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*Research Article*

# The IQ Motif is Crucial for $Ca_v1.1$ Function

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$Ca^{2+}$-dependent modulation via calmodulin, with consensus CaM-binding IQ motif playing a key role, has been documented for most high-voltage-activated $Ca^{2+}$ channels. The skeletal muscle $Ca_v1.1$ also exhibits $Ca^{2+}$-/CaM-dependent modulation. Here, whole-cell $Ca^{2+}$ current, $Ca^{2+}$ transient, and maximal, immobilization-resistant charge movement ($Q_{max}$) recordings were obtained from cultured mouse myotubes, to test a role of IQ motif in function of $Ca_v1.1$. The effect of introducing mutation (IQ to AA) of IQ motif into $Ca_v1.1$ was examined. In dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$, neither $Ca^{2+}$ currents nor evoked $Ca^{2+}$ transients were detectable. The loss of $Ca^{2+}$ current and excitation-contraction coupling did not appear to be a consequence of defective trafficking to the sarcolemma. The $Q_{max}$ in dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ was similar to that of normal myotubes. These findings suggest that the IQ motif of the $Ca_v1.1$ may be an unrecognized site of structural and functional coupling between DHPR and RyR.

## 1. Introduction

Calcium entering the cell through voltage-gated $Ca^{2+}$ channels plays an important role in mediating a wide variety of cellular events and includes feedback processes that regulate activity of the channel itself. The $Ca^{2+}$-dependent modulation of channel activity mediated by the $Ca^{2+}$-binding protein calmodulin (CaM) is found in many ion channels including the $Ca_v1$ family [1]. $Ca^{2+}$-dependent inactivation (CDI) of $Ca_v1.2$ is mediated by CaM, and its structural determinants have been assigned to the proximal region of the C-terminus of $Ca_v1.2$ [1, 2]. Three domains have been identified within this region: a $Ca^{2+}$ binding EF-hand motif, a CaM-tethering site, and a CaM-binding IQ motif. The EF-hand motif, located ~16 residues beyond the end of the last transmembrane segment (IVS6), is absolutely necessary for CDI. The CaM-tethering site, which consists of both $preIQ_3$ and IQ motifs, resides 50 amino acids downstream from the EF-hand motif and binds $Ca^{2+}$-free CaM (apo-CaM) at resting $[Ca^{2+}]_i$. The IQ motif resides downstream from the EF-hand motif and the pre-$IQ_3$ domain, and it binds $Ca^{2+}$-CaM. When the interaction of CaM with either of these domains is compromised, CDI is reduced or eliminated [1, 2].

Recently, it has been demonstrated that the skeletal muscle L-type $Ca^{2+}$ channel ($Ca_v1.1$) also displays CDI mediated by CaM and that CaM associates with $Ca_v1.1$ *in vivo* [3]. The initial 200 amino acids of the C-terminus of the $Ca_v1.1$ are highly conserved and contain the above-described domains including the IQ motif. CaM binding to the IQ motif of $Ca_v1.2$ channel has been shown to be necessary for CDI, and the mutation of the isoleucine (I1624) and glutamine (Q1625) to alanines (AA) in the IQ motif of $Ca_v1.2$ resulted in ablation of CDI and significant reduction of apoCaM binding to $Ca_v1.2$ [1, 2, 4]. Whether the IQ motif in $Ca_v1.1$ plays a similar role remains to be determined.

In the present work, myotubes cultured from normal and dysgenic (lacking endogenous $Ca_v1.1$) mice were used to investigate the role of the IQ motif in the function of $Ca_v1.1$. The results presented demonstrate that the IQ motif in the C-terminus of $Ca_v1.1$ is critical for function of $Ca_v1.1$ as a voltage sensor as well as $Ca^{2+}$ channel. Furthermore, the results indicate that the IQ motif may be a previously unrecognized site of protein-protein interaction between $Ca_v1.1$ and the skeletal muscle ryanodine receptor (RyR1) and may play a role in skeletal muscle excitation-contraction (EC) coupling.

## 2. Experimental Procedures

*2.1. Molecular Biology.* The coding sequence of yellow fluorescent protein- (YFP-) tagged $Ca_v1.1$ channel (YFP-$Ca_v1.1$) was a gift from Dr. K. Beam and is described in detail elsewhere [5]. The residues isoleucine (I) and glutamine (Q) at codons 1529-1530 of rabbit $Ca_v1.1$ [6] were substituted with alanine (A) using the QuikChange II mutagenesis kit (Stratagene, La Jolla, CA), using the YFP-$Ca_v1.1$ as a template. The construct YFP-$Ca_v1.1_{AA}$ was verified by restriction digest analysis and sequencing.

*2.2. Cell Cultures.* Primary myotubes were cultured from normal or dysgenic newborn mouse skeletal muscle as previously described [3]. For confocal microscopy purposes, primary cultures of myotubes were plated onto 35 mm culture dishes with integral no. 0 glass coverslip bottoms (MatTek) instead of Primaria dishes. Approximately one week after plating, dysgenic myotubes were injected with expression plasmids (cDNAs) encoding either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$ at concentrations of 0.2 $\mu$g/$\mu$L, respectively. In experiments assessing the effects of CaM on $Ca^{2+}$ transients, normal myotubes (~one week in culture) were injected with expression plasmids encoding $CaM_{wt}$ or $CaM_{1234}$ (gift of Dr. Yue) and green fluorescent protein (pEGFP-C1, BD Biosciences Clontech, CA) at concentrations of 0.1 and 0.02 $\mu$g/$\mu$L, respectively. Successfully transfected myotubes were identified 36–48 hours after injection by their *yellow* or *green* fluorescence under UV illumination.

