Skeletal muscle junctional membrane protein content in pigs with different ryanodine receptor genotypes

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Skeletal muscle junctional membrane protein content in pigs with different ryanodine receptor genotypes. Am. J. Physiol. 267 (Cell Physiol. 36): C282–C292, 1994.—The content of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, transverse tubule dihydropyridine receptor (DHPR), and SR ryanodine receptor (RyR) was determined in muscle of pigs homozygous for the normal RyR allele and homozygous or heterozygous for the malignant hyperthermia-susceptible (MHS) RyR allele. Total muscle membranes isolated from 1-day-old pigs of the three different genotypes did not differ in the content of any of these proteins. However, at 28 days of age, crude membranes and total muscle homogenates from homozygous MHS pigs exhibited only 61–81% of the [3H]PN 200-110 or [3H]ryanodine binding of identical preparations isolated from normal pigs; these MHS membranes also contained only 50% of the normal content of each of the DHPR subunits. The crude membranes and muscle homogenates from heterozygous pigs were intermediate to both types of homozygotes in terms of [3H]PN 200-110 binding, [3H]ryanodine binding, and the content of the DHPR subunits. However, membrane preparations enriched in triadic junctional proteins isolated from 3- to 4-mo-old pigs of the three different genotypes did not differ in their [3H]PN 200-110 binding, [3H]ryanodine binding, or Ca^{2+}-ATPase activities. We conclude that, although the stoichiometry of the RyR to DHPR is not altered, the presence of the MHS RyR allele during muscle development results in a decreased relative content of these two proteins. This is probably due to a lower junctional membrane content and may be an important ultrastructural consequence of the altered sarcoplasmic Ca^{2+} regulation in MHS muscle.

dihydropyridine receptor; sarcoplasmic reticulum; transverse tubule; malignant hyperthermia

EXCITATION-CONTRACTION COUPLING in mammalian skeletal muscle is mediated by the communication between proteins of the transverse tubule and the sarcoplasmic reticulum (SR) membrane systems at the triadic junction. The dihydropyridine receptor (DHPR) of the transverse tubule is a heteromeric protein of α1, α2/3, β, and γ-subunits (5). This protein effects a slow inward L-type Ca^{2+} current after surface membrane depolarization (34), but its major function in skeletal muscle appears to be as a voltage sensor, where it would transduce surface membrane depolarization into a signal for opening of the SR Ca^{2+}-release channel (31). Associated with the DHPR voltage sensor is an intramembrane charge movement that occurs immediately after depolarization and appears essential for SR Ca^{2+} release (1, 30). The α1-subunit is apparently responsible for the Ca^{2+} channel, charge-movement, and Ca^{2+} antagonist-binding activities of the DHPR (1, 5, 34). The Ca^{2+}-release channel of the SR terminal cisternae is a large homotetramer that binds the plant alkaloid ryanodine (16, 18). The ryanodine receptor (RyR) Ca^{2+} channel activity is modulated by a number of ligands including Ca^{2+}, Mg^{2+}, and adenine nucleotides (16, 18). However, although the RyR may be directly opposed to the DHPR at the triadic junction (4), the mechanism by which the DHPR regulates the RyR activity during excitation-contraction coupling is not yet defined at the molecular level.

Pigs susceptible to the inherited metabolic disorder malignant hyperthermia (MH) have an Arg-615 to Cys-615 mutation within the skeletal muscle RyR (12) that causes alterations in ryanodine binding (8, 15, 24), Ca^{2+} channel activity (10, 33), and excitation-contraction coupling (6, 12). A homologous mutation has also been demonstrated in human MH (14). Interestingly, previous work has demonstrated that transverse tubules isolated from MH-susceptible (MHS) pigs have a decreased maximum binding capacity (B_{max}) for [3H]nitrendipine and [3H]D888 binding to the DHPR compared with that of normal pigs (7). Furthermore, it has also been shown that MHS muscle has a decreased surface membrane L-type Ca^{2+} current and a decreased intramembrane charge movement (19). The reason for these decreased activities associated with the DHPR in MHS muscle is not known, although it is possible that there is an altered subunit composition of the receptor or that some other aspect of the structure or function of the DHPR is abnormal.

To address these possibilities, we determined the content of proteins mediating excitation-contraction coupling in pigs of varying age and all three genotypes with respect to the MHS RyR mutation. Crude membranes isolated from 1-day-old pigs of the three different genotypes did not differ in the content of any of these proteins. However, by 28 days of age, as well as at 3–4 mo, total membrane fractions isolated from homozygous Cys-615 (MHS) animals had only 61–75% of the DHPR and RyR content of homozygous Arg-615 (normal) animals; the ratios between the DHPR subunits as well as between the DHPR and the RyR were not affected by RyR genotype. Membrane preparations enriched in proteins of the triad junction (heavy SR), isolated from the three different genotypes, did not differ in their [3H]PN 200-110 binding, [3H]ryanodine binding, Na^{+}-K^{+}-ATPase, or Ca^{2+}-ATPase activities. We conclude that the stoichiometry of the junctional proteins mediating excitation-contraction coupling is not affected by RyR genotype. However, our data indicate that the relative content of the triadic junctional mem-
brane proteins is decreased by one or two copies of the MHS RyR allele. This may be an important consequence of the altered excitation-contraction coupling and sarcoplasmic Ca\(^{2+}\) homeostasis that results from the MHS RyR mutation.

