THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 263, No. 2, Issue of January 15, pp. 994-1001, 1988
Printed in U.S.A.

Biochemical and Ultrastructural Characterization of the 1,4-Dihydropyridine Receptor from Rabbit Skeletal Muscle

EVIDENCE FOR A 52,000-Da SUBUNIT*

(Received for publication, August 13, 1987)

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The 1,4-dihydropyridine receptor purified from rabbit skeletal muscle contains four polypeptide components of 175,000 Da (nonreduced)/150,000 Da (reduced), 170,000, 52,000, and 32,000 Da (Leung, A. T., Imagawa, T., and Campbell, K. P. (1987) *J. Biol. Chem.* 262, 7943–7946). A monoclonal antibody specific to the 52,000-Da polypeptide component of the dihydropyridine receptor has been produced and used in immunoprecipitation and immunoblotting experiments to demonstrate that the 52,000-Da polypeptide is an integral subunit of the purified dihydropyridine receptor. Peptide mapping experiments with ^{32}P-labeled dihydropyridine receptor have also demonstrated that the 52,000-Da polypeptide is distinct from and not a proteolytic fragment of the 170,000-Da subunit. Densitometric scanning of Coomassie Blue-stained sodium dodecyl sulfate-polyacrylamide gels of the purified dihydropyridine receptor has demonstrated that the 52,000-Da polypeptide exists in a 1:1 stoichiometric ratio with the 170,000-, 175,000/150,000-, and 32,000-Da subunits of the dihydropyridine receptor.

Electron microscopy of the freeze-dried, rotary-shadowed dihydropyridine receptor has shown that the preparation contains a homogeneous population of 16 × 22-nm ovoidal particles large enough to contain all four polypeptides of the dihydropyridine receptor. The particles have two distinct components of similar size which may represent the location in the molecule of the two larger subunits.

The receptor for the 1,4-dihydropyridine class of Ca^{2+} channel blockers is most abundant in the transverse tubular membranes of skeletal muscle (1). Unlike cardiac and smooth muscle, however, the number of functional Ca^{2+} channels in skeletal muscle represents fewer than 5% of the total number of dihydropyridine receptors (2), and the influx of Ca^{2+} through these Ca^{2+} channels is not required for contraction (3). Due to its location and the sensitivity of depolarization-dependent charge movement to the 1,4-dihydropyridines, it has been suggested that the 1,4-dihydropyridine receptor may function in skeletal muscle not only as a Ca^{2+} channel but also as the voltage sensor in excitation-contraction coupling (4).

We have recently shown that the purified 1,4-dihydropyridine receptor from rabbit skeletal muscle contains four protein components of 175,000 (α), 170,000 (δ), 52,000 (β), and 32,000 Da (γ) when analyzed by SDS-PAGE[1] under nonreducing conditions (5–7). Several lines of evidence have shown that the 175,000- and 170,000-Da subunits are distinct proteins. Immunoblotting experiments with monoclonal antibodies and WGA-peroxidase staining have shown that the 175,000-Da subunit is a WGA-positive glycoprotein that undergoes a shift in apparent molecular mass to 150,000 under reducing conditions, whereas the 170,000-Da subunit does not exhibit these properties, and it is recognized by specific monoclonal antibodies (5). Furthermore, the 170,000-Da (δ) subunit has been shown by photoaffinity labeling with [^{3}H]azidopine and [^{3}H]PN200-110 to contain the dihydropyridine binding site of the receptor (6). We and others have also demonstrated that the 170,000-Da (δ) subunit (7, 8) and the 52,000-Da (β) subunit (7–10) are substrates for a cAMP-dependent protein kinase (7–10), a Ca^{2+}/calmodulin-dependent kinase (7, 10), and a protein kinase intrinsic to skeletal muscle triads (7), whereas the 175,000- and 32,000-Da subunits are not phosphorylated by any of these kinase systems.

Despite the existence of a substantial amount of data on the composition of the dihydropyridine receptor, little is known about the relationship between the 175,000/150,000-Da glycoprotein and 170,000-Da dihydropyridine-binding subunits and the low molecular mass components of the receptor. The association of these smaller polypeptides with the dihydropyridine receptor has been demonstrated only by their presence on SDS-polyacrylamide gels of the purified dihydropyridine receptor (5, 10–12), their stoichiometry also has not been determined unequivocally, and the 52,000-Da polypeptide has not been reported in certain preparations of purified receptor (13, 14).

In this report, we describe the production of monoclonal antibodies to the 52,000-Da subunit of the dihydropyridine receptor and the results of immunoblotting, immunoprecipitation, and peptide mapping experiments on this protein. Our

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** Supported by Grant HL-15835 from the National Institutes of Health to the Pennsylvania Muscle Institute.

§§ Established Investigator of the American Heart Association and recipient of Grant HL-37187 from the National Institutes of Health.

[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; [^{3}H]PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)pyridine-3-carboxylate; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

results have demonstrated that the 52,000-Da polypeptide is an integral subunit of the dihydropyridine receptor that is distinct from the 170,000-Da dihydropyridine-binding subunit. Additionally, we also report here the ultrastructural characterization of the purified dihydropyridine receptor.

EXPERIMENTAL PROCEDURES

Preparation of Skeletal Muscle Membranes—Triads were purified from adult rabbit skeletal muscle in the presence of protease inhibitors by a modification of the method of Mitchell *et al.* (15) as described previously (6). Light sarcoplasmic reticulum vesicles were isolated from adult rabbit skeletal muscle in the presence of protease inhibitors by the method of Campbell *et al.* (16). Protein was quantitated by the method of Lowry *et al.* (17) as modified by Peterson (18). [^{3}H]PN200-110 binding was determined according to the method of Glossman and Ferry (19).

