The calcium channel \(\alpha_2\delta_1\) subunit interacts with ATP5b in the plasma membrane of developing muscle cells

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García J. The calcium channel \(\alpha_2\delta_1\) subunit interacts with ATP5b in the plasma membrane of developing muscle cells. Am J Physiol Cell Physiol 301: C44–C52, 2011. First published April 13, 2011; doi:10.1152/ajpcell.00405.2010.—The \(\alpha_2\delta_1\) and \(\alpha_1,1.1\) subunits are present at a 1:1 ratio in the dihydropyridine receptor (DHPR) from adult skeletal muscle. In contrast, during early myotube development \(\alpha_2\delta_1\) is present at higher levels than \(\alpha_1,1.1\) and localizes at the ends of the cells, suggesting that \(\alpha_2\delta_1\) may have a role independent from DHPRs. We sought to identify binding partners of \(\alpha_2\delta_1\) at a period when levels of \(\alpha_1,1.1\) are low. Analysis of protein complexes in their native configuration established that \(\alpha_2\delta_1\) may be associating with ATP5b, a subunit of a mitochondrial ATP synthase complex. This interaction was confirmed with fluorescence resonance energy transfer and coimmunoprecipitation. The association of \(\alpha_2\delta_1\) and ATP5b occurs in intracellular membranes and at the plasma membrane, where they form a functional signaling complex capable of accelerating the rate of decline of calcium transients. The acceleration of decay was more evident when myotubes were stimulated with a train of pulses. Our data indicate that the \(\alpha_2\delta_1\) subunit is not only part of the DHPR but that it may interact with other cellular components in developing myotubes, such as the ATP5b in its atypical localization in the plasma membrane.

skeletal muscle; signaling

THE L-TYPE CALCIUM CHANNEL or dihydropyridine receptor (DHPR) from skeletal muscle is formed by the \(\alpha_1,1,1.1\) and \(\beta,\gamma\) subunits and is involved in excitation-contraction coupling. However, the involvement of the \(\alpha_2\delta_1\) subunit in this mechanism has not been clearly determined. Studies examining the function of the \(\alpha_2\delta_1\) subunit have not revealed a definitive role for this subunit in the modulation of calcium channel or calcium release. Reducing \(\alpha_2\delta_1\) levels with small interfering RNA in muscle cells resulted in an acceleration of the calcium current with dissimilar effects on current conductance (15, 25), and the calcium current with dissimilar effects on current conductance (15, 25).

Thus we performed experiments aimed at finding binding partners of the \(\alpha_2\delta_1\) subunit in skeletal muscle cells early during development, the period when the levels of \(\alpha_1,1.1\) are low. We found that the \(\alpha_2\delta_1\) subunit associates with ATP5b, a subunit of a mitochondrial ATP synthase complex. The association of \(\alpha_2\delta_1\) and ATP5b occurs in intracellular membranes and, more importantly, at the level of the plasma membrane, where they form a functional signaling complex capable of modulating calcium release. The modulation was more evident during repetitive electrical stimulation of myotubes. The data indicate that the \(\alpha_2\delta_1\) subunit is not only part of the DHPR but that it may interact with other cellular components in nascent myotubes.

MATERIALS AND METHODS

Muscle cell preparation. All experiments using animals were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Mice were deeply anesthetized by methoxyflurane inhalation and then decapitated. Experiments were...
performed on muscle cells in culture as described (14). Skeletal muscle of newborn mice (postnatal day 0, P0) was dissociated for cell culture or used for protein extraction (see Blue native polyacrylamide gel electrophoresis). Dissociation of muscle was performed in Ca²⁺-, Mg²⁺-free rodent Ringer solution made up of (in mM): 155 NaCl, 5 KCl, 11 glucose, 10 HEPES, pH 7.4, containing 0.3% trypsin type XI and 0.01% DNase I (Sigma). Dissociated cells were plated on primaria dishes, T75-cm² flasks, or collagen I-coated glass coverslips in Dulbecco’s modified essential medium supplemented with 4.5 g/l glucose, 10% horse serum, and 10% fetal calf serum. Fusion and differentiation were promoted after 2 days in culture by removing the fetal calf serum. This day corresponds to experimental day 0.

