Communication

Specific Absence of the α1 Subunit of the Dihydropyridine Receptor in Mice with Muscular Dysgenesis*

(Received for publication, October 24, 1988)

C. Michael Knudson†, Nirupa Chaudhari‡, Alan H. Sharp¶, Jeanne A. Powell†, Kurt G. Beam†**, and Kevin P. Campbell† ‡

From the †Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, the ‡Department of Physiology, Colorado State University, Fort Collins, Colorado 80523, and the ¶Department of Biological Sciences, Smith College, Northampton, Massachusetts 01063

Muscular dysgenesis is a lethal mutation in mice that results in a complete absence of skeletal muscle contraction due to the failure of depolarization of the transverse tubular membrane to trigger calcium release from the sarcoplasmic reticulum. In order to determine whether the defect in muscular dysgenesis leads to a specific loss of one of the components of excitation-contraction coupling or to a generalized loss of all components of excitation-contraction coupling, we have analyzed skeletal muscle from control and dysgenic mice for the sarcoplasmic reticulum and transverse tubular proteins which are believed to function in excitation-contraction coupling. We report that the proteins involved in sarcoplasmic reticulum calcium transport, storage, and release ([(Ca²⁺ + Mg²⁺)-ATPase, calsequestrin, and calcium release channel) are present in dysgenic muscle. Also present in dysgenic muscle is the 175/150-kDa glycoprotein subunit (α₂) of the dihydropyridine receptor. However, the 170-kDa dihydropyridine binding subunit (α₁) of the dihydropyridine receptor is absent in dysgenic muscle. These results suggest that the specific absence of the α₁ subunit of the dihydropyridine receptor is responsible for the defects in muscular dysgenesis and that the α₁ subunit of the dihydropyridine receptor is essential for excitation-contraction coupling in skeletal muscle.

The molecular mechanisms involved in coupling transverse tubular membrane depolarization to sarcoplasmic reticulum calcium release are not understood. Recently the protein responsible for releasing calcium from the junctional sarcoplasmic reticulum, the calcium release channel, has been identified and purified from rabbit skeletal muscle (1-4). The calcium release channel is identical to the junctional "foot" protein in skeletal muscle (2) and has been shown to consist of a single polypeptide of approximately 450,000 Da (1). The dihydropyridine receptor of the transverse tubular membrane has recently been proposed to be a voltage sensor for skeletal muscle excitation-contraction coupling (5, 6) and thus may be involved in the activation of the calcium release channel. The dihydropyridine receptor has been purified from rabbit skeletal muscle and shown to consist of two higher molecular weight protein subunits and at least two lower molecular weight subunits (7). The high molecular weight subunits include the 170-kDa dihydropyridine binding subunit named α₁ and the 175/150-kDa glycoprotein subunit named α₅, which shifts from 175 to 150 kDa upon reduction (7).

Muscular dysgenesis (mdg) (8, 9) is a lethal autosomal recessive mutation in mice that results in the complete lack of excitation-contraction coupling in all skeletal muscle from these mice (10). Dysgenic muscle resembles normal muscle with respect to the ability of the sarcolemma to generate action potentials (10), the sarcoplasmic reticulum to release calcium in response to caffeine, and the contractile proteins to respond to calcium (11). Dysgenic muscle differs from normal muscle in that depolarization fails to induce the release of calcium from the sarcoplasmic reticulum. Consistent with this functional defect, electron microscopy of embryonic dysgenic skeletal muscle has shown that triads, the membranous region at which excitation-contraction coupling occurs, are reduced in number and abnormal in morphology (12). Attempts to further characterize the defect in dysgenic muscle has shown that the dihydropyridine-sensitive calcium current is missing in dysgenic myotubes (13) and that there is a reduction in high affinity dihydropyridine binding (14) in dysgenic skeletal muscle homogenates. Thus, the mdg mutation clearly and specifically prevents excitation-contraction coupling and provides an excellent model with which to study and identify the molecular components of excitation-contraction coupling.