Production of Monoclonal Antibodies to the Dihydropyridine Receptor—Monoclonal antibodies against the dihydropyridine receptor from rabbit skeletal muscle were prepared as described previously (5). The specificity of the monoclonal antibodies to the 52,000-Da subunit of the dihydropyridine receptor was determined by immunodot, immunoblot, and immunoprecipitation assays as described (5–7).

Purification of Dihydropyridine Receptor from Triads—Dihydropyridine receptor was purified from triads using WGA-Sepharose affinity chromatography and DEAE-cellulose ion exchange chromatography as described previously (6) with only slight modifications as listed below. All buffers used in the preparation contained 0.5 M sucrose, and the solubilization buffer contained the following protease inhibitors: pepstatin A (0.6 μg/ml), aprotinin (0.5 μg/ml), iodoacetamide (18.5 μg/ml), leupeptin (0.5 μg/ml), benzamidine (0.75 mM), and phenylmethylsulfonyl fluoride (0.1 mM). All other buffers contained 0.1 mM phenylmethylsulfonyl fluoride and 0.75 mM benzamidine. In some cases, the dihydropyridine receptor was solubilized from triads using 1% CHAPS with 0.5% asolecithin (L-α-phosphatidylcholine from soybean, Type II-S, Sigma), and all other buffers used in the preparation contained 0.3% CHAPS and 0.15% asolecithin. The purification of the dihydropyridine receptor from phosphorylated triads was carried out as described (7). Detergent-solubilized proteins were quantitated by the method of Lowry *et al.* (17) as modified by Peterson (18) after the proteins were precipitated with 5% trichloroacetic acid in the presence of 0.5 mg of sodium deoxycholate. The purified dihydropyridine receptor was analyzed by SDS-PAGE on 5–16% gradient gels according to the method of Laemmli (20) under both nonreducing (5 mM *N*-ethylmaleimide in sample buffer) and reducing (10 mM dithiothreitol in sample buffer) conditions.

Peptide Mapping of the Phosphorylated Subunits of the Dihydropyridine Receptor—The purified dihydropyridine receptor from triads phosphorylated with [γ-^{32}P]ATP by an intrinsic kinase (7) and the void fraction of the WGA-Sepharose column were subjected to SDS-PAGE on 5–16% gradient gels. The SDS-polyacrylamide gels were stained with Coomassie Blue, destained, and washed with distilled water. The bands corresponding to the 170,000-Da subunit, the 52,000-Da subunit, and the 52,000-Da phosphopeptide in the void fraction of the WGA-Sepharose column were excised. The gel pieces were incubated with trypsin (1 mg/ml in 50 mM NH_4HCO_3, pH 7.85), *Staphylococcus aureus* V8 protease (1 mg/ml in 50 mM NH_4HCO_3, pH 7.85), or cyanogen bromide (2 mg/ml in formic acid) for 4 h at 37 °C. The supernatants were lyophilized and dissolved in Laemmli sample buffer, and the samples were subjected to SDS-PAGE (16–22% gradient gel). The gels were stained with Coomassie Blue, dried, and subjected to autoradiography on Kodak X-Omat AR film with intensifying screens (Du Pont Lightning Plus).

Immunoprecipitation of the Phosphorylated Dihydropyridine Receptor—Monoclonal antibody-goat anti-mouse-IgG-Sepharose beads were prepared as described previously (5). Triads were phosphorylated with [γ-^{32}P]ATP and solubilized with 1% digitonin (7). The solubilized membranes (100 μg of protein) were then incubated with 50 μl of mAb-goat anti-mouse-IgG-Sepharose at 4 °C for 2–4 h with gentle mixing. The mAb-goat anti-mouse-IgG-Sepharose beads containing bound phosphorylated dihydropyridine receptor were washed three times with 500 μl of 0.3% digitonin, 0.5 M NaCl, 0.5 M sucrose, 20 mM NaF, 0.75 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl, pH 7.4, or twice with the above buffer and once with 500 μl of 0.1% SDS in 100 mM Tris-HCl, pH 7.5, 200 mM LiCl, and 20 mM NaF. The immunoprecipitates were extracted from the Sepharose beads by boiling for 1 min in 150 μl of Laemmli sample buffer containing 130 mM Tris-HCl, pH 6.8, 6% SDS, 20% glycerol, 2% 2-mercaptoethanol, and a trace of bromphenol blue. Samples were centrifuged to remove the Sepharose beads and subjected to SDS-PAGE. SDS gels were stained with Coomassie Blue and dried, followed by autoradiography.

Determination of the Subunit Stoichiometry of the Dihydropyridine Receptor—Ten μg of the purified dihydropyridine receptor from nine preparations, isolated using either digitonin or CHAPS for solubilization, were analyzed by SDS-PAGE (5–16% gradient gel) under both reducing and nonreducing conditions. The Coomassie Blue-stained gel was scanned with a Hoefer Model GS-300 scanning densitometer to determine the relative densities of the various bands. The density data was analyzed using the GS-350H Data System software from Hoefer.