*2.3. Electrophysiology.* Patch pipettes were constructed of borosilicate glass and had resistances of 1.8–2.5 MΩ when filled with the standard internal solution, which contained (in mM) 145 Cs-aspartate, 10 $Cs_2$-EGTA, 5 $MgCl_2$, and 10 HEPES (pH 7.4 with CsOH). The external solution contained (in mM) 145 tetraethylammonium chloride (TEA-Cl), 10 $CaCl_2$, 0.003 tetrodotoxin, and 10 HEPES (pH 7.4 with TEA-OH). The holding potential was –80 mV, and test pulses were preceded by a 1-s prepulse to –30 mV to inactivate endogenous T-type $Ca^{2+}$ currents. Recorded membrane currents were corrected off line for linear components of leakage and capacitance by digitally scaling and subtracting the average of 10 preceding control currents, elicited by hyperpolarizing voltage steps (30 mV amplitude) from –50 mV. $Ca^{2+}$ currents were normalized by linear cell capacitance (expressed in pA/pF). Values for $G_{max}$, the maximal $Ca^{2+}$ conductance, were obtained by fitting the measured currents according to the following equation:

$$I_{peak} = \frac{G_{max}\,(V - V_R)}{\{1 + \exp\,[-(V - V_{1/2})/k]\}}, \quad (1)$$

where $I_{peak}$ is the peak current activated at the test potential $V$, $V_R$ is the extrapolated reversal potential, $V_{1/2}$ is the potential for half-maximal activation of the $Ca^{2+}$ conductance, and $k$ is a slope factor.

The fraction of current remaining at the end of an 800 ms test pulse ($r_{800}$) was determined by dividing the current remaining at the end of test pulse by the peak current, and this ratio was used to quantify the level of inactivation

$$r_{800} = \frac{I_{end}}{I_{peak}}. \quad (2)$$

For measurements of charge movement, 0.5 mM $Cd^{2+}$ and 0.1 mM $La^{3+}$ were added to the external solution to block $Ca^{2+}$ currents. Charge movements were elicited in response to a prepulse protocol that consisted of a 1-s prepulse to −30 mV and a subsequent 40 ms repolarization to a pedestal potential (−50 mV), followed by a 25 ms depolarization to +40 mV. The maximum amount of charge that can be moved ($Q_{max}$) was obtained by integrating the charge movement current at test potential of +40 mV. Linear leak and capacity currents were subtracted on line using −P/4 delivered from the holding potential (−80 mV) before each pulse. Charge movements were normalized to total cell capacitance (nC/$\mu$F).

To measure relative changes in voltage-gated $Ca^{2+}$ release from the SR, the $Ca^{2+}$ indicator $K_5$-Fluo-3 (0.5 mM) (Molecular Probes) was included in the pipette solution. After rupture of the cell membrane and entry into the whole cell configuration, cells were allowed to dialyze for about 5 min before recording in order to achieve adequate loading with indicator dye. Fluorescent emission was measured by a photomultiplier system (Biomedical Instrumentation Group, University of Pennsylvania). The set of filters used to record the fluorescent signal from Fluo-3 was as follows: excitation band-pass filter of 470/20 nm; dichroic long-pass mirror (510 nm); emission long-pass filter of 520 nm. After rupture and dye loading into the cell, the baseline fluorescence ($F_{base}$) was monitored. The increase in fluorescent signal during depolarization was expressed as $\Delta F/F$, where $\Delta F$ represents the increase in fluorescence above baseline fluorescence ($\Delta F = F_{transient} - F_{base}$), and $F$ is $F_{base}$. Peak fluorescence during each test pulse was plotted as a function of test potential V and fitted according to the following equation:

$$\frac{\Delta F}{F} = \frac{(\Delta F/F)_{max}}{\{1 + \exp[(V_{F1/2} - V)/k_F]\}}, \quad (3)$$

where $(\Delta F/F)_{max}$ is the maximal fluorescent change, $V_{F1/2}$ is the potential for half-maximal activation of the $Ca^{2+}$ transient, and $k_F$ is a slope factor.

All recordings were performed at room temperature (~20°C), and data are reported as mean ± SEM; $n$ indicates the number of myotubes tested. Data sets were statistically compared by an unpaired, two-sample Student's $t$-test, with a confidence interval of at least 95%.

*2.4. Confocal Microscopy.* Cells were bathed in rodent ringer (in mM: 146 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 10 HEPES; pH 7.4 adjusted with NaOH) and examined with an LSM 510 META laser scanning microscope (Zeiss, Thornwood, New York) with 40X oil immersion objective. The laser line (514 nm) of the argon laser (30 mW maximum output, operated at 50% or 6.3 A) was used to excite YFP