Blue native polyacrylamide gel electrophoresis. Proteins extracted in their native configuration were examined with blue native polyacrylamide gel electrophoresis (BN-PAGE). We followed procedures previously described (30, 34, 40) with few modifications. Material for BN-PAGE was obtained from cells in culture and at postnatal P0. Fifty micrograms of specimen were homogenized in 500 μl sucrose buffer (250 mM sucrose and 20 mM imidazole-HCl, pH 7.0) using a Dounce homogenizer. Homogenized samples were then aliquoted (15 mg wet wt) and centrifuged for 10 min at 15,000 g to obtain a pellet containing membrane. Samples were solubilized in 70 μl of 1× NativePage sample buffer (Invitrogen) containing 2% digitonin and incubated on ice for 1 h. The lysate was centrifuged at 20,000 g for 30 min at 4°C. The supernatant was mixed gently with 5 μl of loading buffer, aliquoted into sterile microcentrifuge tubes, and stored at −80°C until use. Forty to one hundred micrograms of sample were loaded into each well of NativePage Novex 4%-16% bis-Tris gels (Invitrogen). Electrophoresis was carried at 4°C and 100 V for about 2 h. The blue cathode buffer was exchanged with a colorless buffer after 1 h of electrophoresis. For further separation in a second dimension (2D) with SDS-PAGE (7% or 10%), the lanes of the first dimension (1D) BN-PAGE were cut out and equilibrated for 30–60 min in SDS Laemmli loading buffer at room temperature. The lane was positioned horizontally between the glass plates of the SDS gel touching the top surface of the SDS stacking gel. After the run was over, proteins were transferred to polyvinylidene difluoride membranes equilibrated in methanol for 1 min at room temperature followed by equilibration in Towbin buffer. Proteins were detected with the appropriate primary antibody (mouse anti-ATP5b) and horseradish peroxidase conjugated secondary antibodies. The signal was detected with the SuperSignal West Pico ECL Kit (Pierce) following manufacturer’s instructions.

Western blot analysis. For immunodetection of proteins that were not subjected to BN-PAGE, cells were rinsed twice with phosphate-buffered saline (PBS), scraped from the culture dishes, and pelleted. The pellet was resuspended in 100 μl of sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, 0.01% bromophenol blue, pH 6.8). The suspension was heated for 5 min at 95°C and centrifuged at 14,000 g for 10 min. The supernatant was collected and protein quantified by Bradford assay. Proteins (50 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Protein transfer was verified with Ponceau S staining. The membranes were incubated with the primary antibody, washed, and blocked overnight. Membranes were incubated with a secondary antibody conjugated to horseradish peroxidase at a 1:20,000 dilution. Antibody binding was detected with chemiluminescence using the SuperSignal West Femto Detection Kit (Pierce) following manufacturer’s instructions.

Mass spectrometry analysis. Gels for protein identification with mass spectrometry were run in parallel with the gels used for immunodetection. Gels were stained with Coomassie blue G-250, and the bands or spots of interest were excised. Samples were processed in the Protein Research Lab of the University of Illinois at Chicago. Proteins were digested with trypsin using a 1:20 ratio of trypsin to protein in 40 mM NH₄HCO₃, pH 7.8, and 10% AcN and left to incubate overnight at 37°C. The resultant fragments were analyzed with a high-resolution matrix-assisted laser desorption/ionization coaxial tandem time-of-flight (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems; 4700 Proteomics Analyzer). Once the spectrum was obtained, it was analyzed with Data Explorer and Mascot. The experimental and theoretical masses of protein peptides were analyzed with Mascot against the NCBI nonredundant database and Mus musculus as a taxonomy filter. Search parameters included trypsin as a cleavage rule, carbamidomethyl (C) as a fixed modification, maximum of one missed cleavages, and a significance level of P < 0.05 for protein scores.