Electron Microscopy of the Purified Dihydropyridine Receptor—A 10–20-μl drop of solution containing 10–50 μg/ml of purified dihydropyridine receptor was applied to the surface of freshly cleaved mica. The mica was either gently rinsed with 10 drops of 50 mM Tris-HCl, pH 7.3, or directly exposed to 7–8 drops of 2% uranyl acetate containing 20 mg/ml bacitracin for 30 s and finally rinsed in 3% glycerol with bacitracin. This thin layer of solution was frozen by quick immersion of the mica in liquid nitrogen. The specimen was freeze-dried at −100 °C for 30 min and gradually brought to room temperature over 30 min in a Balzers 400 freeze-etch unit, at a vacuum of 2×10^{-6} torr or higher. The molecules were rotary-shadowed at 15° with carbon-platinum and imaged in a Philips 410 electron microscope at a magnification of 75,300 and tilt angles of ±15°. Micrographs taken under eucentric conditions were used for measurements.

Materials—[^{3}H]PN200-110 and [γ-^{32}P]ATP were from Amersham Corp. and Du Pont-New England Nuclear, respectively. Electrophoretic reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Protease inhibitors and peroxidase-conjugated WGA were obtained from Sigma. Digitonin was from Fisher and Sigma and prepared as described previously (7). All other reagents were of reagent grade quality.

RESULTS

Production of A Specific Monoclonal Antibody to the 52,000-Da Subunit of the Dihydropyridine Receptor—Monoclonal antibodies to the dihydropyridine receptor were produced as described previously (5). The immunodot assay positive hybridoma supernatants were then tested on nitrocellulose transfers of SDS-polyacrylamide gels of partially purified dihydropyridine receptor and the void fraction of the WGA-Sepharose column, which has been shown to be devoid of dihydropyridine receptor activity. The monoclonal antibody $VD2_1$ was found to stain a 52,000-Da polypeptide in partially purified dihydropyridine receptor under both nonreducing and reducing conditions but was not reactive against the void fraction of WGA-Sepharose (Fig. 1). This antibody was also tested for its ability to immunoprecipitate the [^{3}H]PN200-110-labeled receptor from solubilized triads. Fig. 2*A* shows the results of an immunoprecipitation assay of mAb $VD2_1$ in comparison with previously described monoclonal antibodies to the 170,000-Da subunit of the dihydropyridine receptor (mAbs IIC12, IIF7, and IIID5), an unrelated mAb, $IXE12_1$, and WGA-Sepharose. Monoclonal antibody $VD2_1$ is also capable of immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor in a dose-dependent manner, as shown in Fig. 2*B*.

Immunoblot Analysis of the Fractions in the Purification of the Dihydropyridine Receptor—Fig. 3 shows the immunoblot staining of the various fractions from the purification of the dihydropyridine receptor. The 170,000- and 52,000-Da subunits of the dihydropyridine receptor were detected by immunoblot staining with mAb IIC12 (Fig. 3*A*) and mAb $VD2_1$ (Fig. 3*B*), respectively. The 52,000-Da component co-purified with the 170,000-Da subunit at all steps of the purification. These two proteins are present in triads and solubilized triads, absent in the void of the WGA-Sepharose column, enriched

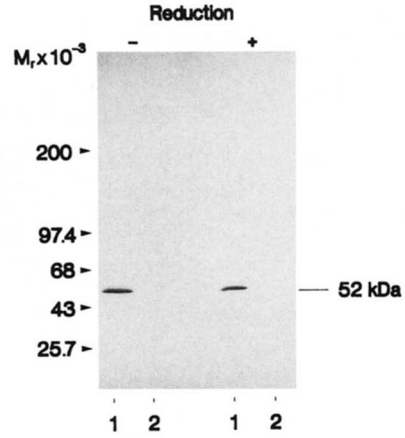

FIG. 1. **Immunoblot staining of partially purified dihydropyridine receptor and the void from WGA-Sepharose with mAb $VD2_1$.** Partially purified dihydropyridine receptor (*lane 1*) and the void from WGA-Sepharose (*lane 2*) were subjected to SDS-PAGE on a 5–16% polyacrylamide gel under reducing and nonreducing conditions and transferred to nitrocellulose as described under "Experimental Procedures." The nitrocellulose transfers were stained by the indirect immunoperoxidase method using mAb $VD2_1$.

in the peak fractions from the WGA-Sepharose column, absent in the void of the DEAE-cellulose column, and once again enriched in the peak fractions of the DEAE-cellulose column. The intensity of staining of each subunit in the various fractions also appear to parallel each other. We have also used CHAPS for the solubilization and purification of the dihydropyridine receptor from triads and have shown the presence of the 52,000-Da polypeptide in the CHAPS-purified dihydropyridine receptor by immunoblot staining with mAb $VD2_1$ (not shown).

Purification of the Phosphorylated Dihydropyridine Receptor—Triads were phosphorylated with [γ-^{32}P]ATP by its intrinsic kinase system, and the dihydropyridine receptor was purified from the phosphorylated triads (7). Fig. 4 shows the autoradiographs of ^{32}P-labeled phosphoproteins in triads (*lane 1*), the void from the WGA-Sepharose column (*lane 2*), and the purified dihydropyridine receptor (*lane 3*). The 170,000- and 52,000-Da subunits of the dihydropyridine receptor are phosphoproteins. The 170,000-Da subunit is absent in the void of the WGA-Sepharose, but a band at 52,000 Da remains in the void of the WGA-Sepharose as well as being present in triads and the purified dihydropyridine receptor.