**EXPERIMENTAL PROCEDURES**

**Materials.** Porcine longissimus dorsi muscle was utilized for all experiments. The animals were killed, after which the muscles were rapidly excised, cut into small pieces, frozen in liquid nitrogen, and stored at \(-80^\circ\)C. All pigs (36–45 kg) were obtained from the Department of Animal Science and maintained at the University of Minnesota Experimental Farm. MHS Pietrain pigs were known to be homozygous for the MHS gene (also known as the halothane or FSS gene), while normal Yorkshire pigs were known to be homozygous for the normal allele (13). Pigs heterozygous for the MHS gene were produced by crosses of Yorkshire boars with Pietrain females.

All experimental animals were genotyped for the presence of the normal and MHS RyR alleles (11). The polymerase chain reaction (PCR) was utilized to amplify the region containing nucleotides 1,811–1,884 of the skeletal muscle RyR coding sequence from white blood cell crude DNA preparations. This 74-base pair RyR PCR fragment was then analyzed by allele-specific restriction endonuclease digestion (11). All Pietrain pigs were homozygous for the Cys-615 RyR allele (T at nucleotide 1,843), all Yorkshire pigs were homozygous for the Arg-615 allele (C at nucleotide 1,843), and all Yorkshire × Pietrain progeny contained both alleles. These genotypes are designated Cys/Cys (MHS), Arg/Cys (homozygous), and Arg/Arg (normal).

The preparation and characterization of antibodies to the various muscle membrane proteins are described elsewhere (28). The following antibodies were used: sheep polyclonal antibodies to the rabbit muscle RyR; monoclonal antibodies (MAb) IVE12 to the fast-twitch and MAb IIDS to the slow-twitch/cardiac muscle SR Ca\(^{2+}\)-ATPase; MAb IIC12 and IIDD6 to the \(\alpha_2\)-subunit of the DHPR; MAb VB2 to the \(\beta\)-subunit of the DHPR, and guinea pig polyclonal antibodies to the \(\alpha_2\)- and \(\gamma\)-subunits of the DHPR. MAb MC2B to the skeletal muscle Na\(^+-K^+\)-ATPase was the gift of Dr. Kathleen Swadener, Department of Cellular and Molecular Physiology, Harvard Medical School.

(+)-\(\beta\)-Methyl-\(^{3}H\)PN 200-110 (80 Ci/mmol) was obtained from Amersham, and nifedipine was a gift from Miles Laboratories. All protease inhibitors were obtained from Sigma. Secondary antibody conjugates were from Boehringer Mannheim. \(^{3}H\)-labeled secondary antibodies and \(^{3}H\)-ryanodine were purchased from Du Pont-New England Nuclear.

**Preparation of muscle homogenates.** One gram of muscle was homogenized in 10 ml ice-cold 10% sucrose, 20 mM tris(hydroxyethyl)aminomethane (Tris)-maleate (pH 7.0), 0.5 mM EDTA, 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupetin, 1 mM iodoacetamide, 0.5 \(\mu\)g/ml pepstatin, 0.2 mM PMSF, and 0.8 mM benzamidine. The homogenate was centrifuged at 2,600 \(g\) for 30 min at 4°C, the supernatant retained, and the pellet homogenized again in 5 vol of the same buffer. This second homogenate was centrifuged at 2,600 \(g\), after which both supernatants were combined and centrifuged at 140,000 \(g\) for 60 min. The resulting pellet was resuspended in 0.6 M KCl, 10% sucrose, 50 mM Tris (pH 7.0), 0.1 mM PMSF, and 0.8 mM benzamidine and was gently agitated for 30 min at 4°C. After centrifugation at 140,000 \(g\) for 60 min, the crude membrane pellet was resuspended in 1 ml 10% sucrose, 20 mM Tris (pH 7.4), 0.1 mM PMSF, and 0.8 mM benzamidine and was frozen in liquid nitrogen. Total recovery of the RyR and DHPR activities in the crude membrane fractions was 25–40% of activity originally present in muscle homogenates, indicating 25–40% membrane recovery in the crude membrane fractions.

**Preparation of membrane fractions enriched in junctional proteins.** Membrane fractions enriched in the junctional proteins were prepared by sucrose gradient centrifugation. Briefly, the 2,600 \(g\) supernatant of a muscle homogenate was centrifuged at 10,000 \(g\) for 30 min. The resulting pellet was resuspended in 0.6 M KCl, 10% sucrose, 10 mM Tris-2-(N-morpholino)ethanesulfonic acid (pH 6.8), incubated on ice for 1 h, and centrifuged at 100,000 \(g\). The pellets were resuspended in 10% sucrose, 0.4 M KCl, 10 mM PIPES, 0.1 mM N,N'-bis(2-ethanesulfonic acid) (PIPES; pH 6.8) and placed on discontinuous sucrose density gradients containing 0.4 M KCl and 10 mM PIPES in all layers. The membranes that banded at the 36–45% sucrose interface after 5 h centrifugation at 85,000 \(g\) were collected and used in all experiments. All solutions contained the protease inhibitors 0.1 mM PMSF, 1 \(\mu\)g/ml leupetin, 1 \(\mu\)g/ml aprotinin, and 0.8 mM benzamidine.

**Partially purified DHPR preparation.** A preparation enriched in DHPR was prepared from the crude membranes as described previously (32). Briefly, 10 mg membrane protein were solubilized at 4°C overnight in 1 ml of 1% digitonin, 0.5 M NaCl, 0.4 M sucrose, 50 mM Tris (pH 7.4), and a protease inhibitor cocktail, and the soluble material was incubated overnight with 0.5 ml wheat germ agglutinin (WGA)-Sepharose 6 MB. After washing the beads to remove unbound material, the bound proteins were eluted with 1 ml of 300 mM N-acetyl-d-glucosamine, 0.1% digitonin, 0.5 M sucrose, 50 mM Tris (pH 7.4), 0.75 mM benzamidine, and 0.1 mM PMSF. Ten milligrams of membrane protein from each of the different MHS, heterozygote, and normal membrane preparations were utilized and processed simultaneously; identical volumes of the final WGA eluate from each preparation were then analyzed on immunoblots.