Immunolocalization. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. Cells were simultaneously labeled with an anti-α₂/δ1 affinity purified polyclonal antibody (1:500, Protein Research Lab, UIC) and the ATP5b monoclonal antibody 3D5 (1:400, Abcam). The secondary antibodies were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-mouse, both at 1:1,000 (Molecular Probes). For simultaneous localization of ATP5b and mitochondria, cells were labeled with a chicken anti-ATP5b antibody (1:250, Sigma) and an antibody directed against the succinate dehydrogenase (SDH) complex, anti-OxPhos Complex II 70 kDa subunit; mouse monoclonal 2E3 (1:250, Molecular Probes). The secondary antibodies for these experiments were Alexa Fluor 488 goat anti-chicken and Alexa Fluor 555 goat anti-mouse, both at a dilution of 1:500 (Molecular Probes). Controls omitting the primary antibody or using mismatched secondary antibodies were routinely performed. Nuclei were stained with TO-PRO3 (Molecular Probes). Cells were examined with a Bio-Rad Radiance 2100 confocal microscope attached to a Nikon TE300 inverted microscope and using a ×60 water-immersion objective with 1.2 numerical aperture. Alexa Fluor 488 was excited with the 488 line of a krypton/argon laser, and the emission was measured at wavelengths of 515 ± 30 nm. Alexa Fluor 568 and 555 were excited with the 568-nm line of the laser, and the emission was measured at 600 ± 40 nm. TO-PRO3 was excited at 637 nm and the emission measured at wavelengths >660 nm.

Images were analyzed with ImageJ. Colocalization was determined from 8-bit images using the plugin of C. Laumonnerie and J. Mutterer (Institut de Biologie Moléculaire des Plantes, Strasbourg, France). For fluorescence resonance energy transfer (FRET) we took advantage of the low emission overlap of the green- and red-labeled secondary antibodies mentioned above and followed a procedure described in Erickson et al. (9). Fluorescence intensity was recorded below saturation in both channels followed by photobleaching of Alexa Fluor 568. Photobleaching was achieved by scanning repetitively with the 568-nm laser line at 60–80% intensity. Fluorescence intensity was measured again after photobleaching. FRET efficiency (E) was determined from the stack of images (8 bits) before and after photobleaching and calculated from E = 1 − (donor fluorescence intensity with acceptor/donor fluorescence intensity without acceptor).

Labeling of membrane proteins with biotin. Protein biontynylation was performed using the Cell Surface Protein Isolation kit according to manufacturer’s instructions (Pierce). Cells were grown on T75 flasks and washed twice with PBS. Cells were incubated in sulfo-NHS-SS-biotin dissolved in ice-cold PBS for 30 min on a rocking platform at 4°C. The reaction was quenched, and the cells were scraped and pelleted at 500 g for 3 min. Cells were rinsed twice with Tris-buffered saline, lysed, and disrupted by sonication. The homogenate was incubated on ice for 30 min with sonication every 10 min to improve solubilization efficiency. Cell lysate was centrifuged at 10,000 g for 2 min at 4°C and the supernatant transferred to a new tube. Labeled proteins were immobilized on a NeutrAvidin Gel column. The column was washed three times and the flow-through collected and pooled; this fraction represents unlabeled intracellular proteins. To elute labeled proteins bound to the avidin gel, the column was incubated with sample buffer containing 50 mM DTT for 1 h at
Immunoblotting. Whole cell extract (0.7–0.8 mg) was incubated with 1 μg of anti-α2/δ1 affinity purified polyclonal antibody and coimmunoprecipitation buffer (50 mM Tris·HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mix) in a rotating microcentrifuge tube overnight at 4°C. The sample was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was transferred to a clean tube. Fifty microliters of protein G-Sepharose slurry were added to the supernatant and incubated at 4°C with gentle rotation. After 4 h, the protein G-Sepharose was centrifuged for 30 s at 1,000 rpm and washed three times with coimmunoprecipitation buffer. After the last wash, 50 μl of sample buffer were added and the sample was stored at −80°C until further use. Proteins were then separated as described above under Western blot analysis. ATP5b was detected in immunoblots with a chicken anti-ATP5b antibody (Sigma). All antibodies used in this study have been previously characterized.