Peptide Mapping of the 170,000- and 52,000-Da Phosphoproteins—Peptide maps of the 170,000-Da subunit, the 52,000-Da subunit, and the 52,000-Da polypeptide in the void of the WGA-Sepharose column were obtained to determine their structural relationships and to determine whether the 52,000-Da polypeptide in the void of the WGA-Sepharose column is similar to the 52,000-Da subunit of the dihydropyridine receptor. The dihydropyridine receptor purified from [γ-^{32}P]ATP-phosphorylated triads and the void fraction from the WGA-Sepharose column were resolved on a SDS-polyacrylamide gel, and the bands corresponding to the 170,000- and 52,000-Da subunits of the receptor and the 52,000-Da polypeptide in the void fraction were cut out from the gel and subjected to digestion with trypsin, *S. aureus* V8 protease, and cyanogen bromide. The proteolytic fragments were analyzed on a 16–22% SDS-polyacrylamide gel. Fig. 5 shows the distinct peptide maps of the 170,000-Da subunit (*lanes 1, 4,* and *7*), the 52,000-Da subunit (*lanes 2, 5,* and *8*), and the 52,000-Da polypeptide from the void of the WGA-Sepharose column (*lanes 3, 6,* and *9*). *Lanes 1–3* were trypsin digests,

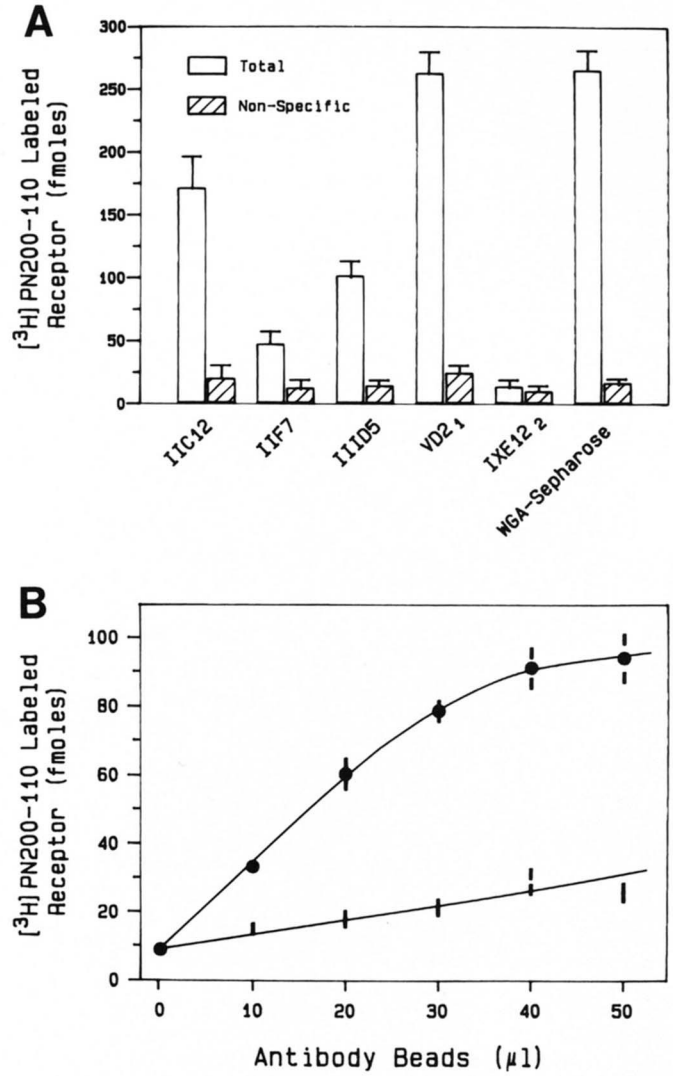

FIG. 2. **Immunoprecipitation assays of mAb $VD2_1$.** *A*, hybridoma supernatants were tested for their ability to immunoprecipitate the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads as described under "Experimental Procedures." The mAbs *IIC12, IIF7, IIID5* (described previously), and *$VD2_1$* are antibodies to the dihydropyridine receptor. *$IXE12_2$* is an unrelated antibody used as a control, and WGA-Sepharose is used as a positive control. The assay mixture contains 311 fmol of dihydropyridine binding activity as determined by a polyethylene glycol precipitation assay of the solubilized triads. The nonspecifically labeled receptor was determined in the presence of 10 μM nitrendipine. The *error bars* represent the standard error of the mean from three independent repeats of the experiments. *B*, various amounts of mAb $VD2_1$-goat anti-mouse-IgG-Sepharose were used, and the volume of the beads was kept constant with Sepharose CL-4B. The amount of specifically labeled dihydropyridine receptor is represented by the *upper curve*, and the nonspecifically labeled receptor determined in the presence of 10 μM nitrendipine is represented by the *lower curve*. The maximum amount of [^{3}H]PN200-110-labeled receptor immunoprecipitated in the experiment corresponds to 91.3% of the total dihydropyridine receptor in the assay mixture.

lanes 4–6 were *S. aureus* V8 protease digests, and *lanes 7–9* were cyanogen bromide digests. Distinct peptide maps were obtained with all three proteins in here and in other experiments (not shown), indicating that the 170,000-Da subunit, the 52,000-Da subunit, and the 52,000-Da polypeptide in the void of the WGA-Sepharose are structurally unrelated.