**Polyacrylamide gel electrophoresis and immunoblotting.** Membrane fractions (10–250 \(\mu\)g), partially purified DHPR preparations (50–100 \(\mu\)l), or whole muscle homogenates (500 \(\mu\)g) were electrophoretically fractionated on 3–12% gradient polyacrylamide gels in the presence of 0.1% SDS and 1% \(\beta\)-mercaptoethanol. The fractionated proteins were then either stained with Coomassie blue or transferred to nitrocellulose membranes. All blocking solutions or solutions containing antibodies consisted of 0.9% NaCl, 50 mM sodium phosphate, and 5% nonfat dry milk. Nitrocellulose blots were incubated with primary antibody as described in the figure legends and then with a horseradish peroxidase-conjugated secondary antibody. After incubation, blots were washed and developed with 0.015% \(\text{H}_2\text{O}_2\) and 0.5 mg/ml 4-chloronaphthol. At a later time, the blots were incubated with \(^{125}\text{I}\)-labeled secondary antibody, washed, and exposed to Kodak XAR-5 film for autoradiography.
Ligand-binding assays. [3H]PN 200-110 binding to muscle membranes was performed in a medium containing 50 mM Tris HCl (pH 7.4), 200 μg membrane protein, and 10 nM [3H]PN 200-110, in a volume of 0.5 ml at 22°C. Nonspecific binding was determined in the presence of 1 μM mifepristone. After 45 min incubation, samples were filtered onto Whatman GF/B filters. The dissociation constant (Kd) for [3H]PN 200-110 binding to the DHPR in crude muscle membranes, heavy SR membranes, and muscle homogenates was in the range 0.16–0.25 nM and did not differ for the three genotypes (data not shown). Thus, using a PN 200-110 concentration (10 nM) that was ~40 times the Kd of the pig DHPR for this ligand should be sufficient for estimating the total DHPR content in the samples.

[3H]Ryanodine binding to muscle membranes was performed in a medium containing 1 M NaCl, 10 mM PIPES (pH 7.0), 100 nM ryanodine (consisting of 10 nM [3H]ryanodine and 90 nM nonradioactive ryanodine), 2 mM CaCl2, 1.85 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetracetic acid (EGTA), 1.85 mM nitritrocetic acid (ionized Ca2+ = 6 μM), 10 mM ATP, and 50 μg membrane protein, in a volume of 0.25 ml. Incubation was for 90 min at 37°C. Nonspecific binding was determined in the presence of 20 μM nonradioactive ryanodine. The Kd of the RyR for [3H]ryanodine in crude membranes, heavy SR, and muscle homogenates was in the range 8–10 nM and did not differ for the three genotypes (Ref. 33 and data not shown) Thus the total ryanodine concentration chosen for binding to crude membranes was ~10 times the Kd of the pig RyR for this ligand under these conditions and could be utilized to estimate the total RyR content in the samples.

The protein concentration for ligand binding determinations on muscle homogenates was 2 mg/ml. Assay conditions for ligand binding were the same as those described above for membrane fractions, except that the [3H]PN 200-110 concentration was 2.5 nM and the [3H]ryanodine concentration was 20 nM. Muscle homogenate [3H]ryanodine binding activity at this ryanodine concentration was ~75% of the Bmax for all three genotypes (data not shown).

ATPase assays. Ca2+-ATPase activity was measured in 100 mM KCl, 10 mM PIPES (pH 7.0), 10 μM A-23187, 5 mM MgATP, and 12.5 μg membrane protein in a volume of 0.5 ml at 22°C; Ca2+−ATPase activity was defined as the difference between P1 liberation in the presence of 0.1 mM CaCl2 and that in the presence of 1 mM EGTA.

Na+−K+−ATPase activity was measured in (in mM) 100 NaCl, 10 KCl, 40 histidine (pH 7.0), 1 EGTA, and 5 MgATP, as well as 0.2 mg/ml saponin and 50 μg membrane protein, in a volume of 0.25 ml at 37°C. Na+/K+−ATPase activity was defined as the difference between P1 liberation in the presence and that in the absence of 1 mM ouabain.

Immunofluorescence microscopy. Longissimus dorsi muscle was dissected to 2- to 3-mm-diameter bundles (5-mm length), embedded in optimum cutting temperature compound, and frozen in liquid nitrogen-cooled isotopane. Samples were stored at ~80°C until use. Fresh cryosections were cut and processed for indirect immunofluorescent analysis as described previously (29). Serial sections were first stained for 1 h at 37°C with primary antibodies to the DHPR (Mab IIID5) or antibodies to either the fast-twitch (Mab VE12) or to the slow-twitch/cardiac (Mab IID8) muscle SR Ca2+−ATPase. After washing in 0.9% NaCl and 50 mM sodium phosphate (pH 7.4), the sections were further incubated for 30 min at 37°C with affinity-purified fluorescent isothiocyanate-labeled goat F(ab)2 anti-mouse immunoglobulin G (Boehringer Mannheim) and subsequently examined in a Zeiss Axiosplan fluorescence microscope.

