits Conduct Current Better than Full-length Dihydropyridine Receptors

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Abstract  Skeletal muscle dihydropyridine (DHP) receptors function both as voltage-activated Ca²⁺ channels and as voltage sensors for coupling membrane depolarization to release of Ca²⁺ from the sarcoplasmic reticulum. In skeletal muscle, the principal or α₁S subunit occurs in full-length (~10% of total) and post-transcriptionally truncated (~90%) forms, which has raised the possibility that the two functional roles are subserved by DHP receptors comprised of different sized α₁S subunits. We tested the functional properties of each form by injecting oocytes with cRNAs coding for full-length (α₁S) or truncated (α₁SAC) α subunits. Both translation products were expressed in the membrane, as evidenced by increases in the gating charge (Q_max, 80–150 pC). Thus, oocytes provide a robust expression system for the study of gating charge movement in α₁S, unencumbered by contributions from other voltage-gated channels or the complexities of the transverse tubules. As in recordings from skeletal muscle, for heterologously expressed channels the peak inward Ba²⁺ currents were small relative to Q_max. The truncated α₁SAC protein, however, supported much larger ionic currents than the full-length product. These data raise the possibility that DHP receptors containing the more abundant, truncated form of the α₁S subunit conduct the majority of the L-type Ca²⁺ current in skeletal muscle. Our data also suggest that the carboxyl terminus of the α₁S subunit modulates the coupling between charge movement and channel opening.

Key words: Ca²⁺ channels • skeletal muscle • Xenopus oocyte expression • cut-open oocyte voltage clamp • gating charge movement

Introduction  Skeletal muscle dihydropyridine (DHP) receptors are L-type Ca²⁺ channels that serve a dual role in muscle, functioning both as Ca²⁺-conducting pores and as the voltage sensors for excitation-contraction (E-C) coupling (Ríos and Brum, 1987; Tanabe et al., 1988; Melzer et al., 1995). These channels are heteromeric complexes containing the pore-forming α₁S subunit plus the β₃, α₂δ, and γ regulatory subunits (Höfmann et al., 1994). Two forms of the α₁S subunit have been detected in skeletal muscle: a 212-kD full-length translation product (α₁SL), present as a minor component, and a more abundant 175-kD form (α₁SAC, 90% of α₁S subunit recovered from transverse tubule membrane preparations) created by post-translational cleavage of 175 amino acids from the COOH terminus (De Jongh et al., 1989, 1991). While the COOH termini of other cloned Ca²⁺ channel α₁S subunits have been shown to play a role in channel regulation (Wei et al., 1994; Lee et al., 1999; Zühlke et al., 1999), the functional significance of the COOH-terminal truncation of α₁S is unknown.

One long-standing hypothesis holds that some DHP receptors in skeletal muscle are specialized voltage sensors for E-C coupling, while others are capable of both voltage sensing and Ca²⁺ conduction. Comparison of DHP binding to L-type Ca²⁺ current amplitude in intact muscle fibers suggested that only a small percentage of DHP receptors are functional Ca²⁺ channels (Schwartz et al., 1985). The discovery of two size forms of α₁S occurring in a 90%:10% ratio led to the hypothesis that the rarer full-length form is capable of both sensing the voltage and passing current, while the more abundant truncated form is a dedicated voltage sensor for E-C coupling (De Jongh et al., 1989).

Injection of cDNAs encoding either α₁S or a COOH-terminally truncated form of α₁S (at Asn 1662) into the nuclei of dysgenic mouse myotubes lacking α₁S restores contraction, gating charge movement, and L-type Ca²⁺ current (Tanabe et al., 1988; Adams et al., 1990; Beam et al., 1992). While the version of α₁SAC used in these experiments was artificially truncated slightly upstream of the native cleavage site, these results clearly suggest that α₁SAC can function as both an ion channel and a voltage sensor for E-C coupling. However, the role of full-length α₁S remains unknown, since it is possible that an undetermined proportion of the α₁S subunits were cleaved to α₁SAC in the myotubes. One recent study has addressed this issue. When the green fluores-
The rabbit α15 cDNA was obtained in the pKCRH, pBluescript, pcDNA3, and pHindIII sites, while the β1a, α15SC, β1b, α15, α8, and γ subunits into Xenopus oocytes were recently found to give rise to robust DHPR-sensitive L-type currents, whereas injection of full-length α15 cDNA gave little or no current (Ren and Hall, 1997). While these results suggest a difference in channel function for α15 and α15SC, they leave open the possibility that full-length α15 simply expresses poorly in oocytes, but conducts Ca\(^{2+}\) just as well as α15SC. Moreover, measurements of ionic current alone cannot address the crucial question of whether α15 and α15SC function differently as voltage sensors.