Immunoprecipitation of the Phosphorylated Dihydropyridine Receptor—Triads were phosphorylated, solubilized with digitonin, and then immunoprecipitated with mAb-goat anti-

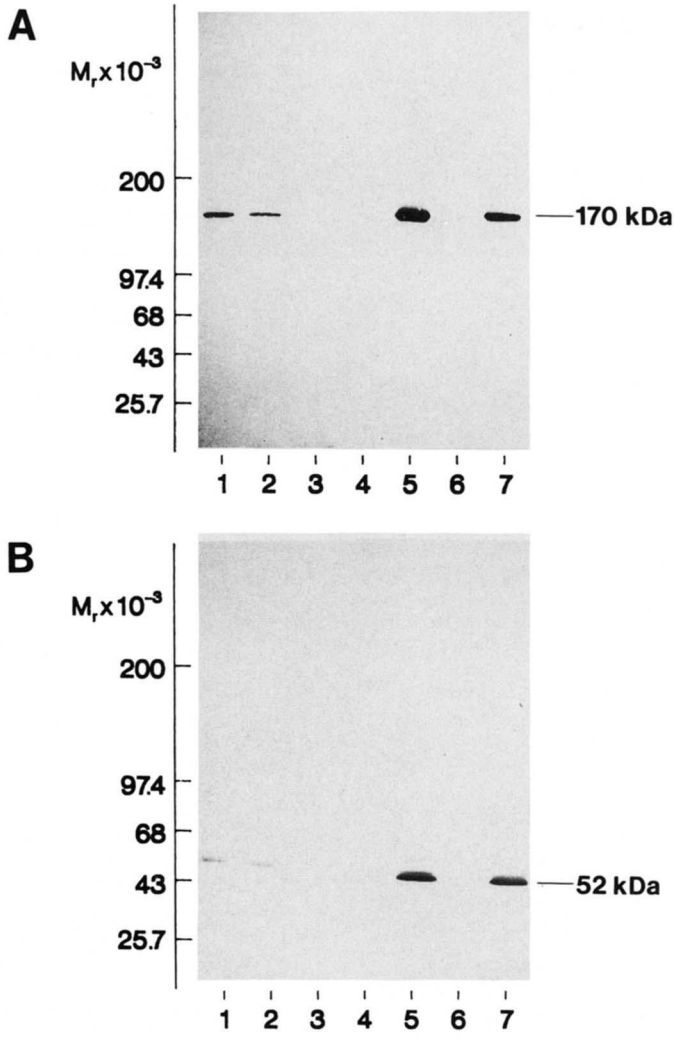

FIG. 3. **Purification of the dihydropyridine receptor from skeletal muscle triads.** The various fractions from the purification of the dihydropyridine receptor from triads were subjected to SDS-PAGE on a 5–16% gradient gel and transferred to nitrocellulose as described under "Experimental Procedures." The immunoblots were stained with mAb IIC12, anti-170,000-Da protein (*A*) and mAb $VD2_1$, anti-52,000-Da protein (*B*). The samples on the transfers are: triads, 150 μg (*lane 1*); digitonin-solubilized triads, 150 μg (*lane 2*); first void from WGA-Sepharose column, 150 μg (*lane 3*); second void from WGA-Sepharose column, 150 μg (*lane 4*); eluate from WGA-Sepharose, 10 μg (*lane 5*); void from DEAE-cellulose, 10 μg (*lane 6*); peak fractions from DEAE-cellulose, 7.5 μg (*lane 7*).

mouse-IgG-Sepharose beads. The 170,000- and 52,000-Da subunits of the dihydropyridine receptor were co-immunoprecipitated by both mAb IIC12 and mAb $VD2_1$. Fig. 6 shows that immunoprecipitation of the phosphorylated receptor with mAb IIC12, a monoclonal antibody specific to the 170,000-Da subunit of the dihydropyridine receptor, also co-immunoprecipitated the 52,000-Da subunit (*lane 2*). However, when the dihydropyridine receptor-bound mAb IIC12-goat anti-mouse-IgG-Sepharose beads were washed with a buffer containing 0.1% SDS, the 52,000-Da subunit was dissociated from the immunoprecipitated complex, while the 170,000-Da subunit remained on the beads (*lane 3*). Conversely, when mAb $VD2_1$-goat anti-mouse-IgG-Sepharose beads were used to immunoprecipitate the phosphorylated dihydropyridine receptor, both the 170,000- and 52,000-Da subunits were immunoprecipitated (*lane 4*). However, washing with buffer containing 0.1% SDS removed the 170,000-Da subunit from the immunoprecipitated complex (*lane 5*).

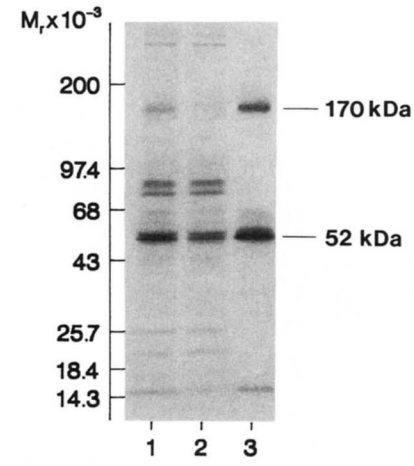

FIG. 4. **Purification of the phosphorylated dihydropyridine receptor.** The digitonin-solubilized triads (*lane 1*), the void fraction of the WGA-Sepharose column (*lane 2*), and the purified dihydropyridine receptor (*lane 3*) were subjected to SDS-PAGE on a 5–16% gradient gel under reducing conditions as described under "Experimental Procedures." The gel was then stained with Coomassie Blue, dried, and subjected to autoradiography.