EXPERIMENTAL PROCEDURES

Isolation of Normal and Dysgenic Skeletal Muscle Membranes—Adult mouse skeletal muscle and newborn normal or dysgenic skeletal muscle were dissected, frozen in liquid nitrogen, and stored at −70 °C. The samples were then homogenized using a Brinkmann Polytron PTA-7 in 10 volumes of buffer A containing sodium pyrophosphate (20 mM), sodium phosphate monobasic (20 mM), MgCl₂ (1 mM), EDTA (0.5 mM), sucrose (300 mM), and the following protease inhibitors: aprozin (7.6 μM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and PMSF (0.2 mM). Homogenates were centrifuged for 10 min in a TJ-6R Beckman centrifuge at 1500 × g. Supernatants were removed, and pellets were homogenized in buffer A and centrifuged two additional times as described above. The three supernatants were combined and centrifuged at 150,000 × g in a Beckman Type 50.2 Ti rotor. Membranes were resuspended in a minimum volume of buffer A. Adult rabbit microsomes were isolated by a modification of the method of Mitchell et al. (15, 16). Protein was determined by the method of Lowry et al. (17) as modified by Peterson (18).

* This work was supported by grants from the Muscular Dystrophy Association (to J. A. P., K. G. B., and K. P. C.), National Institutes of Health Grant NS-24444 (to K. G. B.), and National Institutes of Health Grants HL-37187, HL-14388, and HL-39265 (to K. G. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† Supported by the Blakeslee Endowment to Smith College.
‡ Recipient of National Institutes of Health Research Career Development Award NS 01190.
¶ Established Investigator of the American Heart Association.

The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
Culturing and Solubilization of Myotubes—Primary cultures of myoblasts were prepared from fore- and hindlimbs of newborn normal and dysgenic mice as previously described (13, 19). Briefly, limb muscle is minced, digested with collagenase, filtered, and cells are then plated onto 35-mm Falcon "primaria" dishes or 25-mm primaria flasks (Falcon). Myotubes were allowed to form and mature (10–12 days). Cultures were washed with calcium- and magnesium-free Ringer's solution (155 mM NaCl, 5 mM KCl, and 10 mM HEPES, pH 7.4 with NaOH), scraped from the dishes into calcium- and magnesium-free Ringer's solution containing 10 μM aprotinin, pelleted, and rapidly frozen for storage at -70°C.

Myotubes were thawed, weighed, and quickly solubilized in 20 volumes (v/w) of solubilization buffer (6% SDS, 50 mM dithiothreitol, 10 mM EDTA, 100 μg/ml benzamidine, 40 μg/ml PMSF, 185 μg/ml iodoacetamide, 0.5 M sucrose, and 130 mM Tris, pH 6.8, with HCl). Samples were vortexed, heated for 5 min in boiling water, and centrifuged to remove any remaining particulate matter. Protein was quantitated by the method of Lowry et al. (17) as modified by Peterson (18) after precipitation with 5% trichloroacetic acid in the presence of 0.5 mg of sodium deoxycholate.

SDS-PAGE and Immunoblot Analysis—Skeletal muscle membranes or isolated myotubes were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (20) and stained with 0.05% Coomassie Blue or transferred to nitrocellulose according to Towbin et al. (21). Nitrocellulose blots were stained with various antibodies as described (22–25) using secondary antibodies conjugated to either horseradish peroxidase or alkaline phosphatase.

Monoclonal antibodies against calcequastrin, the (Ca2+ + Mg2+)-ATPase, and the 170-kDa dihydropyridine binding subunit of the dihydropyridine receptor have been previously prepared (23–25). Polyclonal antibodies directed against the calcium release channel were previously prepared (25) by injecting guinea pigs with purified receptor according to the method of Tung (26). Polyclonal antibodies against the α2 subunit were prepared using SDS gel slices according to the method of Tung (26) as previously described (25).

Myotubes—Electrophoretic reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Protease inhibitors were from Sigma. Peroxidase and alkaline phosphatase-conjugated secondary antibodies were obtained from Boehringer Mannheim and Cappel. All other chemicals were of reagent grade quality.