To study the current-carrying and voltage-sensing capabilities of the two size forms of α15 in parallel, we expressed α15 and α15SC with the β1b, α8, and γ auxiliary subunits in Xenopus oocytes. We examined the β1a and β1b splice variants, both of which are expressed in muscle tissue (Hofmann et al., 1994; Ren and Hall, 1997), because of a previous report that the less-abundant β1b variant was critical for forming channels capable of conducting Ba\(^{2+}\) current (Ren and Hall, 1997). The cut-open oocyte voltage clamp technique (Stefani and Bezanilla, 1998) was used to record ionic currents (in 10 mM Ba\(^{2+}\)) and gating charge movement (in 2 mM Co\(^{2+}\)) in parallel. We found that both size forms of the α15 subunit supported gating charge movements in 2 mM Co\(^{2+}\) when expressed with auxiliary subunits in oocytes, even though only the truncated form conducted appreciable L-type currents in 10 mM Ba\(^{2+}\). This result was independent of whether the β1a or β1b splice variant was used. We conclude that, while α15 and α15SC function almost identically as voltage sensors, α15SC is the form specialized to carry L-type current, while α15 appears to be somehow inhibited from passing substantial ionic current.

**M E T H O D S**

The rabbit α15, rat brain β1b, rabbit α8, and rabbit γ Ca\(^{2+}\) channel cDNAs were obtained in the pkCRH, pBluescript, pcDNA3, and pcD-x vectors, respectively, as a gift from Dr. Kevin Campbell (University of Iowa, Iowa City, IA). The rabbit β1a subunit was obtained in the pNKS2 vector as a gift from Dr. Bernhard Flucher (University of Innsbruck, Innsbruck, Austria). α15SC and γ subclones were obtained from the pGEMHE oocyte expression vector (gift of Dr. Emily Liman, Harvard University) at the HindIII and BamHI polylinker sites, respectively. β1b was subcloned into pGEMHE between the SacII and HindIII sites, while α8 and β1a were left in pcDNA3 and pNKS2, respectively. α15SC-PGEMHE was made from α15-PGEMHE using the Clontech Transformer site-directed mutagenesis kit (CLONTECH Laboratories, Inc.). The mutagenic primer caused a loop-out deletion of base pairs 5320–5844 (amino acids 1698–1893) of the rabbit α15 sequence, while the selection primer mutated the MfeI site at position 2788 of the PGEMHE sequence to a unique NdeI site. For in vitro synthesis of RNA, the plasmids containing the Ca\(^{2+}\) channel subunits were linearized using the following restriction enzymes: Sse8387I for α15 and α15SC, XbaI for β1b, NotI for α8, PvuII for γ, and NheI for γ. Capped cRNA was synthesized using the mMessage mMACHINE T7 kit (for α15, α15SC, β1b, α8, and γ) or SP6 kit (for β1a, Ambion Corp.) and extracted using the RNeasy purification kit (Bio101).

Stage V and VI oocytes were harvested from egg-bearing female Xenopus laevis frogs under anesthesia with 3-aminobenzoic acid ethyl ester (1 mg ml\(^{-1}\) in a cold water bath for 25 min; Sigma-Aldrich), in accordance with the guidelines of the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Oocytes were removed into Ca\(^{2+}\)-free OR-2 solution containing (mM): 82.5 NaCl, 2.5 KCl, 1 MgCl\(_2\), and 5 HEPES, pH 7.6. The egg sacs were manually torn open using forceps, and the oocytes were incubated in OR-2 containing 2 mg ml\(^{-1}\) collagenase (GIBCO BRL) for 2.5 h in a room temperature shaker (60 rpm) to remove the follicular membrane. The oocytes were then washed four times in OR-2 solution and transferred for storage to ND-96 solution containing (mM): 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 2.5 pyruvate, 5 HEPES, and 50 μg ml\(^{-1}\) gentamicin (GIBCO BRL), pH 7.0. Oocytes were injected with 50–100 nl of a 1:1:1:1 mixture of α15 (or α15SC), β1b (or β1a), α8, and γ cRNA using injection pipettes pulled from thin-wall capillary glass (World Precision Instruments) and were stored at 18°C for 5–7 d before recording.