Quantitation of labeling. Autoradiograms obtained from [35S]labeled immunoblots were scanned with a Molecular Dynamics 300S computing densitometer and analyzed using Image Quant version 1.24 data analysis software. For RyR image quantitation, scans of the Coomassie blue-stained gel and the horseradish peroxidase-stained immunoblot were obtained. After determination of the image intensity of a component of an individual preparation, the mean intensity ± SD of the MHS, heterozygote, and normal sample populations was calculated. The values were then normalized, with the population (usually normals) containing the highest mean content set to 100%.

Statistical analysis. Comparisons of the mean values for the normal, heterozygote, and MHS populations were made with Student's t test.

RESULTS

Analysis of crude membrane fractions isolated from muscle of 1-day, 28-day, and 3- to 4-mo-old pigs of the three different RyR genotypes. Skeletal muscle crude membrane preparations were isolated from pigs of all three RyR genotypes at different ages. Crude membrane fractions were utilized to obtain a representative preparation of the SR and T-tubule membranes in high yield (see EXPERIMENTAL PROCEDURES). The total yield of membrane protein obtained per gram of muscle was similar for the three different RyR genotypes. The content of Ca2+−ATPase, DHPR, and RyR (Fig. 1, A, B, and C) in the crude membrane fractions increased markedly (~3- to 5-fold) from 1 to 28 days of age, after which there was little or no further increase in the content of these proteins by 3–4 mo of age (Fig. 1).

There were no differences in the Ca2+−ATPase activities or [3H]PN 200-110 or [3H]ryanodine binding activities per milligram membrane protein isolated from 1-day-old pigs of the three different RyR genotypes (Fig. 1). There was also no difference in Ca2+−ATPase activity between the three genotypes at any age (Fig. 1A). However, by 28 days of age, there were significant differences between the different genotypes in the maximal [3H]PN 200-110 binding (Fig. 1B) and maximal [3H]ryanodine binding (Fig. 1C) activities, with Arg/Arg membranes having greater ligand-binding activity than Cys/Cys membranes.

The significant differences in ligand-binding activities between MHS and normal membranes were maintained in the 3- to 4-mo-old pigs, with MHS membranes exhibiting 61% of the [3H]PN 200-110 binding and 75% of the [3H]ryanodine binding of normal membranes (Table 1). [3H]PN 200-110 and [3H]ryanodine binding activity of Arg/Cys pigs 28 days old and 3–4 mo of age were typically intermediate to both types of homozygotes (Fig. 1 and Table 1). Furthermore, there was no difference in the Na+−K+−ATPase activity between the 3- and 4-mo-old pigs of the three different RyR genotypes (Table 1).

Linear correlation analyses were performed to determine whether, in the individual membrane preparations, the content of one of these proteins was closely correlated with the content of another protein. This analysis revealed a high correlation between the [3H]PN 200-110 binding and [3H]ryanodine binding activity for
a given preparation ($r = 0.720$). However, the correlation coefficients between all other activities measured on the individual preparations were $< 0.18$.

**Immunoblot analysis of crude membrane fractions from 3- to 4-mo-old pigs.** When proteins from the crude membrane fractions of 3- to 4-mo-old pigs of all three RyR genotypes were electrophoretically fractionated on gradient polyacrylamide gels, the protein-staining patterns observed were quite similar (Fig. 2). Also shown in Fig. 2 are the immunoblots when nitrocellulose trans-


ters were stained with a panel of antibodies to muscle membrane proteins. Each of these antibodies recognized a single immunostaining component in all three RyR genotypes, indicating minimal proteolysis of the samples. It is evident from these immunoblots that there was little difference in the immunostaining intensity of the samples when antibodies to the fast-twitch Ca$^{2+}$-ATPase or Na$^+$/K$^+$-ATPase were utilized. There was also no difference between the RyR genotypes when an antibody to the slow-twitch SR Ca$^{2+}$-ATPase was utilized (data not shown).

There were, however, consistent differences among the 3- to 4-mo-old pig crude membrane samples in the immunostaining intensities of the α$_1$- and α$_2$-subunits of DHPR, with Cys/Cys and heterozygote samples having less staining intensity with each of these antibodies than the Arg/Arg samples (Fig. 2). This was the case for every sample load analyzed (50–250 μg/lane). Similar results were obtained when another antibody to the α$_1$-subunit (MAb III-D5) was utilized for the immunoblot analysis (data not shown).

When antibodies to the RyR were utilized for immunostaining, there was also variation in staining intensity between the different RyR genotypes (Fig. 2). As with the DHPR immunostaining pattern, Cys/Cys samples consistently demonstrated less RyR immunostaining than did the Arg/Arg samples. This pattern was also observed on the Coomassie blue-stained polyacrylamide gel (Fig. 2, top), where the intensity of the high relative molecular weight RyR band in MHS samples was consistently less than the corresponding band in normal

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**Table 1. Analysis of crude membrane fractions from 3- to 4-month-old pigs with different RyR genotypes**

<table>
<thead>
<tr>
<th></th>
<th>Cys/Cys</th>
<th>Arg/Cys</th>
<th>Arg/Arg</th>
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<tr>
<td><strong>[3H]PN 200-110</strong></td>
<td></td>
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<tr>
<td>binding, pmol/mg (MHS)</td>
<td>$3.0 \pm 0.5^*$</td>
<td>$3.5 \pm 0.7^*$</td>
<td>$4.9 \pm 0.7$</td>
</tr>
<tr>
<td>(71%)</td>
<td>(71%)</td>
<td>(100%)</td>
<td></td>
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<tr>
<td><strong>[3H]ryanodine binding</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pmol/mg</td>
<td>$4.6 \pm 0.5^*$</td>
<td>$5.3 \pm 0.4^*$</td>
<td>$6.1 \pm 0.8$</td>
</tr>
<tr>
<td>(75%)</td>
<td>(87%)</td>
<td>(100%)</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$-ATPase, μmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_0$, mg$^{-1}$ min$^{-1}$</td>
<td>$0.49 \pm 0.12$</td>
<td>$0.50 \pm 0.15$</td>
<td>$0.47 \pm 0.12$</td>
</tr>
<tr>
<td>Na$^+$/K$^+$-ATPase, μmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_0$, mg$^{-1}$ min$^{-1}$</td>
<td>$4.4 \pm 0.7$</td>
<td>$3.4 \pm 0.6$</td>
<td>$3.8 \pm 0.6$</td>
</tr>
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</table>