Identification of binding protein partners of α2/δ1 with mass spectroscopy. We then proceeded to identify the components of the MPC containing the α2/δ1 protein. The band where α2/δ1 was localized in blue native gels was cut and analyzed with mass spectroscopy. Peptide mass fingerprint analysis identified six peptides with a Mowse score of 69 (P < 0.05) corresponding to the β subunit of the ATP synthase mitochondrial F1 complex β subunit ATP5b (gi 31980648). The identified peptides and their mass are shown in Table 1. The presence of ATP5b in the same band where α2/δ1 was found was intriguing since the ATP synthase complex is normally found in the inner mitochondrial membrane where it transports H⁺ and catalyzes the synthesis of ATP. However, recent

**Table 1. Peptides identified from blue native-polyacrylamide gels**

<table>
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<tr>
<th>Start-End</th>
<th>Mrt</th>
<th>ppm</th>
<th>Sequence</th>
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<td>1405.6739</td>
<td>8</td>
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<tr>
<td>110-124</td>
<td>1574.7757</td>
<td>1574.8086</td>
<td>21</td>
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<tr>
<td>95-109</td>
<td>1649.9115</td>
<td>1649.9101</td>
<td>1</td>
</tr>
<tr>
<td>125-143</td>
<td>1918.0907</td>
<td>1918.0888</td>
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</table>

The peptides correspond to ATP5b. Mrt, Relative molecular mass; Expt, experimental; Calc, calculated; ppm, parts per million.
evidence has demonstrated an anomalous presence of the ATP5b protein in the plasma membrane of endothelial and tumor cells (5, 17, 26, 39, 41). Thus our results seem to indicate that the ATP synthase is also present in the plasma membrane of developing skeletal muscle cells.

To corroborate further a possible interaction of the ATP5b with the α/β1 subunit, the individual components of the MPC were separated in a second dimension with SDS-PAGE. Two lanes from a blue native gel were cut and placed at the top of denaturing gels, as described in MATERIALS AND METHODS. The two denaturing gels were run simultaneously. One denaturing gel was used to transfer proteins and immunoblotting while the other was used for isolation of spots and analysis with mass spectroscopy (Fig. 2). Immunodetection of α/β1 revealed the presence of this protein in two places, probably due to proteolysis (Fig. 2B). The second gel was stained with Coomassie blue to show the individual components of the MPC (Fig. 2A). The corresponding spots where α/β1 was localized with immunoblotting were marked on this gel and cut. Peptide mass fingerprinting positively identified the α/β1 subunit in both of these spots. The same procedure used for identification of α/β1 was followed to identify the ATP5b protein. Immunodetection of ATP5b (Fig. 2D) shows that this protein is strategically located right below the α/β1 subunit and at the correct molecular mass of ~52 kDa. Analysis of the corresponding spot on the gel shown in Fig. 2C confirmed the identity as the ATP5b. Thus these results further suggest that the α/β1 subunit and ATP5b form part of the same MPC.

In the previous experiments, we were not able to detect the α1.1 subunit, which suggested that its levels were too low or that it did not form part of the same MPC where α/β1 and ATP5b were found. To examine this issue, we searched for the presence of the α1.1 subunit in 2D gels since the proteins can be examined separately under these conditions, thus making it possible to detect α1.1 even if its levels are lower. For these experiments we increased the concentration of protein to 100 μg. Figure 2E, left, shows the immunodetection of α/β1 at ~250 kDa (Fig. 2E, right). However, the signal corresponding to α1.1 was considerably small, indicating that the amount of the α1.1 subunit is lower than that of α/β1.