Oocytes were voltage clamped in the cut-open configuration, with active clamp of the upper and guard compartments, using an oocyte clamp (CA-1B; Dagan Corp.) under control of a custom stimulation/recording program written in AxoBASIC and running on an IBM-compatible computer. Voltage-sensing electrodes, fabricated from borosilicate capillary glass (1.65-mm outer diameter; VWR Scientific) using a multi-stage puller (Sutter Instrument Co.), were filled with 3 M KCl and had resistances of 0.2–1 MΩ. The leads of the amplifier headstage, attached to Ag/AgCl pellets in plastic wells containing 2 M NaCl solution, were connected to the upper, guard, and lower oocyte compartments using glass agar bridges containing 110 mM Na-methanesulfonic acid and 10 mM HEPES, in 3% agarose, pH 7.0, and threaded with a platinum wire. Current traces, evoked by depolarizing test pulses from a holding potential of −90 mV, were corrected for linear leak and capacity currents (minimized by the analog compensation of the amplifier) by addition of control currents evoked by hyperpolarizing pulses of equal amplitude from the holding potential (P/5−1 protocol). Since the charge movement was not noticeably altered by changing the holding potential to −110 or −120 mV (data not shown), we judged our leak subtraction protocol to be sufficient to selectively subtract linear components. Signals were filtered at 1 kHz and sampled at 20 kHz (for gating currents) or 2 kHz (for ionic currents). Analysis and curve fitting was performed using a combination of custom AxoBASIC routines and SigmaPlot (Jandel Scientific). Student’s t test was used to make pairwise comparisons between sets of data (P values are reported in the text; P = 0.05 was taken as the limit of statistical significance).

Solutions for recording Ba\(^{2+}\) currents and Ca\(^{2+}\) channel gating currents were prepared following O’Cise et al. (1996) and Stefani and Bezanilla (1998) and were Ca\(^{2+}\)-free to avoid contamination with oocyte Cl\(^{−}\) currents (including Ba\(^{2+}\)-activated Cl\(^{−}\) currents). For gating current measurements, the external solution (applied in the external and guard compartments) contained (mM): 2 Ca(OH)\(_2\), 96 NaOH, and 10 HEPES, adjusted to pH 7.0 with methanesulfonic acid. For ionic current measurements, the external solution contained 10 mM Ba(OH)\(_2\) instead of 2 mM Ba\(^{2+}\).
Co(OH)₂, but was otherwise identical. The lower chamber in contact with the part of the oocyte permeabilized with 0.1% saponin (Sigma-Aldrich) contained 110 mM potassium glutamate and 10 mM HEPES, adjusted to pH 7.0 with NaOH. To avoid possible contamination with nonlinear charge movement related to the oocyte Na⁺–K⁺ pump (Neely et al., 1993; Olcese et al., 1996; Stefani and Bezanilla, 1998), 0.1 mM ouabain (Sigma-Aldrich) was present in all external solutions.

**RESULTS**

Oocytes injected with either α₁S or α₁SAC plus the auxiliary subunit cRNAs showed transient currents at the beginning and end of short depolarizing pulses, while un.injected oocytes and oocytes injected with auxiliary subunit cRNAs alone lacked these currents (Fig. 1 A). These transient currents had the properties expected of gating currents arising from the movement of charged elements in the ion channel protein: (a) they depended on the presence of the pore-forming α subunit (Fig. 1 A); (b) they always followed the direction of the voltage step (A); (c) the integral of the current at the onset of a voltage step (Qon) matched the integral of the current at the offset of the step (Qoff) (B); and (d) Qon and Qoff approached limiting values at both positive and negative potentials, with a voltage dependence that followed a Boltzmann distribution (C).