Values are means ± SD of preparations from 5 different animals of each genotype. Numbers in parentheses indicate value relative to normal (100%). Crude membrane fractions were prepared and analyzed for $[3H]$PN 200-110 binding, [3H]ryanodine binding, Ca$^{2+}$-ATPase and Na$^+$/K$^+$-ATPase as described in EXPERIMENTAL PROCEDURES. MHS, malignant hyperthermia susceptible. RyR, ryanodine receptor. *Significantly different from normal, $P < 0.05$. †Significantly different from heterozygote, $P < 0.05$. 

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*Fig. 1. Dihydropyridine receptor (DHPR), ryanodine receptor (RyR), and Ca$^{2+}$-ATPase content of crude membrane fractions isolated from pigs of 3 different RyR genotypes at various ages. Membrane preparations were isolated from malignant hyperthermia-susceptible (MHS; ), heterozygous (‡), and normal (□) pigs at 1 day, 28 days, and 3–4 mo of age. Ca$^{2+}$-ATPase (A), [3H]PN 200-110 binding (B), and [3H]ryanodine binding (C) activities were then determined as described in EXPERIMENTAL PROCEDURES. Symbols represent mean ± SD for duplicate determinations on membrane preparations from 4 different animals. *Significantly different from normal, †significantly different from heterozygotes, $P < 0.05$. 

Fig. 2. Electrophoretic and immunoblot analysis of homozygous MHS, heterozygote, and homozygous normal muscle membranes. Membrane preparations were prepared and electrophoresis performed as described in EXPERIMENTAL PROCEDURES. Gels were then stained with Coomassie blue or transferred to nitrocellulose membranes for incubation with antibodies. Immunoblots shown were developed with horseradish peroxidase-conjugated secondary antibody. Only pertinent areas of immunoblots are shown. CB, Coomassie blue-stained gel, 50 μg/lane; RyR, sheep polyclonal anti-rabbit RyR antibody (1:1,000), 25 μg/lane; Ca-ATPase, monoclonal antibody (Mab) IVB12 to fast-twitch muscle sarcoplasmic reticulum (SR) Ca2+-ATPase (1:20), 10 μg/lane; Na+-K+-ATPase, MAb MCB2 to skeletal muscle Na+-K+-ATPase (1:4), 250 μg/lane; α1, MAb IIIC12 to α-subunit of DHPR (1:10), 250 μg/lane; α2, guinea pig polyclonal antibody to α2-subunit of rabbit DHPR (1:1,000), 250 μg/lane. Lanes contain homozygous MHS (M), heterozygote (H), or homozygous normal (N) samples. Position and relative molecular weight of markers are indicated on left.
samples. The Coomassie blue-stained RyR band intensity for each of the samples was obtained by densitometry. The means of the three populations were then determined and normalized. MHS crude membrane RyR content determined by this method was 75 ± 20%, heterozygote RyR content was 91 ± 12%, and normal RyR content was 100 ± 20%. The MHS content was significantly less than normal (P < 0.05).

To further quantify the content of all these proteins in the crude membrane fractions, the immunoblots that had been stained with Na⁺-K⁺-ATPase, Ca²⁺-ATPase, and α₁- and α₂-subunits of DHPR antibodies were probed with ¹²⁵I-labeled secondary antibodies, and the resulting autoradiograms were scanned for labeling intensity; the horseradish peroxidase-stained immunoblot of the RyR was scanned directly. The optical densities of the labeled bands were determined, these values averaged, and the genotype exhibiting the highest autoradiographic labeling intensity for a given antibody was normalized to 100% (Table 2). This analysis revealed no significant difference in Na⁺-K⁺-ATPase labeling between the different pig RyR genotypes (Table 2), whereas a slightly lower intensity of SR Ca²⁺-ATPase labeling in the MHS membranes relative to the normal membranes was observed.

A highly significant difference in the content of both the α₁- and α₂-subunits of the DHPR was observed, with both the Cys/Cys and heterozygote crude membranes having a significantly lower content of these two proteins than did the Arg/Arg membranes (Table 2). Although the mean values for the content of the α₁- and α₂-subunits in heterozygous membranes were intermediate to the MHS and normal mean values, they were significantly different only from the normal membranes. The ratio of the α₁- and α₂-subunit contents (based on immunostaining for each individual preparation) was close to 1 and was not significantly different for the three different RyR genotypes (Table 1).

The immunostaining intensity for the RyR in the crude membrane fractions was also significantly different between the three different RyR genotypes (Table 2). In this case MHS membranes had significantly less staining intensity than either the heterozygote or normal membranes, whereas the heterozygote RyR staining intensity did not differ significantly from the normal value.