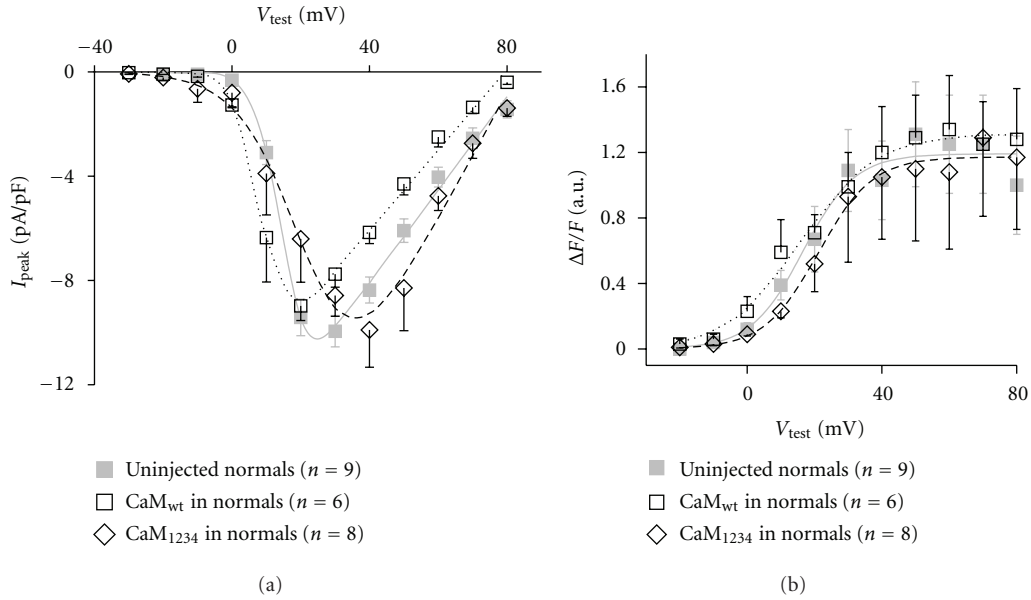


FIGURE 1: $Ca^{2+}$-binding ability of CaM does not affect skeletal muscle EC coupling. (a) The average peak current density ($I_{peak}$) is plotted as a function of membrane potential ($V_{test}$). Data were obtained from the indicated number of myotubes for each group. The smooth lines through the data were generated by using equation (1) (see Methods) and the average values. (b) The average peak fluorescence ($\Delta F/F$) is plotted as a function of membrane potential ($V_{test}$). Data were obtained from the indicated number of myotubes for each group. The smooth lines through data represent Boltzmann fits to the average data using equation (3) (see Methods).

fluorophore. Emissions of YFP were recorded in single-track configuration with a long-pass filter of 530 nm (Chroma, Rockingham, Vermont). Fluorescence signals were analyzed by the 510 LSM Image Examiner software (Zeiss, Thornwood, New York).

*2.5. Immunocytochemistry.* Primary cell cultures were plated onto 35 mm culture dishes with integral no. 0 glass coverslip bottoms (MatTek). Myotubes expressing constructs of $Ca_v1.1$ were identified by fluorescence. The cultures will be fixed with 100% methanol at −20°C for a minimum of 20 min. Cells were then incubated for 1 hour in PBS (phosphate-buffered saline) containing 1% BSA (bovine serum albumin) and 10% goat serum to block unspecific labeling. After 3 washes with PBS/BSA (.2%), cell cultures were incubated with specific primary antibody against the RyR1 (34C, Developmental Studies Hybridoma Bank (DSHB), UI) (dilution 1 : 4000) overnight at 4°C. Cells were washed out 3 times with PBS/BSA (.2%), followed by 1 hour of incubation with secondary antibody conjugated with Alexa 568 (at final dilution 1 : 5,000, goat anti-rabbit IgG, Invitrogen). Cells were then washed 3 times with PBS/BSA (.2%) to remove unbind secondary antibody and assessed with a confocal microscope.

## 3. Results

*$Ca^{2+}$-binding ability of CaM does not affect skeletal muscle EC coupling.* First, I addressed the question whether the $Ca^{2+}$-binding ability of CaM plays any role in skeletal muscle EC coupling. Overexpressed mutant CaM which does not bind $Ca^{2+}$ ($CaM_{1234}$) can displace approximately 70% of endogenous CaM, as reflected by abolishment of CDI of $Ca_v1.1$ [3]. However, overexpression of either $CaM_{wt}$ or $CaM_{1234}$ in normal myotubes did not significantly affect either current-voltage ($I/V$) relationship (Figure 1(a)) or voltage-gated $Ca^{2+}$ release from SR as indicated by similar peak fluorescence-voltage relationship ($\Delta F/F$-$V$) in comparison with uninjected normal myotubes (Figure 1(b)). This result suggests that either the $Ca^{2+}$-binding ability of CaM or CaM itself does not play a role in skeletal muscle EC coupling. However, CaM associates with $Ca_v1.1$ *in vivo* [3] and that indicates the possibility that CaM may still serve as a structural subunit of $Ca_v1.1$, that is, that interaction between CaM and $Ca_v1.1$ can stabilize the DHPR complex. By doing so, it may also ensure proper structural and functional coupling between DHPR and RyR1.

Therefore, I examined whether CaM association with $Ca_v1.1$ is necessary for its function as a voltage sensor for EC coupling. The IQ motif of $Ca_v1.1$ has been shown to bind CaM similar to IQ motifs of $Ca_v1.3$ and $Ca_v2$ channels [7]. Introduction of the mutation IQ/AA in the IQ motif of the cardiac L-type $Ca^{2+}$ channel ($Ca_v1.2$) resulted in abolishment of CDI and significant reduction of apoCaM binding to $Ca_v1.2$ [2, 4]. Thus, corresponding IQ motif mutation in the C-terminus of $Ca_v1.1$ was obvious place to start.