Fig. 2, which compares the magnitude and voltage dependence of the charge movement measured in oocytes expressing α₁S and α₁SAC, shows that both constructs were clearly expressed abundantly at the surface membrane regardless of which β₁ subunit isoform was coexpressed. Qoff was used for the comparison instead of Qon because of the faster kinetics (and hence greater signal/noise ratio at small test depolarizations) of the OFF response. The maximum Qoff appeared larger in oocytes expressing α₁SAC than in oocytes expressing α₁S (Fig. 2, A and B). While this effect on Qoff,max was not statistically significant in the presence of β₁a (Fig. 2 A; P = 0.13), it was statistically significant in the presence of β₁b (B; P = 0.008). The normalized Qoff versus voltage (Q-V) curves for α₁S and α₁SAC had roughly the same midpoints and slopes (Fig. 2 C). As has been observed for other voltage-dependent ion channels and for native L-type Ca²⁺ channels in skeletal muscle, the Q-V curve started at significantly more negative potentials and was less steep than the G-V curve for L-type current (Fig. 2 C, ▼). This is consistent with the idea that skeletal muscle Ca²⁺ channels undergo rapid voltage-dependent transitions among several closed conformations before opening, some of which are presumably involved in excitation–contraction coupling (Feldmeyer et al., 1990; Ma et al., 1996).

**Figure 1.** Gating currents from oocytes expressing the truncated and full-length forms of the rabbit skeletal muscle L-type Ca²⁺ channel α₁ subunit (α₁SAC and α₁S). (A) Voltage-clamp traces recorded in six oocytes during 20-ms depolarizing steps to the values shown on the left from a holding potential of −90 mV. The combination of cRNAs injected is shown above each set of traces. Each trace is the average of four responses filtered at 1 kHz and sampled at 20 kHz. The dotted lines represent zero current. (B) Qon vs. −Qoff plot for the α₁SAC, β₁a-injected oocyte from A, middle right, shows charge conservation. A straight line with a slope of 1 is shown as a reference. Values of Qon and Qoff were obtained by integrating the gating currents during and after the pulse. 10 datum points (0.5 ms) near the end of the pulse were used to define the baseline for Qon, while 10 datum points near the end of the trace (3–5 ms after the end of the pulse) were used to define the baseline for Qoff. Qon was integrated throughout the pulse to allow slow components of charge movement to be included. Qoff did not have prominent slow components and was integrated over the first ~3 ms after the pulse. A number of cells showed small, slow tail currents at depolarized potentials (see +20 mV traces in A, top right and middle left) that were excluded from Qoff by baseline subtraction. (C) Qon (●) and Qoff (▼) versus voltage for the same oocyte were fitted by Boltzmann distributions of the form: Q-V = Qmax/[1 + exp{(V − V1/2)/kD}] where Qmax is the maximal charge moved at depolarized voltages, V1/2 is the voltage at which charge movement is half maximal, and kD describes the steepness of the distribution. The solid line shows the average of the Qon and Qoff fits: Qmax = 175 pC, V1/2 = −25.9 mV, and kD = 15.6 mV.