Linear correlation analyses of the immunonquantitation analyses for each component within a preparation demonstrated a high positive correlation between the α₁- and α₂-subunit contents (r = 0.841), between the RyR and the α₁- and α₂-subunits of the DHPR (r = 0.767 and 0.659, respectively), as well as a negative correlation between the RyR, α₁- or α₂-subunit contents, and the Ca²⁺-ATPase (r = -0.750, -0.861, and -0.708, respectively). The other correlation coefficients between these components of each preparation were <0.41.

Immunoblot analysis of partially purified DHPR preparations from 3- to 4-mo-old pigs. Partially purified DHPR preparations were obtained by digitonin solubilization of the crude membrane preparations of the three different RyR genotypes followed by WGA-Sepharose affinity chromatography. Identical starting membrane protein amounts were utilized, the purification of all samples was done in parallel, and identical sample volumes were analyzed on the immunoblots, allowing samples to be directly compared. As in crude membranes, antibodies for the α₁-, α₂-, β-, and γ-subunits of the DHPR again recognized only a single component (data not shown).

Quantitation of the immunolabeling using ¹²⁵I-labeled secondary antibodies (Table 3) indicated that the content of α₁- and α₂-subunits in the partially purified Cys/Cys preparations was ~50% that of Arg/Arg preparations, which is similar to our data analyzing the content of these proteins in crude membranes (61%, Table 2). Furthermore, the content of the β- and γ-subunits in the MHS samples was ~50% of normal (Table 3). For the heterozygote samples, the contents of the different subunits were intermediate to both MHS and normal samples; however, this difference was not statistically significant due to a large SD. For example, the α₂-subunit content of the heterozygote samples was significantly lower than normal but not significantly different than that of MHS; the α₂-subunit content of heterozygote samples was significantly different from both MHS and normal; and the β- and γ-subunit content of the heterozygote samples was not significantly different from either MHS or normal (Table 3).

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**Table 2. Immunoblot analysis of crude membrane fractions from 3- to 4-mo-old pigs with three different RyR genotypes**

<table>
<thead>
<tr>
<th></th>
<th>Cys/Cys (MHS)</th>
<th>Arg/Cys (Heterozygote)</th>
<th>Arg/Arg (Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺-K⁺-ATPase</td>
<td>99 ± 9</td>
<td>98 ± 23</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>100 ± 9*</td>
<td>97 ± 9</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>RyR</td>
<td>62 ± 15*†</td>
<td>88 ± 15</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>α₁</td>
<td>45 ± 14*</td>
<td>64 ± 25*</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>α₂</td>
<td>43 ± 9*</td>
<td>58 ± 13*</td>
<td>100 ± 24</td>
</tr>
<tr>
<td>α₁/α₂</td>
<td>1.2 ± 0.6</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SD in % (except for α₁/α₂) of preparations from 4 different MHS, heterozygote, and normal pigs each and are normalized relative to group with highest content (100%). Crude membranes were analyzed for immunodetection and quantitation of Ca²⁺-ATPase, Na⁺-K⁺-ATPase, RyR, and α₁- and α₂-subunits of the dihydropyridine receptor (DHPR) as described in EXPERIMENTAL PROCEDURES. *Significantly different from normal, P < 0.05. †Significantly different from heterozygote, P < 0.05.

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**Table 3. Immunoblot analysis of partially purified DHPR preparations from 3- to 4-mo-old pigs with three different RyR genotypes**

<table>
<thead>
<tr>
<th></th>
<th>Cys/Cys (MHS)</th>
<th>Arg/Cys (Heterozygote)</th>
<th>Arg/Arg (Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁</td>
<td>48 ± 24*</td>
<td>70 ± 24*</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>α₂</td>
<td>47 ± 21*</td>
<td>71 ± 13*</td>
<td>100 ± 22</td>
</tr>
<tr>
<td>β</td>
<td>46 ± 17*</td>
<td>68 ± 24</td>
<td>100 ± 52</td>
</tr>
<tr>
<td>γ</td>
<td>55 ± 33*</td>
<td>81 ± 31</td>
<td>100 ± 52</td>
</tr>
</tbody>
</table>

Values are means ± SD in % of preparations from 4 different MHS, heterozygote, and normal pigs each. DHPR from crude membranes was solubilized, partially purified, and analyzed for immunodetection of receptor subunits as described in EXPERIMENTAL PROCEDURES. *Significantly different from normal, P < 0.05. †Significantly different from both normal and MHS, P < 0.05.
Analysis of muscle homogenates from 3- to 4-mo-old pigs. To ensure that we were not biasing our data by selecting a specific membrane fraction, muscle homogenates were prepared from all three RyR genotypes and analyzed for [3H]PN 200-110 binding and [3H]ryanodine binding activities. As observed with crude membranes, [3H]PN 200-110 binding, as well as [3H]ryanodine binding to Cys/Cys muscle homogenates was significantly less than was binding to Arg/Arg muscle homogenates (Table 4). For both ligands, binding to the MHS homogenates was ~80% of the binding to normal homogenates. [3H]PN 200-110 and [3H]ryanodine binding to the heterozygote muscle homogenates was not significantly different from binding to homogenates of either type of homozygote, although it was numerically intermediate. The ratio of [3H]PN 200-110 binding to [3H]ryanodine binding was ~1 for homogenates of all three types of muscle (Table 4).

Samples of each of the homogenates were electrophoresed, transferred to nitrocellulose paper, and immunostained with an antibody to the fast-twitch muscle SR Ca2+-ATPase. After quantitation by densitometry and normalization of the data, this analysis indicated no significant differences in Ca2+-ATPase content between whole muscle homogenates of the three different genotypes (Table 4). There was a very high SD for this whole muscle homogenate immunooquantification analysis.