RESULTS AND DISCUSSION

To determine the defect in muscular dysgenesis we have examined normal and dysgenic muscle for the protein constituents of the sarcoplasmic reticulum and transverse tubular membrane involved in excitation-contraction coupling. Since the quantity of neonatal dysgenic muscle is limited (approximately 0.1 g/mouse), we have used indirect immunoperoxidase staining of protein blots (Western or immunoblots) from normal and dysgenic muscle for this analysis. Initially, we tested monoclonal and polyclonal antibodies previously prepared in our lab against adult rabbit proteins for cross-reactivity with proteins of similar molecular weight in newborn and adult mouse muscle. Fig. 1 shows that the calcium release channel, (Ca2+ + Mg2+)-ATPase, calcequastrin, and the α1 subunit of the dihydropyridine receptor are present in newborn and adult mouse muscle and are recognized by various antibodies. The α2 subunit of the dihydropyridine receptor was also recognized by a polyclonal antibody against rabbit α2, but antibodies to the rabbit β and γ subunits did not cross-react with normal mouse membranes.

In Fig. 2, membranes were prepared from newborn normal (+/mdg?) mice and dysgenic (mdg/mdg) mice and were tested for the presence of the sarcoplasmic reticulum calcium release channel, the (Ca2+ + Mg2+)-ATPase, and calcequastrin. Each of these proteins is present, but in reduced amounts, in dysgenic muscle. The two proteins specific to the triad junction, the sarcoplasmic reticulum calcium release channel and calcequastrin, are diminished more than the (Ca2+ + Mg2+)-ATPase. The reduction in the calcium release channel (junctional foot protein) and in calcequastrin (electron dense con-
tion that may occur in newborn dysgenic mice and of potential vascular tissue contamination which may be present in membranes prepared from limb muscle. Fig. 4 shows that the sarcoplasmic reticulum calcium release channel and the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase are detected in near normal levels in dysgenic myotubes. Even with multiple staining, the 170-kDa protein (a1 subunit) of the dihydropyridine receptor could not be detected in dysgenic myotubes. These results further illustrate the specificity of the defect in muscular dysgenesis for the a1 subunit of the dihydropyridine receptor.

The results described here show that despite the overwhelming loss of one critical component of triads (a1 subunit of the dihydropyridine receptor), expression of other genes crucial for excitation-contraction coupling is only moderately affected. Thus, we propose that all other abnormalities reported in dysgenic muscle can be attributed to the selective loss of the a1 subunit. These include the absence of the dihydropyridine-sensitive calcium current (13), reduced dihydropyridine binding (14), failure of excitation-contraction coupling (10), and abnormal morphology of the triad junction (14). Our results provide strong evidence that the a1 subunit of the dihydropyridine receptor is involved in coupling transverse tubular depolarization to sarcoplasmic reticulum calcium release and support the hypothesis that the dihydropyridine receptor acts as a voltage sensor in the transverse tubular membrane for excitation-contraction coupling.

The muscular dysgenic mutation could lead to depletion of the a1 subunit by several different routes. First, the mutation might alter a regulatory region of the gene for the a1 subunit and thereby decrease transcription. Second, the mutation may alter the structural gene such that the gene produces a non-functional, and therefore labile, product. Third, the primary

![Fig. 2. Immunoblots of newborn normal (+/mdg?) and dysgenic (mdg/mdg) mouse skeletal muscle membranes. Each panel contains normal membranes (lane 1) and dysgenic membranes (lane 2) stained with antibodies against the calcium release channel (panel A), the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (panel B), and calsequestrin (panel C). A, blots are stained with polyclonal guinea pig anti-calcium release channel antibodies followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase (100 pg of protein/lane). B, blots are stained with monoclonal mouse antibody VE12 against (Ca$^{2+}$ + Mg$^{2+}$)-ATPase followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (150 pg of protein/lane). Molecular weights (Mr, $\times 10^5$) are indicated on the left.](image2)

![Fig. 3. Immunoblots of normal (+/mdg?) and dysgenic (mdg/mdg) neonatal mouse skeletal muscle membranes. Each panel contains normal membranes (lane 1) and dysgenic membranes (lane 2) stained with antibodies against the a1 subunit (panel A) and the a1 subunit (panel B) of the rabbit skeletal muscle dihydropyridine receptor. A, blots are stained with polyclonal guinea pig antibodies against the a1 subunit followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase. All lanes contain 250 pg of protein. Protein samples were electrophoresed under nonreducing conditions containing 20 mM N-ethylmaleimide (−) or under reducing conditions containing 10 mM dithiothreitol (+) for 10 min at room temperature. B, blots are stained with polyclonal antibody against the a1 subunit followed by incubation with goat anti-guinea pig IgG conjugated to horseradish peroxidase (100 pg of protein/lane). Molecular weights (Mr, $\times 10^5$) are indicated on the left.](image3)