Localization of the α/β1 subunit and ATP5b at the cellular level. Since the experiments with blue native gels and mass spectroscopy were performed using total cell protein, they did not distinguish the membrane where α/β1 and ATP5b could be located. In mature muscle the α/β1 subunit is located in the transverse tubular membrane and the ATP5b subunit in the mitochondrial membrane. Thus we sought to determine whether these two proteins were present in the plasma membrane and/or in an intracellular compartment. We first examined the localization of ATP5b by immunocytochemistry in 2-
to 5-day-old primary myotubes. Figure 3A shows a myotube stained with the anti-ATP5b monoclonal antibody 3D5 (Fig. 3Aa) and the corresponding overlaid transmission image (Fig. 3Ab). The ATP5b was found inside along the myotube and possibly associated with the plasma membrane. We then performed further experiments to examine the spatial relationship of ATP5b with mitochondria. Cells were costained with a chicken anti-APT5b antibody and a mouse monoclonal antibody against the mitochondrial SDH complex. Nuclei were stained with TO-PRO3. As shown in Fig. 3B, the localization of ATP5b as determined with a second antibody is identical to the localization presented in Fig. 3A. ATP5b was found again inside the myotube and possibly associated with the plasma membrane. In contrast, SDH was found solely inside the cell and far from the border of the cell. Interestingly, ATP5b colocalized with SDH within the cell as illustrated in the merged image.

To determine conclusively the presence of ATP5b in the plasma membrane, we then labeled plasma proteins from intact primary myotubes with biotin. Figure 3C shows that both the α2δ1 subunit and ATP5b are present in the plasma membrane fraction (eluate, E) and in the intracellular membrane fraction (flow through, F). The ribosomal protein L26 (RPL26) was used as a marker for the intracellular fraction. Note that RPL26 was absent from the eluate fraction, indicating that the sample corresponded solely to plasma membrane proteins. These data confirm that ATP5b localizes both in intracellular membranes and in the plasma membrane in muscle cells. See also the supplemental material showing colabeling of ATP5b and the plasma membrane with wheat germ agglutinin. The presence of the ATP5b in the plasma membrane represents an atypical location for this protein in developing muscle cells and suggests that it may participate in unidentified signaling mechanisms by interacting with the α2δ1 subunit.

To determine the spatial relationship of the ATP5b with the α2δ1 subunit, primary myotubes were examined by double labeling with an anti-α2δ1 polyclonal antibody and an anti-ATP synthase monoclonal antibody. Figure 4A shows an example of a double-labeled, 5-day-old myotube. Figure 4Ad shows a binary threshold mask from colocalization analysis of images in Fig. 4, Aa and Ab. Colocalization was higher at the ends of the cell as seen from the merged image (Fig. 4Ac) and the binary mask, which agrees with the distribution of α2δ1 in young myotubes we previously reported (2). For this particular cell, the Pearson’s correlation coefficient ($R_p$) was 0.69 and the overlap coefficient ($R_o$) was 0.967. Different degrees of colocalization were observed in all cells examined ($n = 143$); of those, 95 cells did not show colocalization, while in the other 48 cells $R_o$ varied from 0.12 to 0.71 and $R_p$ varied from 0.84 to 0.97.

The proximity of the α2δ1 subunit and ATP5b was further examined with FRET measurements. The secondary antibodies used to label the α2δ1 subunit and ATP5b for FRET experiments were Alexa-Fluor 488 and Alexa-Fluor 568, respectively. Figure 4, Ba–b, shows the upper end of the cell in Fig. 4A before photobleaching of the acceptor fluorophore (568 nm). This region of the cell was scanned repetitively with the 568-nm line at 60% laser intensity until the fluorophore was almost completely bleached. Figure 4Bd shows the image of the full cell with the bleached region. Fluorescence recorded from the same area as in Fig. 4Ba after photobleaching showed