Analysis of heavy SR membranes from 3- to 4-mo-old pigs. We previously examined [3H]ryanodine binding to porcine skeletal muscle heavy SR membranes that are enriched in junctional protein components (27, 28). Confirming our previous reports, heavy SR membranes prepared from pigs of the three different RyR genotypes did not differ in maximal [3H]ryanodine binding (Table 5). Furthermore, these heavy SR membranes isolated from the three RyR genotypes did not differ in [3H]PN 200-110 binding, Ca2+-ATPase activity, or Na+K+-ATPase activity (Table 5). It was noted that the heavy SR membranes had a two- to threefold increase in the

<table>
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<tr>
<th>Table 5. Analysis of heavy SR membranes from 3- to 4-mo-old pigs with three different RyR genotypes</th>
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<td><img src="image_url" alt="Table content" /></td>
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</tbody>
</table>

Values are means ± SD of 3 H9 MHS, 3 heterozygote, and 6 normal preparations. Heavy sarcoplasmic reticulum (SR) fractions were prepared and analyzed for [3H]PN 200-110 binding, [3H]ryanodine binding, Ca2+-ATPase, and Na+K+-ATPase as described in EXPERIMENTAL PROCEDURES.

![Image](image_url)

Immunofluorescence microscopy of the DHPR in MHS and normal muscle. Analysis of the DHPR distribution in Cys/Cys and Arg/Arg longissimus dorsi muscle was made in fresh cryostat serial sections from 3- to 4-mo-old pigs stained with antibodies to both the fast-twitch and slow-twitch SR Ca2+-ATPase enzymes, as well as the α1-subunit of the DHPR. MHS and normal samples were then simultaneously processed for immunofluorescence microscopy. Figure 3 shows representative fluorescent micrographs for a normal (Fig. 3, A, C, E) and a MHS pig (B, D, F). The results confirm our previous conclusions (8) that the pig longissimus dorsi has a high content of fast-twitch (type II) fiber when determined with antibodies to both types of SR Ca2+-ATPase isoforms, with no difference in the fiber type distribution between the MHS and normal muscles (Fig. 3, compare A-C and B-D). Significantly, the cross-sectional areas of the fibers from the homozygous MHS muscle were greater than those of the normal fibers. This was a consistent finding in all samples from two MHS and two normal pigs and is also in agreement with a previous study (9).

Figure 3, E and F, shows that the DHPR α1-subunit immunofluorescence staining intensity varied in individual fibers. However, by analyzing the serial sections, it is clear that this variation is due to the higher immunofluorescence intensity in the fast-twitch fibers than the slow-twitch fibers for both MHS and normal muscles. Figure 3, E and F, also shows that there was no gross abnormality in the distribution of α1-immunofluorescence intensity in the MHS fibers. Interestingly, there was a consistently lower immunofluorescence intensity when using antibodies to α1 in the MHS muscle sections compared with identically prepared normal samples (2 different samples from each of 2 different MHS and 2 different normal pigs).

**DISCUSSION**

MHS pigs have an Arg-615 to Cys-615 mutation in the skeletal muscle SR RyR/Ca2+-release channel (11) that is responsible for abnormal sarcoplasmic Ca2+ regula-
tion in this tissue (20). The effects of this mutation are variously observed as abnormalities in excitation-contraction coupling (6, 12), SR Ca^{2+} release (17, 25, 27), SR Ca^{2+} channel regulation (10), and [H]ryanodine binding (8, 15, 24). The abnormality in the single-channel activity of the MHS RyR observed with native SR vesicles has now been reconstituted from purified preparations of this protein (33). Evidence to date also indicates that the skeletal muscle RyR is a candidate gene for many pedigrees of human MH (14, 22, 23). Thus it is of interest that there are reports of decreased dihydropyridine binding in isolated MHS pig transverse
tubules (7) and decreased intramembrane charge movement and surface membrane L-type Ca\(^{2+}\) currents (19) in MHS pig muscle fibers. It is very likely that an alteration in the content or regulatory properties of DHPR activity would also have significant effects on the structure and function of the triad junction in MHS muscle. For example, it is known that, in other muscle alterations, such as denervation (35) or an altered frequency of stimulation associated with fiber type conversion (28), the membrane content of excitation-contraction coupling proteins can also be altered.

Using the muscle of 3- to 4-mo-old pigs, we had previously shown that there was no difference in the affinity of the DHPR for its ligands when purified T-tubule membranes of Cys/Cys and Arg/Arg pigs were compared (7). In the present report our data indicate that all subunits (α1, α2, β, and γ) of the DHPR are proportionately decreased in the membrane preparations from older animals (Fig. 2 and Tables 2 and 3) and that the immunofluorescence staining pattern of MHS and normal pig muscle (Fig. 3) revealed no abnormality in the distribution of the DHPR in the MHS pig fibers. Therefore the decreased DHPR activities in MHS muscle membrane preparations cannot be ascribed to an abnormal subunit composition of this protein. Rather, it is likely that there is a physical decrease in the quantity of DHPR protein in the T-tubule membrane or a decreased amount of this DHPR-containing membrane in MHS muscle.