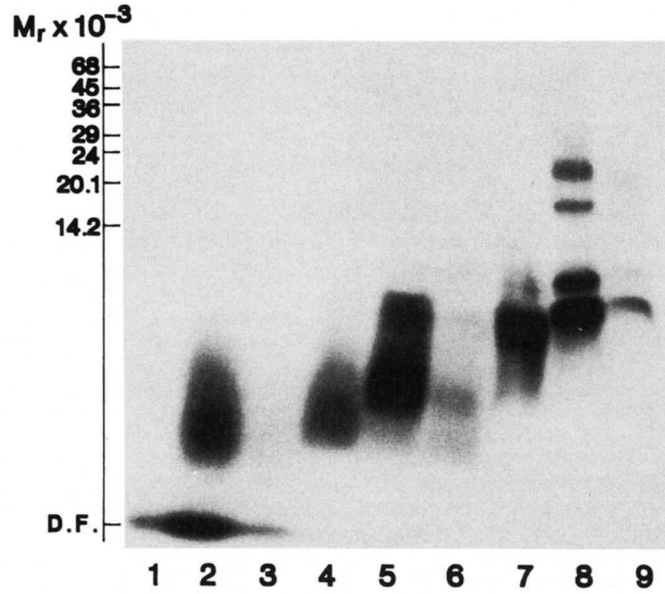

FIG. 5. **Peptide mapping of the protein components of the dihydropyridine receptor.** Dihydropyridine receptor was purified from phosphorylated triads as described under "Experimental Procedures." The purified dihydropyridine receptor and the void fraction of the WGA-Sepharose column were subjected to SDS-PAGE (5–16% gradient gel). The SDS gel was stained with Coomassie Blue, destained, and washed with distilled water. The bands corresponding to the 170,000-Da subunit (*lanes 1, 4,* and *7*), the 52,000-Da subunit (*lanes 2, 5,* and *8*), and the 52,000-Da phosphopeptide in the void fraction of the WGA-Sepharose column (*lanes 3, 6,* and *9*) were excised. The gel pieces were incubated with trypsin (*lanes 1–3*), *S. aureus* V8 protease (*lanes 4–6*), or cyanogen bromide (*lanes 7–9*) as described under "Experimental Procedures." The supernatants were subjected to SDS-PAGE (16–22% gradient gel) followed by autoradiography with intensifying screen. *D.F.*, dye front.

Determination of the Subunit Stoichiometry of the Purified Dihydropyridine Receptor—The purified dihydropyridine receptor has been shown by Coomassie Blue staining of SDS-polyacrylamide gels to contain four protein components of molecular masses 175,000, 170,000, 52,000, and 32,000 Da under nonreducing conditions and molecular masses 170,000, 150,000, 52,000, and 32,000 Da under reducing conditions

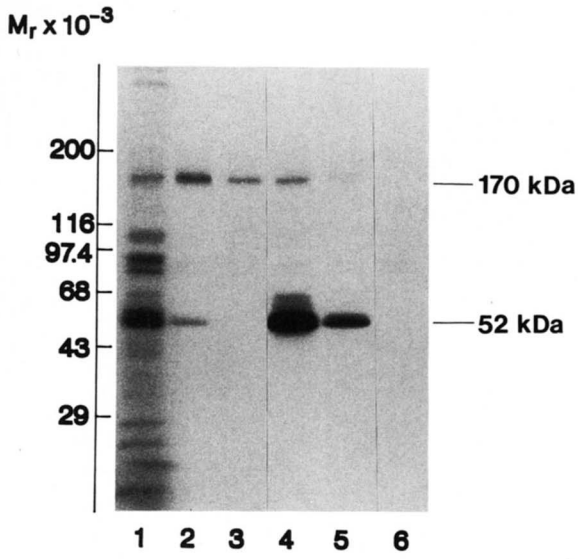

FIG. 6. **Immunoprecipitation of the phosphorylated dihydropyridine receptor from solubilized triads.** Isolated triads were phosphorylated with 50 μM [γ-^{32}P]ATP and solubilized with 1% digitonin as described under "Experimental Procedures." *Lane 1* contains 10 μg of digitonin-solubilized triads. Digitonin-solubilized triads (100 μg) were incubated with mAb-goat anti-mouse-IgG-Sepharose, mAb IIC12 (*lanes 2* and *3*), mAb VD2$_1$ (*lanes 4* and *5*), or goat anti-mouse-IgG-Sepharose (*lane 6*) at 4 °C for 4 h. The Sepharose beads were washed with 0.3% digitonin and 0.5 M NaCl in Buffer A (*lanes 2* and *4*) or 0.1% SDS in 100 mM Tris-HCl, pH 7.5, 200 mM LiCl, and 20 mM NaF (*lanes 3* and *5*) as described under "Experimental Procedures." Samples were subjected to SDS-PAGE on a 5–16% gradient gel under reducing conditions. The gel was stained with Coomassie Blue, dried, and subjected to autoradiography.