The mutation (IQ/AA) in the CaM-binding site of $Ca_v1.1$ disables function of $Ca_v1.1$ as a $Ca^{2+}$ channel and voltage sensor for EC coupling. I introduced the IQ/AA mutation in the C-terminus of $Ca_v1.1$ and investigated how this

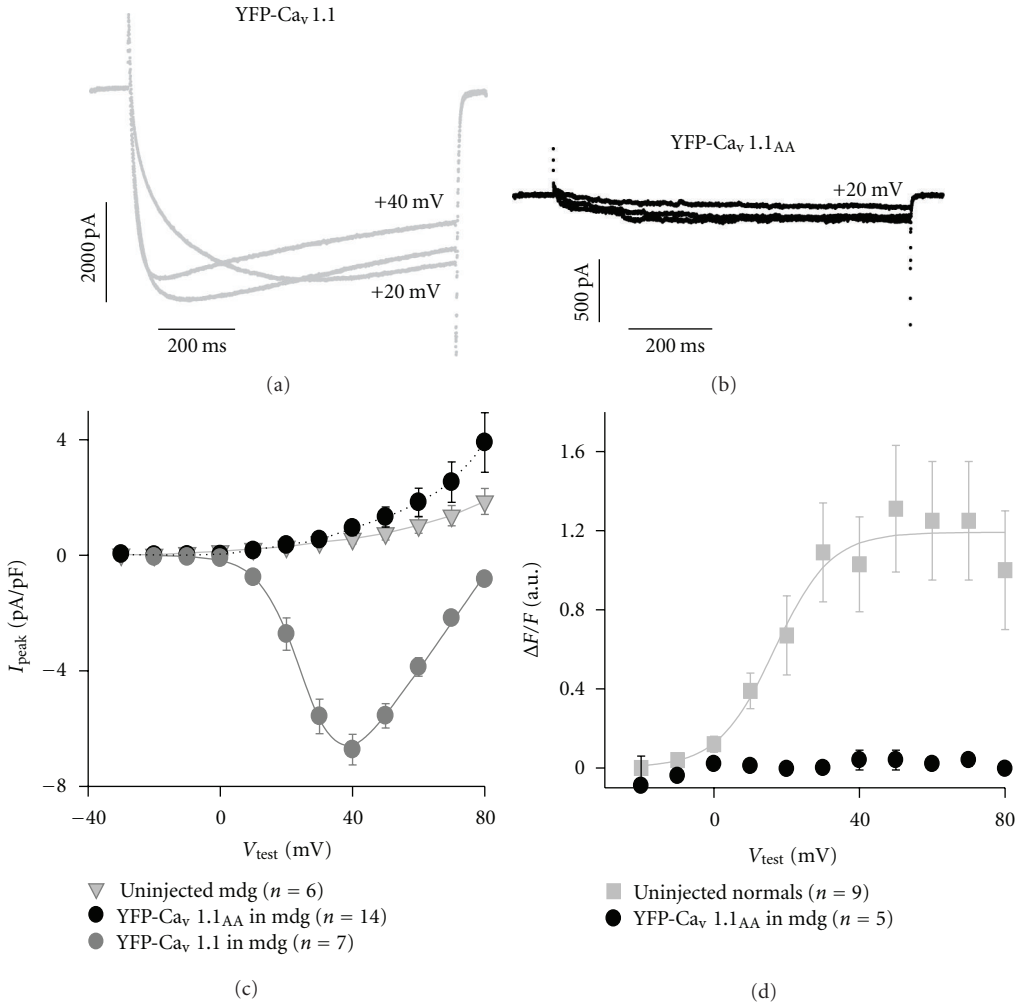


FIGURE 2: The IQ/AA mutation disables function of $Ca_v1.1$ as a $Ca^{2+}$ channel and voltage sensor for EC coupling. Representative whole-cell L-type $Ca^{2+}$ currents recorded from dysgenic myotubes expressing either (a) YFP-$Ca_v1.1$ (cell 1 (05-10-02); linear capacitance $C = 587$ pF) or (b) YFP-$Ca_v1.1_{AA}$ (cell 1 (01-13-03); linear capacitance $C = 201$ pF). Note that the current scales are different in (a) and (b). (c) The average peak current density ($I_{peak}$) is plotted as a function of membrane potential ($V_{test}$). Data were obtained from the indicated number of myotubes for each group. The smooth lines through the data were generated by using equation (1) (see Methods) and the average values. (d) The average peak fluorescence ($\Delta F/F$) is plotted as a function of membrane potential ($V_{test}$). Data were obtained from the indicated number of myotubes for each group. The smooth lines through data represent Boltzmann fits to the average data using equation (3) (see Methods).

mutation will alter $Ca_v1.1$ function as $Ca^{2+}$ channel and voltage sensor for EC coupling. Introduction of the mutation IQ/AA in the IQ motif of $Ca_v1.2$ resulted in abolishment of CDI and significant reduction of apoCaM binding to $Ca_v1.2$ [2, 4]. Whether IQ motif in the $Ca_v1.1$ plays a similar role is unknown. Dysgenic myotubes expressing either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$ were used to examine the role of IQ motif in $Ca^{2+}$-dependent inactivation (CDI) of $Ca_v1.1$. Injections of plasmids encoding various constructs of $Ca_v1.1$ into dysgenic myotubes at concentrations of 0.2–0.5 $\mu g/\mu L$ have been previously demonstrated to produce a similar extent of maximal, immobilization-resistant charge movement and similar $Ca^{2+}$ current densities as normal myotubes, which corresponds to similar protein expression levels [3, 6, 8–10].

Figure 2(a) shows $Ca^{2+}$ currents mediated by YFP-$Ca_v1.1$ expressed in dysgenic myotube. The fraction of current remaining at the end of the pulse ($r_{800}$) displayed a U-shaped voltage dependence (data not shown), consistent with a current-dependent inactivation process. In such a process, the extent of inactivation varies in proportion with the amplitude of the inward calcium current, which in turn depends on the number of conducting channels and the electrochemical driving force on calcium. Inactivation was minimal at a test potential of +10 mV, as reflected by an $r_{800}$ value of $0.9 \pm 0.08$ ($n = 7$), and maximal at a test potential of +40 mV, as reflected by a minimum $r_{800}$ value of $0.74 \pm 0.03$ ($n = 7$). Correspondingly, the $Ca^{2+}$ current attained its maximum conductance at +40 mV (Figure 1(c)). Thus, $Ca^{2+}$ currents mediated by YFP-$Ca_v1.1$