![Fig. 3. Localization of ATP5b and α2δ1 in cellular membranes. Aa: staining of myotubes with a mouse monoclonal anti-ATP5b antibody. Ab: transmission image of the myotube in (Aa) with the overlapping fluorescence image corresponding to ATP5b. The ATP5b protein was found inside along the myotube and at the periphery of the cell. Image size for A and B: 104 × 104 μm. B: costaining of ATP5b and mitochondria. ATP5b was detected with a primary chicken antibody and a secondary Alexa Fluor 488 goat anti-chicken antibody. Mitochondria were labeled with a mouse monoclonal anti-SDH and Alexa Fluor 555 goat anti-mouse. Nuclei were stained with TO-PRO3. The figure shows separate images for ATP5b, mitochondria, and nuclei labeling. The merged image shows a high degree of colocalization of ATP5b and SDH inside the cells but not at the plasma membrane where ATP5b is found by itself. The size of each image is 42 × 42 μm. C: biotinylation labeling of proteins from myotubes shows α2δ1 and ATP synthase in the plasma membrane (E, eluate fraction), demonstrating the presence of both proteins in this cellular membrane. Nonlabeled protein was recovered in the cytoplasmic fraction (F, flow through). RPL26, used as a control for fraction purity, was absent from the eluate fraction.](image-url)
an increase in donor intensity and shown in Fig. 4Bc. The change in normalized fluorescence before and after photobleaching is shown in the graph in Fig. 4C. Note the increase in donor fluorescence and a concomitant decrease in the acceptor fluorescence, which was bleached. Calculation of FRET efficiency $E$ gave a value of 8.24% and a Foerster distance ($R_0$) of $\sim$7.4 nm. The average $E$ obtained from 13 cells that showed colocalization was $7.8 \pm 1.2\%$. This result suggests that both the $\alpha_2/\delta_1$ subunit and ATP5b are present in the same membrane in myotubes.

Although these experiments delineate the regions of the cell where the proteins colocalize, they do not prove conclusively that there may be a physical interaction between them. To this end, we used coimmunoprecipitation to examine this issue. Immunoprecipitation of $\alpha_2/\delta_1$ was followed by denaturing the proteins in SDS-PAGE and immunoblotting of ATP5b. Figure 4D shows the results obtained with protein isolated from skeletal muscle from P0 mice. A band of $\sim$52 kDa corresponding to ATP5b was consistently found in several preparations. Similar results were obtained with protein isolated from 5- to 6-day-old myotubes. Thus the colocalization results together with FRET measurements indicate that the intermolecular distance between the $\alpha_2/\delta_1$ subunit and ATP5b is close enough to allow for direct interaction between the two proteins, and the coimmunoprecipitation studies indicate a physical interaction of the proteins. Taken together, these results provide further support for the idea that these two proteins form part of a macromolecular complex in membranes of muscle cells.

**Functional significance of the interaction between $\alpha_2/\delta_1$ subunit and the ATP5b.** Because the previous experiments indicated a physical association between the $\alpha_2/\delta_1$ subunit and ATP5b in myotubes, we sought to determine the physiological relevance of this interaction. Calcium transients were measured from 5- to 6-day-old myotubes loaded with the calcium indicator Fluo-4 AM as previously reported (14). Transients were elicited in response to electrical stimulation to ensure a complete and uniform depolarization of the cells examined since few myotubes exhibit spontaneous contractions at this time in culture. Calcium transients were recorded in response to a single pulse or a train of stimuli under different experimental conditions: 1) in the presence of the anti-ATP5b mAb3D5 antibody ($n = 22$ myotubes); 2) in the presence of a control monoclonal antibody raised against hemagglutinin of the same isotype (IgG1) as the anti-ATP5b antibody ($n = 14$ myotubes); 3) in the absence of any antibody (control untreated; $n = 17$ myotubes). Myotubes were exposed to the antibodies (1:50 dilution) for at least 1 h before the beginning of the experiment and throughout the recordings. The anti-ATP5b antibody was the same used for immunolocalization of ATP5b in cells and Western blots. Figure 5 shows representative calcium transients in response to a single stimulus (Fig. 5A) or to a train of 10 Hz for 2 s (Fig. 5B). Exposure of myotubes to the control antibody did not modify the properties of calcium transients compared with transients recorded from untreated myotubes. The main effect of the ATP5b antibody was to reduce the time constant of decay after both single pulse stimulation or a train of stimuli 

$\text{max rate of decay} = 0.01$) only for the train of stimuli, as shown by the traces on the right column. For train stimulation, the maximum rate of decay was significantly larger in the presence of the ATP5b antibody compared with either control condition. Other properties of the