The unexpected finding in this study was that not only was the DHPR content of MHS pig crude muscle membranes reduced to 61–80% of normal muscle but that the RyR content of MHS muscle was also reduced (75–81% of normal) in these preparations (Figs. 1 and 2 and Tables 1–3). There was a high correlation between the content of these two proteins in the individual crude membrane preparations. The difference in both [\(^3\)H]PN 200-110 binding and [\(^3\)H]ryanodine binding between MHS and normal samples was also observed in muscle homogenates, with a ratio of DHPR to RyR of ~1 (Table 4). Our previous results using heavy SR membranes enriched in proteins of the triad junction had not indicated an altered RyR content in MHS membranes (8, 24, 25), and this was confirmed for the heavy SR membranes isolated in the present study (Table 5). However, the data presented here demonstrate that heavy SR membranes from the three pig RyR genotypes also do not differ in DHPR activity. Thus it appears that MHS muscle and crude MHS membranes have a slightly reduced content of both of the triadic junctional proteins (DHPR and the RyR) but that the stoichiometric relationship between these proteins is not altered (Tables 1, 4, and 5).

The activity of the RyR in intact muscle bundles (13) and SR vesicles (25) isolated from pigs that are heterozygous for the RyR alleles displays properties intermediate between that of MHS and normal pigs, indicating that both alleles of the RyR gene are being expressed. It is therefore interesting to note that, in the crude membrane preparations isolated from heterozygous pigs, the maximal [\(^3\)H]PN 200-110 binding, the content of the α1- and α2-subunits of DHPR, and the maximal [\(^3\)H]ryanodine binding were significantly less than that of normal pigs (Fig. 1). Although the mean values for DHPR content of crude membranes isolated from heterozygote pigs were typically intermediate to membranes isolated from both types of homozygote pigs, they generally were not significantly greater than the contents determined for the respective MHS preparations (Figs. 1 and 2 and Tables 1–4). The high sample variation in this analysis probably contributed to our inability to accurately determine whether the DHPR content of the crude membranes isolated from heterozygote pigs was indeed significantly different from both types of homozygote pigs. However, our data indicate that only one abnormal RyR allele is necessary to cause an altered content of the DHPR and RyR in these preparations.

A possible explanation for the proportionally decreased DHPR and RyR content of MHS muscle crude membrane fractions and whole muscle homogenates in this report is that the junctional membrane content per unit mass of muscle is lower than in normal muscle. This hypothesis is supported by the lower than normal [\(^3\)H]PN 200-110 and [\(^3\)H]ryanodine binding activities in MHS muscle homogenates (Table 4), and particularly the lower [\(^3\)H]PN 200-110 and [\(^3\)H]ryanodine binding activities in MHS crude membrane fractions (Tables 1–3) where the data are expressed relative to total protein, most of which is the SR Ca\(^{2+}\)-ATPase. Such a hypothesis could also explain why membrane preparations that are highly enriched in the junctional proteins have similar triadic protein contents per milligram membrane protein in all three genotypes (Table 5). We propose that this possible structural difference could also explain the previously described lower DHPR content of purified T-tubule preparations isolated from MHS muscle (7), where the MHS T-tubules would have a decreased content of DHPR-containing junctional T-tubule membrane relative to nonjunctional T-tubule membrane lacking the DHPR. We have been unable to demonstrate significant differences in the mRNA levels for the DHPR subunits or the RyR between the pigs of varying genotype, indicating that there are unlikely to be large differences in the rate of transcription of these proteins in the different types of muscle (data not shown). Furthermore, that the properties and content of other components of the surface membranes are unaltered by the RyR mutation is indicated by the similarity in their Na\(^+\)-K\(^-\)-ATPase content (Tables 1, 2, and 5 and Fig. 1), as well as other marker proteins in the various preparations (7, 26).

A lower relative content of junctional-to-nonjunctional T-tubule membranes in MHS tissue may also explain the decreased nonlinear charge movement and surface membrane Ca\(^{2+}\) currents reported in MHS pig muscles (19). These latter data are normalized to the membrane capacitance, which in turn reflects total T-tubule membrane surface area within the voltage clamp. If there is a larger amount of nonjunctional T-tubule membrane within the voltage clamp (relative to DHPR-containing junctional T-tubule), this would lead to lower relative Ca\(^{2+}\) current and charge move-
ment in MHS fibers compared with normal fibers, even though there may be no difference between genotypes in the DHPR content at the triad junction itself. In any event, it is notable that, regardless of whether the DHPR or RyR activity is measured or whether it is skinned fibers, isolated membranes, or muscle homogenates being analyzed, the measured activity of MHS preparations is usually in the range of 60–80% of that in normal samples (Refs. 7 and 19 and this report).

We conclude that reduced expression of the DHPR activity in some preparations isolated from MHS muscle does not reflect an altered DHPR content at the triad junction. It instead appears to reflect an intrinsic difference in the subcellular membrane contents between MHS and normal muscles, which in turn influences the relative content of their associated proteins. Significantly, membranes from neonatal pigs of the three RyR genotypes were identical in all regards, with differences in DHPR and RyR content between the genotypes only becoming detectable after several weeks of age (Fig. 1). Thus the influence of the Cys-615 RyR allele on the content of the membrane proteins is correlated with muscle development and may be the result of an adaptive response of the muscle to altered sarcoplasmic Ca\(^{2+}\) regulation (20). The amount of T-tubule area has been shown to increase with fiber diameter and development (3, 21) and the ratio of triadic junctional area to fiber cross-sectional area can vary greatly between fibers (2). It is possible that the larger fiber cross-sectional areas in MHS muscle (9) (Fig. 3) in turn result in an altered relative proportion of the junctional and nonjunctional elements of SR and T-tubule membrane within this tissue. Therefore, although the basis for abnormal Ca\(^{2+}\) regulation in MHS muscle lies in the Arg-615 to Cys-615 RyR mutation (11) causing an abnormal SR RyR/Ca\(^{2+}\)-release channel activity (33), this altered MHS RyR may exert multiple subsequent effects on skeletal muscle biology and excitation-contraction coupling.

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