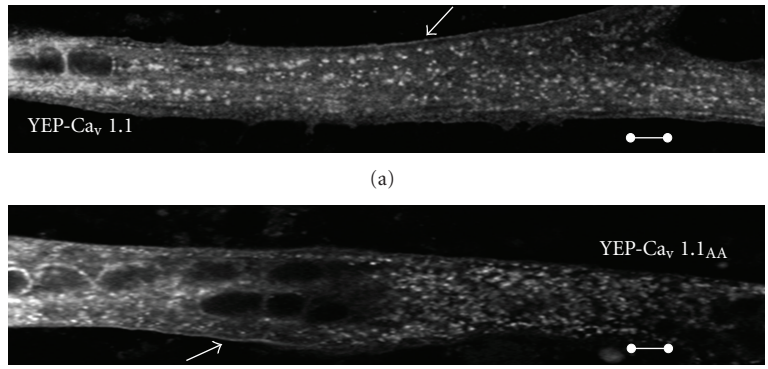


FIGURE 3: YFP-$Ca_v1.1_{AA}$ displays similar expression pattern as YFP-$Ca_v1.1$ in dysgenic myotubes. Confocal images of either YFP-$Ca_v1.1$ (a) or YFP-$Ca_v1.1_{AA}$ (b) *yellow* fluorescence in dysgenic myotubes. *Bar* 50 $\mu$m.

displayed a current-dependent inactivation process, current-voltage ($I$-$V$) relationship (Figure 2(c)), and maximal $Ca^{2+}$ ion conductance ($G_{max}$ = 166 ± 18 nS/nF; $n$ = 7) similar to the endogenous $Ca_v1.1$ of normal myotubes [3]. These results suggest that YFP fused to the N-terminus of $Ca_v1.1$ does not interfere with channel function.

In contrast, dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ (Figures 2(b) and 2(c)) displayed either very small (<1pA/pF) or no measurable $Ca^{2+}$ currents. This is a very dramatic and surprising result considering that the corresponding mutation (IQ/AA) in the IQ motif of $Ca_v1.2$ resulted only in ablation of CDI but did not affect the $I$-$V$ relationship of $Ca^{2+}$ currents mediated by $Ca_v1.2$ [2]. Further voltage-gated $Ca^{2+}$ currents and SR $Ca^{2+}$ release were measured simultaneously from dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ and compared with recordings from uninjected normal myotubes and normal myotubes overexpressing $CaM_{wt}$ or $CaM_{1234}$. The voltage-gated $Ca^{2+}$ release from SR was completely abolished in dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ (Figure 2(d)).

The loss of $Ca_v1.1_{AA}$ function could be a result of several scenarios such as that mutation caused misfolding of protein and insufficient membrane targeting or that protein-protein interaction between RyR1 and $Ca_v1.1$ was significantly disturbed. If the latter possibility is the case, this result suggests that either the IQ motif itself or association of CaM with $Ca_v1.1$ is necessary for *orthograde* signaling from $Ca_v1.1$ to RyR1, which underlies skeletal muscle EC coupling.

*The IQ/AA mutation does not prevent proper targeting of $Ca_v1.1$ into sarcolemma.* The severe loss of function, abolished $Ca^{2+}$ current and orthograde signaling mediated by the $Ca_v1.1_{AA}$, could be explained by compromised targeting of $Ca_v1.1$ to the T-SR junction as a result of incomplete protein folding.

Figure 3 shows confocal images of *yellow* fluorescence from a dysgenic myotube expressing either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$. Expression of YFP-$Ca_v1.1$ (a) or YFP-$Ca_v1.1_{AA}$ (b) resulted in the appearance of small yellow fluorescence puncta located near the cell surface. The small puncta correspond to groups of $Ca_v1.1$ localized to T-SR junctions; these puncta are similar in size and distribution to those of $Ca_v1.1$ foci revealed by immunohistochemistry [11]. There is a similar staining of the membrane and distribution of puncta in both myotubes, suggesting that both constructs are likely targeted to T-SR junctions.

To confirm targeting of YFP-$Ca_v1.1_{AA}$ to the sarcolemma, the $Q_{max}$ was measured at +40 mV (Figure 4). The $Q_{max}$ in dysgenic myotubes expressing $Ca_v1.1_{AA}$ (5.9 ± 0.5 nC/$\mu$F; $n$ = 27) was similar to that of normal myotubes (5.5 ± 0.4 nC/$\mu$F; $n$ = 16), but significantly larger ($P < 0.001$) than in dysgenic myotubes alone (2.5 ± 0.2 nC/$\mu$F; $n$ = 18). This finding suggests that IQ/AA mutation in $Ca_v1.1$ did not prevent the protein from being properly targeted or undergoing voltage-dependent conformational changes, which strongly suggest proper folding as intramembrane segment S4 of $Ca_v1.1$ is responsible for voltage-dependent movement.

To further confirm $Ca_v1.1_{AA}$ proper targeting into T-SR junctions and site of EC coupling, I investigated colocalization of $Ca_v1.1$ and RyR1. Dysgenic myotubes expressing either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$ (*yellow* fluorescence: YFP was artificially assigned as *green*) were incubated with specific primary antibody against the RyR1 followed by incubation with secondary antibody conjugated with Alexa 568 (*red* fluorescence). Colocalization of *green* and *red* fluorescence results in *yellow* pattern suggests colocalization of YFP-$Ca_v1.1$ and RyR1 in T-SR junctions *in vivo* (see Figures 5(g) and 5(h)). Colocalization patterns of YFP-$Ca_v1.1_{AA}$ with RyR1 were compared to YFP-$Ca_v1.1$ and RyR1 patterns in 5 different experiments. Colocalization patterns of either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$ with RyR1 were similar and have been analyzed by MetaMorph 7 software (Molecular Devices). Colocalization in near surface slices of z-stacks of YFP-$Ca_v1.1$ and RyR1 was 83 ± 4% ($n$ = 7), and colocalization of YFP-$Ca_v1.1_{AA}$ and RyR1 was 85 ± 2% ($n$ = 7). These results strongly suggest that YFP-$Ca_v1.1_{AA}$ is targeted into T-SR junctions.