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Co2+ the standard error of the mean and are smaller than the symbol when not visible. Gating currents were recorded in 2 mM extracellular 0.3 pC, V1/2Q dividing each I-V curve by Gmax(V = conductance attained, Erev is the current reversal potential, V1/2G is the half-activation potential, and small Ba2+ injected eggs. Moreover, this difference was observed drive gating charge movements not found in uninjected. Hall, 1997), but that injecting cRNA coding for either full-length or truncated α15 resulted in large voltage-driven gating charge movements not found in un.injected eggs. Moreover, this difference was observed whether β1a (Fig. 3, A and B) or β1b (C and D) cRNA was coinjected. The maximal amplitude of the gating currents measured in each egg was comparable with that of the largest ionic current, consistent with the idea that channel expression was high but the channel open probability was low. Indeed, while the ionic currents, even those carried by α15SC, were small compared with those measured for other cloned Ca2+ channels expressed in oocytes, the gating currents were as large as or larger than those of other Ca2+ channels (Neely et al., 1993; Olcese et al., 1996). This suggests that oocyte expression of the skeletal muscle Ca2+ channels is considerably more robust than has been previously believed. Oocytes injected with cRNA coding for the auxiliary subunits alone had DHP-insensitive inward Ba2+ currents comparable in size with the L-type current, but with rapid kinetics, presumably carried by an endogenous Ca2+ channel (Fig. 3 E, top). These oocytes did not have measurable gating currents (Fig. 3 E, bot-
The primary goal of this study was to determine whether the different magnitudes of L-type Ba$^{2+}$ current observed previously when the full-length $\alpha_{1S}$ and truncated $\alpha_{1SAC}$ subunits were expressed in Xenopus oocytes (Ren and Hall, 1997) represents a function specialization of these two size forms of the skeletal muscle L-type Ca$^{2+}$ channel. Using the cut-open oocyte voltage clamp method, we measured large transient currents from oocytes expressing $\alpha_{1S}$ or $\alpha_{1SAC}$ with either the $\beta_1$ or $\beta_1b$, $\alpha_\delta$, and $\gamma$ subunits that represented gating charge movements in the $\alpha_1$ subunit molecule, demonstrating that both size forms of the channel are expressed in oocytes and constitute working voltage sensors. The range and steepness of the voltage dependence of the charge movement mediated by $\alpha_{1S}$ and $\alpha_{1SAC}$ were essentially the same, independent of which $\beta_1$ subunit was coexpressed. The maximum amount of charge moved at depolarized voltages, $Q_{\text{max}}$, tended to be larger for $\alpha_{1SAC}$ than for $\alpha_{1S}$. However, this difference in $Q_{\text{max}}$ was statistically significant (both $Q_{\text{on, max}}$ and $Q_{\text{off, max}}$) only when the $\beta_1b$ subunit, rather than the $\beta_1a$ subunit, was coexpressed. Measurements of both charge movement and ionic current from the same oocyte showed that: (a) oocytes expressing the truncated subunit plus auxiliary subunits supported both charge movements and L-type Ba$^{2+}$ current; (b) oocytes expressing the full-length subunit plus auxiliary subunits had charge movements but very little L-type Ba$^{2+}$ current; and (c) oocytes expressing the
auxiliary subunits alone had neither detectable charge movements nor L-type Ba\(^{2+}\) current. The stark differences observed between cells expressing \(\alpha_{1S}\) versus \(\alpha_{1SC}\) suggest there was no appreciable COOH-terminal truncation of \(\alpha_{1S}\) in the oocyte.

We conclude that the two naturally occurring size forms of the skeletal muscle DHP receptor \(\alpha\) subunit are functionally specialized when expressed with the auxiliary subunits found in muscle. \(\alpha_{1S}\) and \(\alpha_{1SC}\) are both functional voltage sensors, but \(\alpha_{1SC}\) is a much
more effective Ca^{2+} channel. Only tiny ionic currents were seen when the α_{1S} subunit was coexpressed with either the β_{1a} or β_{1b} subunit (Fig. 4, A and C), and the currents observed when the α_{1S}/β_{1b}/α_{3δ}/γ combination was injected were ~90% insensitive to 5 μM nimodipine (data not shown). This differs from the data of Ren and Hall (1997), who observed what appeared to be L-type current when α_{1S} was expressed with β_{1b}, α_{3δ}, and γ (although this conclusion was based on current kinetics rather than on dihydropyridine block). We observed robust ionic currents when either β_{1a} or β_{1b} cRNAs were coinjected with α_{1SAC} cRNA, in contrast to the original report of a requirement for β_{1b} (Ren and Hall, 1997). Moreover, separate experiments in which 5 μM nimodipine was applied to oocytes expressing the α_{1SAC}/β_{1b}/α_{3δ}/γ combination confirmed that the inward current seen in this case was 80–90% L-type (data not shown). Our results therefore support the hypothesis that the predominant form of native DHP receptors in skeletal muscle, those containing α_{1SAC} and β_{1b}, can function both as voltage sensors and as L-type calcium channels. Given the strong similarity of the kinetics and voltage dependence of the ionic and gating currents measured here in oocytes to those measured previously in mammalian muscle cells (Dulhunty and Gage, 1983; Simon and Beam, 1985; Lamb, 1987; Delbono, 1992; Garcia et al., 1992), we believe that the functional differences we observed for α_{1S} and α_{1SAC} in oocytes are likely to occur in native DHP receptors. Nevertheless, experimental confirmation of the distinct functional roles of the α_{1S} and α_{1SAC} subunits must ultimately be delineated in native cells, especially given the mounting evidence from dyspedic myotubes that both functions of the DHP receptor are strongly influenced by interacting ryanodine receptors (Nakai et al., 1996; Avila and Dirksen, 2000). Definitive proof of distinct roles for the two size forms of the α_{1S} subunit may ultimately require the identification and selective inhibition of the enzyme responsible for COOH-terminal truncation.