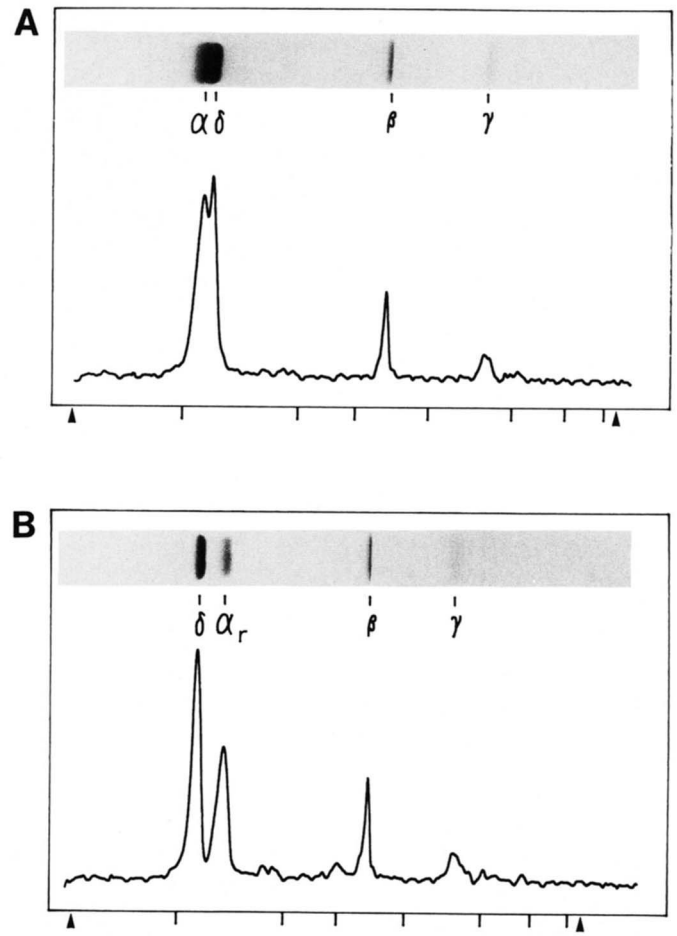

FIG. 7. **Densitometric scans of SDS-polyacrylamide gels of the purified dihydropyridine receptor.** The purified dihydropyridine receptor (10 μg/lane) was separated on a 5–16% SDS-polyacrylamide gel under nonreducing (*A*) and reducing (*B*) conditions as described under "Experimental Procedures." The gels were stained with Coomassie Blue, and following destaining the densitometric scans were obtained with a Hoefer GS-300 scanning densitometer. The top and dye front of the gels are indicated by the *left* and *right arrowheads*, respectively. The *hash marks* indicate the positions of the molecular weight standards: (from *left* to *right*) myosin (heavy chain), 200,000; phosphorylase B, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300.

(Fig. 7). Coomassie Blue-stained polyacrylamide gels of the dihydropyridine receptor purified from either digitonin- or CHAPS-solubilized triads were scanned with a densitometer to determine the relative quantities of the four subunits (Fig. 7). The absorbances of the various bands were integrated and then divided by the apparent molecular mass of the respective band to yield a relative ratio of the subunits. The purified dihydropyridine receptor from nine different preparations was analyzed, and the results are summarized in Table I. The 170,000-, 175,000/150,000-, 52,000-, and 32,000-Da proteins exhibited a stoichiometric ratio of 1.0:0.79:1.0:1.0. With silver staining, the 52,000-Da polypeptide stained rather anomalously and much lighter than the 32,000-Da protein. No difference in the stoichiometric ratio of the subunits was seen between the preparations using CHAPS and those using digitonin for the solubilization of the dihydropyridine receptor.

Electron Microscopy of the Purified Dihydropyridine Receptor—Isolated dihydropyridine receptors are globular, with a round or slightly elongated profile, depending on the position of the molecule on the mica. The more precisely round profiles have a heavier platinum shadow (*i.e.* they appear darker), indicating that they are taller. Their average diameter is 16 ± 0.9 nm (n = 89, from 10 micrographs, two samples). The elongated profiles are less heavily shadowed and have a length of up to 22 nm. We conclude that the molecule has an ovoidal shape, with short and long diameters of 16 and 22 nm. The other major observation is that the molecule is primarily composed of two components of similar size, separated by a small central gap (Fig. 8).

The shadowing can also be used to assess the purity of the preparation. The images contain a homogeneous population of profiles. However, in the background there are smaller

TABLE I

Determination of the subunit stoichiometry of the purified dihydropyridine receptor by densitometric scanning of Coomassie Blue-stained SDS-polyacrylamide gels

The purified dihydropyridine receptor from different preparations was subjected to SDS-PAGE on 5–16% gradient gels (10 μg/lane), stained with Coomassie Blue and scanned with a Hoefer Model GS-300H scanning densitometer as described under "Experimental Procedures." The data is compiled from two scans of each of nine different preparations.

Subunit	M_r	Relative intensity (n = 18)	Stoichiometric ratio
		% ± S.E.	
δ	170,000	28.1 ± 1.0	1.00
α	175,000/150,000	19.6 ± 0.8	0.79
β	52,000	8.8 ± 0.3	1.03
γ	32,000	5.5 ± 0.3	1.04