Together these results suggest that the IQ/AA mutation is not likely to affect protein folding within membrane. Furthermore, much more drastic alternation or deletions

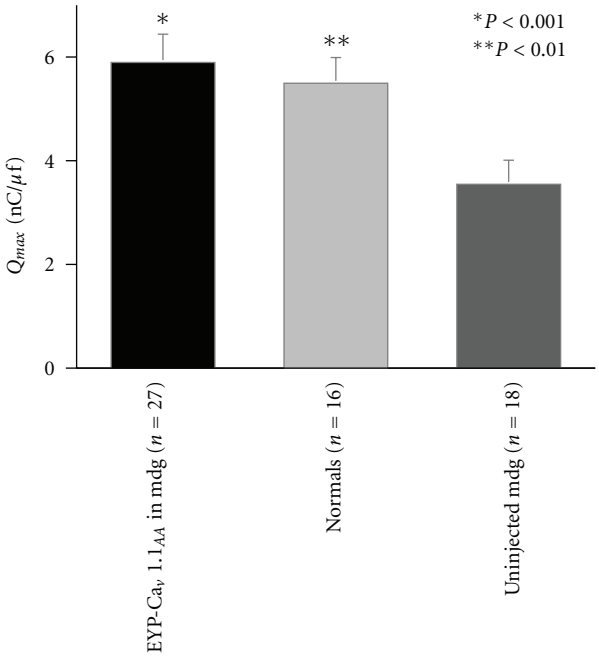


FIGURE 4: $Ca_v1.1_{AA}$ generates normal densities of intramembrane charge movement. The average maximal, immobilization-resistant charge movement at +40 mV ($Q_{max}$) obtained from the indicated number of myotubes for each group. The charge movements were elicited by 25 ms depolarizations from a pedestal potential (−50 mV) to +40 mV. Symbols and error bars represent mean ± SEM.

in $Ca_v1.1$ sequence did not have such dramatic effects [12, 13].

Taking altogether, the loss of both ionic $Ca^{2+}$ current and skeletal muscle EC coupling in $Ca_v1.1_{AA}$ along with charge movement similar to normal myotubes suggests that the IQ motif of the $Ca_v1.1$ may be unrecognized site of protein-protein interaction between $Ca_v1.1$ and RyR1 and play a role in both *orthograde* and *retrograde* signaling.

## 4. Discussion

The present study provides new information about the skeletal muscle L-type $Ca^{2+}$ channel ($Ca_v1.1$). Specifically, the data demonstrate *in vivo* that the IQ motif in the C-terminus of $Ca_v1.1$ is critical for function of $Ca_v1.1$ as a voltage sensor as well as a $Ca^{2+}$ channel. Furthermore, the results indicate that the IQ motif, in addition to II-III loop, may be a previously unrecognized site of protein-protein interaction between $Ca_v1.1$ and RyR1 and, thus, may play a role in skeletal muscle EC coupling.

$Ca_v1.1$ is localized in regions of the T-tubular membrane that are closely apposed to the sarcoplasmic reticulum (i.e., the T-SR junction), and the primary role of $Ca_v1.1$ is to serve as the voltage sensor for skeletal muscle EC coupling. The second protein that plays a major role in this process is the skeletal muscle ryanodine receptor (RyR1). RyR1 is localized in junctional SR membrane and functions as calcium release channel. The mechanism of signal transmission between $Ca_v1.1$ and RyR1 is still incompletely understood, but the most accepted view is that they are mechanically coupled and interact with each other through protein-protein interaction (*orthograde* and *retrograde* signaling). *Orthograde* signaling is the signal from $Ca_v1.1$ to RyR1, in which movement of the voltage sensors in $Ca_v1.1$ trigger opening of RyR1 and release of $Ca^{2+}$ from the SR (EC coupling). *Retrograde* signaling is communication from RyR1 to $Ca_v1.1$, in which RyR1 somehow increases the amount of L-type $Ca^{2+}$ current mediated by $Ca_v1.1$ [8, 9].

The $Ca^{2+}$ conductance of $Ca_v1.1$ channel is not necessary for functional excitation-contraction coupling between RyR1 and $Ca_v1.1$; however, a direct protein-protein interaction between these two proteins in multiple sites is. It has been shown that cytoplasmic loops of $Ca_v1.1$ and several regions of RyR1 play important role for normal physiological EC coupling in skeletal muscle [10, 14–17]. It has been also shown that protein-protein interaction between RyR1 and $Ca_v1.1$ is necessary for $Ca_v1.1$ display of $Ca^{2+}$ conductance (retrograde signaling) [8]. It is clear that there are multiple contact sites between RyR1 and $Ca_v1.1$ and not all of them are recognized and understood, yet. The most investigated region of contact between RyR1 and $Ca_v1.1$ in $Ca_v1.1$ is II-III cytoplasmic loop, but other regions play a role [14, 15].