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**Fig. 4.** Interaction of the $\alpha_2/\delta_1$ subunit and ATP5b in muscle cells. **A:** cellular localization was examined by double labeling of myotubes with antibodies against $\alpha_2/\delta_1$ (a) and ATP5b (b). The merged image is shown in Ac. Ad: binary threshold mask resulting from analysis of images in a and b. Colocalization was higher at the ends of the cell. The size for each image in A is $70 \times 70 \mu m$. B: fluorescence resonance energy transfer (FRET) analysis of the cell shown in A. a and b: fluorescence intensity before photobleaching of the upper end of the cell. c: increase in the fluorescence intensity after the acceptor has been photobleached. d: image of the cell with the photobleached region. C: graphic representation of the average change in normalized fluorescence before and after photobleaching for the donor and the acceptor in all cells examined. D: coimmunoprecipitation of native $\alpha_2/\delta_1$ subunit and ATP5b from skeletal muscle from P0 mice. Samples were immunoprecipitated with an anti-$\alpha_2/\delta_1$ polyclonal antibody (IP). ATP5b was detected with a chicken anti-ATP5b antibody and an anti-chicken IgG peroxidase conjugate antibody (IB, lane marked +). Control experiments in the absence of IgG did not detect ATP5b (lane marked −).
calcium transients were not changed significantly, although a
tendency to increase the maximum rate of rise was observed in
the presence of the ATP5b antibody. These results indicate that
there is a functional interaction between the
\( \alpha \beta_{2,1} \) subunit and
ATP5b in myotubes and, furthermore, they indicate that the
\( \alpha \beta_{2,1}/\text{ATP synthase complex} \) is able to alter calcium handling.

**DISCUSSION**

We have identified a new interaction of the \( \alpha \beta_{2,1} \) subunit of
calcium channels with ATP5b, the \( \beta \) subunit of a mitochondrial
ATP synthase complex, in developing muscle cells. The association
of \( \alpha \beta_{2,1} \) and ATP5b occurs at both the plasma membrane
and in intracellular membranes, as demonstrated by protein label-
ing with biotin and antibodies, and FRET analysis. We have
previously shown that the \( \alpha \beta_{2,1} \) subunit does not always colo-
calize with the \( \alpha_1.1 \) subunit and that it is involved in attachment
and migration of myoblasts (15). Thus we hypothesized that the
\( \alpha \beta_{2,1} \) subunit may be interacting with other cellular components
different from DHPRs early during development. With this idea in
mind, we sought to identify possible binding partners of \( \alpha \beta_{2,1} \).
Experiments extracting protein in its native conformation and
mass spectroscopy established initially that the \( \alpha \beta_{2,1} \) subunit may
be associating with ATP5b. This interaction was confirmed with
FRET, coimmunoprecipitation, and the effect of the anti-ATP5b
antibody on calcium transients. The fact that colocalization of
ATP5b and \( \alpha \beta_{2,1} \) appeared in only some cells while ATP5b was
consistently found in the MPCs and communoprecipitated with α2/61 may be explained by the difference in data provided by each technique; immunolocalization detects all the ATP5b molecules in each individual cell, whereas immunoprecipitation and MPC detect the subset of ATP5bs that are interacting with α2/61 in the whole population of cells. Another interesting issue is that although not all the cells showed colocalization of α2/61 and ATP5b, all the cells that were examined with the ATP5b antibody had a faster decay of the calcium transient. This may indicate that the calcium transient may be modulated by ATP5b or by the interaction of ATP5b with α2/61. It is important to note, however, that the cells with calcium transients were the ones that responded to electrical stimulation. The interaction of α2/61 subunit and ATP5b suggests that the α2/61 subunit has multiple functions in muscle cells with regard to signal transduction mechanisms. Recent evidence indicates that ion channel subunits, including calcium channels, are not only passive elements allowing flow of ions across membranes, but that they have other roles essential for cell functioning (see 8, 19).