![Fig. 4. Immunoblots of normal (+/mdg?) and dysgenic (mdg/mdg) myotubes grown in primary culture and solubilized in SDS. Each panel contains normal myotubes (lane 1) and dysgenic myotubes (lane 2) stained with antibodies against the calcium release channel (panel A), the a1 subunit (panel B), and (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (panel C). A, blots are stained with polyclonal guinea pig anti-calcium release channel antibodies followed by incubation with rabbit anti-guinea pig IgG conjugated to horseradish peroxidase (100 pg of protein/lane. Arrowhead denotes position of calcium release channel. B, blots are stained with monoclonal mouse antibody IIIDS against the a1 subunit of the dihydropyridine receptor followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (200 pg of protein/lane. C, blots are stained with monoclonal mouse antibodies VE12 and VE8 against the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (150 pg of protein/lane). Molecular weights (Mr, $\times 10^5$) are indicated on the left.](image4)
defect may prevent the production, by nonmuscle cells, of a trophic signal necessary for normal muscle development. The third hypothesis is supported by results which show that coculturing myotubes with spinal cord cells restores contractility (28) and calcium currents (29) to a fraction of the myotubes. On this basis Rieger et al. (29) suggest that the mutation prevents motor neurons from supplying a factor which activates a gene required for excitation-contraction coupling in skeletal muscle. The recent demonstration that normal fibroblasts are able spontaneously to fuse with myotubes in culture and bring about functional rescue of dysgenic muscle (30) provides an alternative explanation to the proposal of Rieger et al. (29). Specifically fibroblasts or other non-neuronal cells in the normal spinal cord preparation could fuse with dysgenic myotubes and thus provide the necessary genetic information for proper calcium currents and excitation-contraction-coupling. In fact, we feel it is unlikely that the absence of a trophic factor would produce a complete loss of only the α₁ subunit as we have shown here.

The observation that the sarcoplasmic reticulum calcium release channel is substantially expressed in dysgenic muscle is consistent with caffeine-induced contractures previously demonstrated in dysgenic muscle (11). The near normal levels of the calcium release channel in dysgenic myotubes as compared to the greatly reduced level in mdg muscle suggest that the absence of the α₁ subunit of the dihydropyridine receptor in mdg muscle results in reduced expression of the calcium release channel. Preliminary results suggest that the dihydropyridine receptor may be in close association with the sarcoplasmic reticulum calcium release channel (junctional foot protein) (31, 32), and thus the absence of the α₁ subunit may prevent proper formation of the triadic junction in mdg muscle.

Schneider and Chandler (33) originally proposed that charged groups in the transverse tubule of the sarcolemma would be displaced by changes in voltage and that this displacement could directly regulate the release of calcium from the sarcoplasmic reticulum. Rios and Brum (5) have suggested that the dihydropyridine receptor is the voltage sensor in the transverse tubular membrane originally described by Schneider and Chandler. The lack of spontaneous contraction (in spite of spontaneous action potentials) in dysgenic myotubes suggests that the absence of the α₁ subunit prevents the normal activation of the calcium release channel. Additionally, the relaxed state of dysgenic myotubes suggests that the calcium release channel is closed even in the absence of the α₁ subunit of the dihydropyridine receptor in dysgenic myotubes. Thus, the α₁ subunit of the dihydropyridine receptor cannot act as a gate or “plunger” in the release of calcium from the sarcoplasmic reticulum (33) but instead may regulate calcium release via some other less direct mechanism. Thus, despite the recent identification of two of the molecular components which are required for normal excitation-contraction coupling, the calcium release channel and the α₁ subunit of the dihydropyridine receptor, the mechanism by which these proteins interact to induce sarcoplasmic reticulum calcium release remains unknown.

Acknowledgments—We would like to thank Albert Leung and Mitchell Gaver for their generous contributions of antibodies used in this study.

REFERENCES
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