In the present experiments, normal myotubes and dysgenic myotubes expressing either YFP-$Ca_v1.1$ or YFP-$Ca_v1.1_{AA}$ were used to examine the role of the IQ motif in both functions of $Ca_v1.1$, as a voltage sensor in EC coupling and $Ca^{2+}$ channel. The primary cultures of skeletal muscle myotubes provide a natural cellular environment for $Ca_v1.1$. First, I examined whether a fusion of YFP to $Ca_v1.1$ would interfere with its function. The $Ca^{2+}$ currents mediated by YFP-$Ca_v1.1$ displayed an $I$-$V$ relationship similar to the endogenous $Ca_v1.1$ [3], suggesting that YFP fused on the N-terminus of $Ca_v1.1$ does not interfere with its channel function, as was also shown by others [5]. Endogenous $Ca_v1.1$ also exhibits CaM-mediated $Ca^{2+}$-dependent inactivation (CDI) [3]. The $Ca^{2+}$ currents mediated by YFP-$Ca_v1.1$ also displayed current-dependent inactivation similar to the CDI of endogenous $Ca_v1.1$, further supporting observation that fusion of YFP with $Ca_v1.1$ does not interfere with channel function.

Second, I examined how IQ/AA mutation in $Ca_v1.1$ will affect its function. Surprisingly, the intriguing finding of the present study was that dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ displayed either very small or no measurable $Ca^{2+}$ currents. Significant decrease or abolishment of $Ca^{2+}$ current through $Ca_v1.1$ could have resulted from improper targeting or folding of the protein. If $Ca_v1.1_{AA}$ was retained inside of myotubes due to incorrect folding and targeting, neither $Ca^{2+}$ currents nor $Q_{max}$ would be obtained. The absence of $Ca^{2+}$ currents in some of the dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ would suggest both. However, even though the $Ca^{2+}$ current was absent, $Q_{max}$ comparable with normal myotubes was observed. The $Q_{max}$ measured in dysgenic myotubes expressing $Ca_v1.1_{AA}$ was significantly larger ($P < 0.001$) than in dysgenic myotubes alone, but similar to that of normal myotubes measured here and

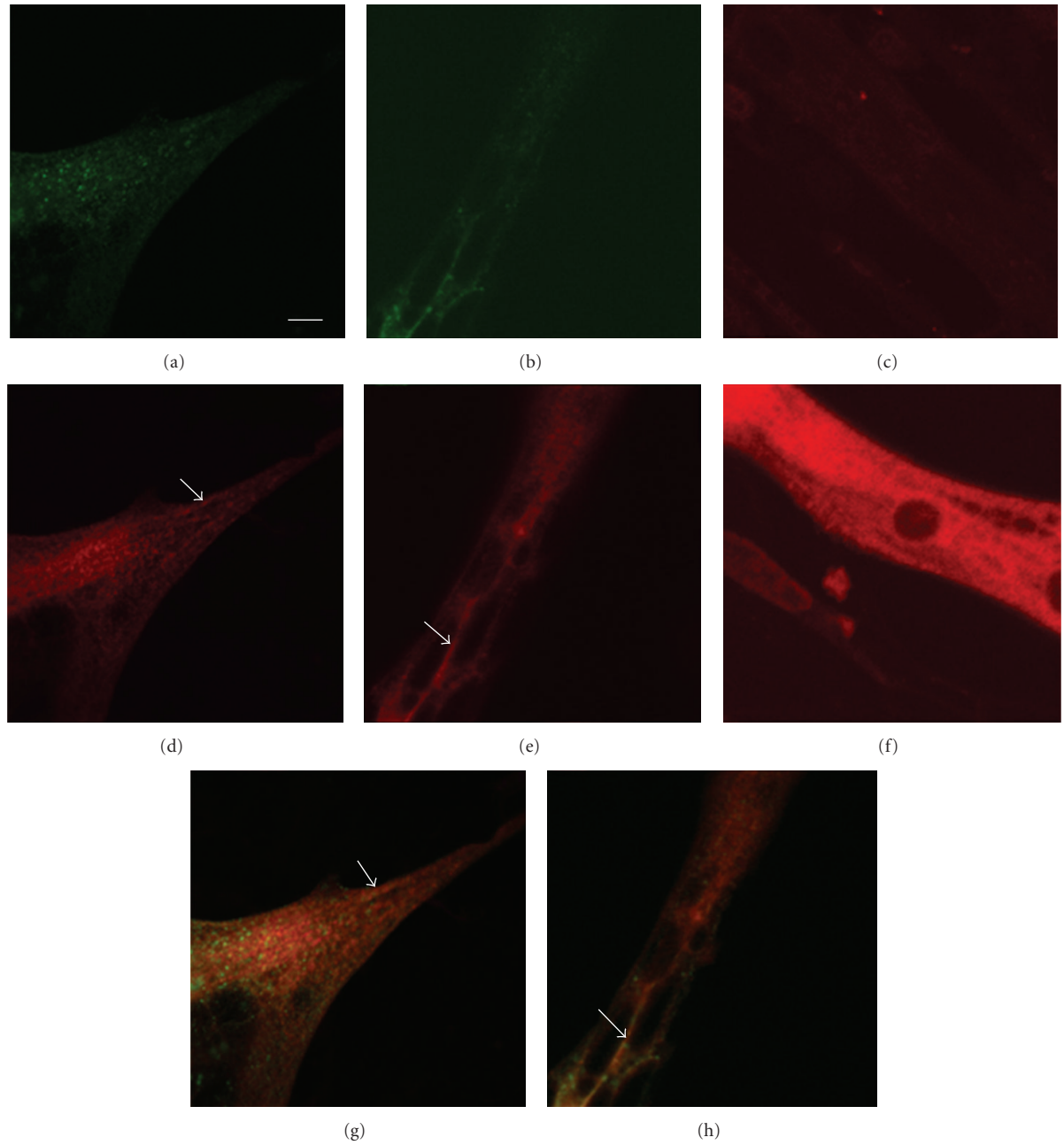


FIGURE 5: $Ca_v1.1_{AA}$ is targeted to T-SR junctions. Confocal images of colocalization of either YFP-$Ca_v1.1$ (a) or YFP-$Ca_v1.1_{<?erm?>AA<?erm?>}$ (b) *green* fluorescence and immunolabeled RyR1 ((d) and (e)) *red* fluorescence in dysgenic myotubes. (g) and (h) represent overlay of (a) and (d), and (b) and (e), respectively. The control dysgenic myotube (no $Ca_v1.1$) immunolabeled without (c) and with (f) primary Ab(34C). *Bar*, 20 $\mu$m.