Association of α2/6 subunits with proteins other than subunits of calcium channels has been reported for isoforms 1 and 2. Studies using rabbit skeletal muscle cells in culture showed that the α2/61 subunit colocalizes with the neural cell adhesion molecule, and the cellular distribution of both these proteins changes concomitantly during myogenesis in vitro, although the functional significance of a putative physical interaction between these two proteins was not examined (37). More recently, Eroglu et al. (10) demonstrated that the α2/61 subunit binds to thrombospondins in the central nervous system. Thrombospondins are proteins in the extracellular matrix secreted by astrocytes and mediate cell-cell and cell-matrix interactions (3). Interestingly, secretion of thrombospondins occurs during synaptogenesis in early postnatal life. The interaction between the α2/61 subunit and thrombospondins proved to be required for the formation of new synapses, most likely independently from calcium channel function. Similarly, the α2/62 subunit has been shown to bind the lipid raft-associated proteins SLP-2, stomatin, and prohibitin and that this interaction may influence the functionality of calcium channels in the nervous system (6).

Our results showing an interaction between the α2/61 subunit and ATP5b in muscle cells are consistent with these previous studies and help establish the α2/61 protein as an important molecule with multiple functions in developing cells. The previously unidentified association of the α2/61 subunit with the ATP5b and the effect of the anti-ATP5b antibody on calcium transients suggest that this complex at the level of the plasma membrane is functional and may be involved in the regulation of calcium handling during early stages of development. The role of ATP in regulating skeletal muscle function has been extensively examined by other laboratories. The effect of the ATP5b antibody on calcium transients may be explained by the presence of the α1.1.1 subunit in 2D denaturing gels. Interestingly, α1.1.1 was detected when the concentration of protein was increased, indicating that only few α1.1.1 subunits form part of the α2/61-ATP5b complex. These data suggest that the α2/61 subunit mediates an interaction between ATP5b and the DHPR in immature myotubes.

It has been reported that the effect of α2/61 on channel kinetics is to favor the transition to the inactivated state of the cardiac DHPR (2, 31). Although the effect of α2/61 on inactivation of the skeletal muscle DHPR has not been examined, recent evidence has shown that the α2/61 subunit is important for maintaining the amplitude of the calcium transients during prolonged depolarizations due to either KCl application or trains of electrical stimulation (13). Since the observed effect of the ATP5b antibody was to enhance the decay of the calcium transient and the antibody would not be acting internally, it is attractive to hypothesize that the inactivation of the DHPR was promoted through the α2/61 rather than causing an increase in calcium removal from the cytoplasm. The experiments presented here, however, do not conclusively distinguish between these two possibilities or a combination of both. It will be interesting to determine whether the ATP5b is also involved in regulating the concentration of ATP in the extracellular fluid as well. The role of ATP in regulating skeletal muscle function has been extensively examined. It has been shown that ATP activates satellite cells for the formation and regeneration of skeletal muscle (1, 4, 35), regulates intracellular calcium concentration and contractility (7, 21, 29), and can influence metabolism (27). Moreover, extracellular ATP is necessary for the repair of skeletal muscle in mdx mice, a model for Duchenne muscular dystrophy (28, 42). Although it has been shown that ATP can be released together with neurotransmitters in the neuromuscular junction (33) and that muscle can release ATP after contractions (12, 21), it may be possible that ATP5b increases ATP extracellularly.

Another important finding reported in this paper is the unconventional localization of ATP5b in the plasma membrane of muscle cells. The usual localization of ATP5b is the inner mitochondrial membrane since it forms part of the mitochondrial ATP synthase complex. However, accumulating experimental evidence indicates that the presence of the ATP5b subunit in the plasma membrane is more common than previously realized (5, 17, 26, 39, 41). Several functions have been attributed to the ATP5b in the plasma membrane, including serving as a receptor for apolipoprotein A-I in hepatocytes (22) and angiotatin in endothelial cells (24), regulation of H+ concentration across the membrane (5), and regulation of flow-induced release of ATP (41). Thus the presence of ATP5b in the plasma membrane and its interaction with the α2/61 subunit suggests that these two proteins form a previously unidentified signaling complex in muscle cells, especially during early development of myotubes. An evaluation of this signaling complex in this paper showed that it is able to regulate calcium release. An important aspect that remains to be determined is whether the α2/61-ATP5b interaction is important for attachment and migration of myoblasts. The experiments presented here are the first direct evidence indicating that α2/61 is able to form complexes with proteins other than subunits of the DHPR in developing skeletal muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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