The mechanism by which the carboxyl terminus modulates the coupling between voltage-dependent gating and channel opening remains unknown. A similar effect has been observed for the cardiac L-type Ca^{2+} channel. Deletion of amino acids 307–472 from the 665-amino acid carboxyl terminus of the α_{1C} subunit increased Ba^{2+} currents four- to sixfold in the oocyte expression system (Wei et al., 1994). The increase in ionic current did not appear to be accompanied by an increase in gating charge, consistent with the notion that the coupling was enhanced, rather than an increase in the number of channels. The confidence in this conclusion is somewhat limited, however, by the fact that charge movements were measured in the presence of large, unblocked inward Ba^{2+} currents. Several motifs important for modulation of Ca^{2+} currents have been identified in the carboxyl tails of α subunits. The carboxyl terminus deletion of α_{1S} studied herein removes several consensus sites for PKC and cAMP-mediated phosphorylation, but leaves intact an EF hand and calmodulin-binding IQ consensus motifs, both of which have been implicated in Ca^{2+}-dependent modulation of inactivation and facilitation of ionic current (Lee et al., 1999; Zühlke et al., 1999). It remains to be determined whether the enhanced coupling of gating charge movement to channel opening we observed in α_{1SAC} is dependent on any of these previously identified modulatory roles of the carboxyl terminus. Our observation that truncation of the α_{1S} subunit significantly increased the magnitude of gating charge movements (an effect not seen with COOH-terminal truncation of α_{1C}) suggests that the COOH-terminus of α_{1S} may play a role in channel expression in the membrane as well as in the coupling of gating to pore opening.

Our experiments give the unexpected result that both size forms of the α_{1S} subunit express as well as or better than other cloned Ca^{2+} channel isoforms in Xenopus oocytes, as judged by gating current amplitudes (Neely et al., 1993; Olcese et al., 1996). This finding contradicts the common belief that the small ionic currents seen by us and others trying to express α_{1S} in heterologous systems (Perez-Reyes et al., 1989; Nargeot et al., 1992; Johnson et al., 1997) reflect an inability of this channel to express stably outside the muscle environment. On the other hand, the local environment of the triadic junction does enhance the efficacy of DHP receptors to function as Ca^{2+} channels, as shown by a decrease in the ratio of ionic to gating current in dyspedic mice lacking the skeletal muscle isoform of the ryanodine receptor (Nakai et al., 1996). The large gating currents we observed relative to the small ionic currents underscore the idea that the coupling of charge movement to pore opening is poor in DHP receptors (Flockerzi et al., 1986; Ma et al., 1991; Dirksen and Beam, 1995), even for channels containing the truncated α_{1S} subunit. Robust expression of the skeletal muscle L-type Ca^{2+} channel in a heterologous system will be useful in elucidating the functional consequences of muscle-specific modulatory mechanisms, which may include modifications to the channel protein (such as phosphorylation or the COOH-terminal truncation studied here) or interactions with other proteins (such as the auxiliary Ca^{2+} channel subunits or other components of the triad junction). In addition, the ability to record skeletal muscle Ca^{2+} channel gating currents in an expression system lacking the electrically complex features of muscle cells, such as t tubules and additional voltage-gated channels, may allow for unprecedented precision in the study of the channel voltage sensor.

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