to the $Q_{max}$ measured in dysgenic myotubes expressing various constructs of wt $Ca_v1.1$ at the similar experimental conditions elsewhere [6, 7].

The amount of $Q_{max}$ in dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ suggests that IQ/AA mutation in $Ca_v1.1$ did not prevent the protein from being properly targeted and that protein can undergo voltage-dependent conformational changes. The size of small measurable $Ca^{2+}$ currents measured in some (6 out of 14) of the dysgenic myotubes expressing $Ca_v1.1_{AA}$ (<1pA/pF) was similar to L-type $Ca^{2+}$ currents measured in dyspedic (lacking a functional gene of RyR1) myotubes [7], suggesting a loss of *retrograde* signaling from RyR1. Endogenous $Ca_v1.1$ channels are present in sarcolemma of the dyspedic myotubes in similar density as in normal myotubes, as was demonstrated by comparable $Q_{max}$ (dyspedic: 4.0 ± 1.4 nC/$\mu$F; normal: 6.4 ± 2.8 nC/$\mu$F) [7]. Thus, the amount of $Q_{max}$ measured in dysgenic myotubes expressing $Ca_v1.1_{AA}$ (5.9 ± 0.5 nC/$\mu$F) is in

good agreement with the previously published values, and indicates that the IQ/AA mutation may have also disrupted *retrograde* signaling between $Ca_v1.1$ and RyR1. The similar expression patterns and comparable colocalization of YFP-$Ca_v1.1$ and YFP-$Ca_v1.1_{AA}$ with RyR1 in dysgenic myotubes obtained by confocal microscopy and immunocytochemistry further support the argument that YFP-$Ca_v1.1_{AA}$ seems to be folded and targeted properly to the T-SR junctions. In addition, much more drastic alternation or deletions in $Ca_v1.1$ sequence did not have such dramatic effects [12, 13].

Third, the IQ/AA mutation in C-terminus of $Ca_v1.1$ had a dramatic effect on its function as a voltage sensor for EC coupling. Even though amount of $Q_{max}$ in dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ is sufficient to support EC coupling (see above), the voltage-gated $Ca^{2+}$ release from SR was completely abolished in these cells. This finding suggests that either tethering of CaM to $Ca_v1.1$ as a structural subunit or the IQ motif itself is necessary for orthograde signaling between $Ca_v1.1$ and RyR1 (EC coupling). Overexpression of $CaM_{wt}$ and $CaM_{1234}$ in normal myotubes did not significantly affect the peak fluorescence-voltage relationship ($\Delta F/F$-$V$) in comparison with uninjected normal myotubes, suggesting that the $Ca^{2+}$-binding ability of CaM does not play a role in skeletal muscle EC coupling in single twitch contractions.

For the first time, the present study shows that the IQ motif plays a role in both *orthograde* (skeletal muscle EC coupling) and *retrograde* ($Ca^{2+}$ current) signaling between $Ca_v1.1$ and RyR1 *in vivo*. Several regions of RyR1 were shown to participate in protein-protein interactions between $Ca_v1.1$ and RyR1. However, until recently only the II-III loop of the $Ca_v1.1$ has been thought to be necessary to convey *orthograde* and *retrograde* signaling between $Ca_v1.1$ and RyR1. The present findings suggest that the C-terminus in addition to the II-III loop participates in and is necessary for the correct transmission of signals between $Ca_v1.1$ and RyR1. These results support previously published *in vivo* findings that in addition to the II-III loop of $Ca_v1.1$ additional intracellular loops of $Ca_v1.1$ are necessary to restore the full extent of *orthograde* and *retrograde* signaling between $Ca_v1.1$ and RyR1 [15]. The present findings also support *in vitro* results from pull-down assays, where it was demonstrated that CaM-binding region of RyR1 (3614–3543) interacts with the proximal C-terminus of $Ca_v1.1$ (1393–1527) in the absence of CaM [18, 19]. It was also shown that CaM binding to the RyR1 is not essential for skeletal EC coupling [20]. This would indicate together with binding studies [18] that CaM association to either $Ca_v1.1$ or RyR1 is not crucial for skeletal muscle EC coupling, but CaM-binding domains of both $Ca_v1.1$ and RyR1 are. For example, it has been shown that CaM-binding region of RyR1 binds to IQ peptide of $Ca_v1.2$ and in pull-down assay binds to $Ca_v1.1$ [18]. It still remains to be determined whether CaM itself needs to be tethered to $Ca_v1.1$ to ensure signaling and more experiments are in progress.

In conclusion, the results from confocal microscopy, immunocytochemistry, charge movement, and $Ca^{2+}$ transients obtained from dysgenic myotubes expressing YFP-$Ca_v1.1_{AA}$ indicate that the IQ motif in the C-terminus of $Ca_v1.1$ plays a crucial role in both *orthograde* (EC coupling) and *retrograde* ($Ca^{2+}$ current) signaling between $Ca_v1.1$ and RyR1.

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