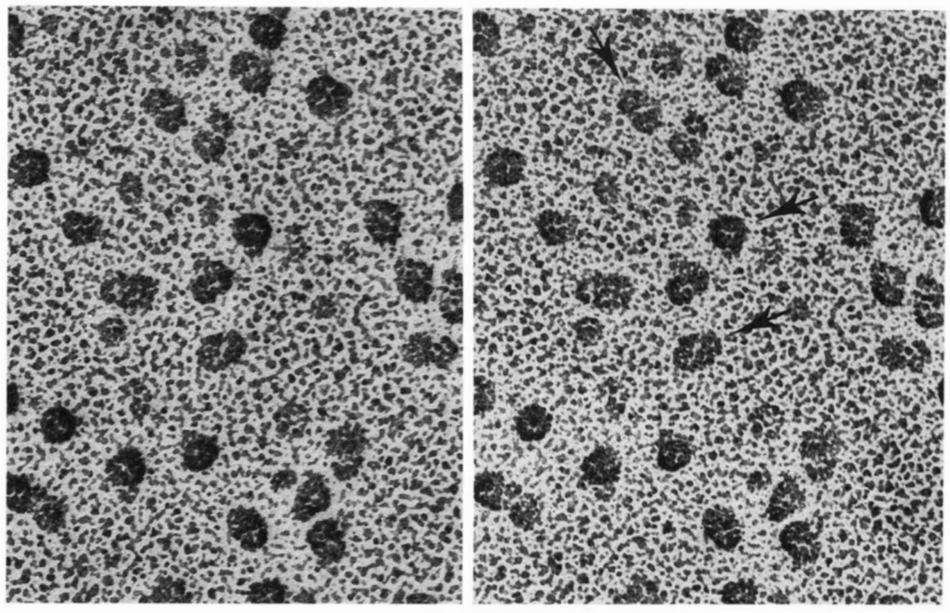

FIG. 8. **Stereomicrographs of freeze-dried, rotary-shadowed dihydropyridine receptor.** The purified dihydropyridine receptor was freeze-dried, rotary-shadowed with carbon-platinum, and imaged in an electron microscope as described under "Experimental Procedures." Note variations in shape, from round to elongated, of the globular molecule and separation into two subunits (*arrows*). A stereo viewer with a magnification of 2–3-fold should be used to fuse the micrographs. (× 300,000, tilt angle ±15°).

particles constituting approximately 20% of the total number of profiles. Most of the background particles may represent dissociated subunits of the complex.

DISCUSSION

We have recently shown that the 1,4-dihydropyridine receptor purified from digitonin-solubilized rabbit skeletal muscle triads contains four polypeptide components of 175,000, 170,000, 52,000, and 32,000 Da. In this study, we describe the ultrastructure of the purified dihydropyridine receptor and report evidence that further demonstrates the structural relationship between the 52,000-Da polypeptide and the dihydropyridine receptor.

A monoclonal antibody specific against the 52,000-Da polypeptide of the dihydropyridine receptor from rabbit skeletal muscle has been produced in our laboratory (Fig. 1). This monoclonal antibody is capable of immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads (Fig. 2). Immunoblot staining of the various fractions from the chromatographic procedures used in the purification of the dihydropyridine receptor revealed that this 52,000-Da polypeptide co-purifies with the 170,000-Da subunit throughout the entire purification process (Fig. 3), demonstrating the tight association between these two proteins. We have previously demonstrated the tight association between the 170,000-Da subunit and the 175,000-Da glycoprotein subunit. Immunoprecipitation of the 170,000-Da subunit also resulted in the co-precipitation of the 175,000-Da glycoprotein subunit (5), and when the digitonin-solubilized dihydropyridine receptor was bound to WGA-Sepharose, a buffer containing 1% SDS was required to separate the 170,000-Da subunit from the 175,000-Da glycoprotein subunit (6). Considered together, these results demonstrate the tight association between the 52,000-Da polypeptide and the 175,000/150,000-Da glycoprotein subunit and the 170,000-Da dihydropyridine-binding subunit of the dihydropyridine receptor.

When the dihydropyridine receptor was purified from triads phosphorylated with [γ-^{32}P]ATP by the intrinsic kinase, the 170,000-Da phosphoprotein subunit of the dihydropyridine receptor was absent from the void of the WGA-Sepharose column (Fig. 4). However, a 52,000-Da phosphoprotein was present in both the eluate and the void of the WGA-Sepharose (Fig. 4). The 52,000-Da polypeptides in these fractions are likely to be distinct proteins, because monoclonal antibody VD2$_1$ recognizes only the 52,000-Da polypeptide in the WGA-Sepharose eluate and not the 52,000 Da polypeptide in the void of the WGA-Sepharose. However, to further clarify the relationship between these two 52,000-Da phosphoproteins and to investigate a possible structural relationship between the 170,000- and 52,000-Da polypeptides in the purified dihydropyridine receptor, peptide mapping was performed on these proteins. The distinct peptide maps (Fig. 5) demonstrate that the 52,000- and 170,000-Da polypeptides are structurally unrelated and that the 52,000-Da polypeptide is not a proteolytic fragment of the 170,000-Da protein. The different peptide maps of the 52,000-Da polypeptide in the purified dihydropyridine receptor and in the void of the WGA-Sepharose column, along with the inability of mAb VD2$_1$ to recognize the 52,000-Da polypeptide in the void of the WGA-Sepharose on immunoblots also demonstrate the distinctiveness of these proteins.

The association of this 52,000-Da polypeptide with the dihydropyridine receptor had only been shown previously by protein staining of polyacrylamide gels of the purified dihydropyridine receptor. We have shown in this report that a monoclonal antibody to the 52,000-Da polypeptide in the purified dihydropyridine receptor is capable of immunoprecipitating dihydropyridine binding activity from digitonin-solubilized triads. Because the 170,000-Da subunit of the dihydropyridine receptor contains the dihydropyridine-binding site (6), the immunoprecipitation of dihydropyridine binding activity by an antibody specific to the 52,000-Da polypeptide demonstrates that the 52,000- and 170,000-Da polypeptides are associated in digitonin-solubilized triads. It also shows that the 52,000-Da polypeptide is not an unrelated polypeptide that co-purifies nonspecifically with the dihydropyridine receptor.

To further demonstrate the association of this 52,000-Da polypeptide with the dihydropyridine receptor in triads, monoclonal antibodies to the 170,000-Da subunit and to the 52,000-Da polypeptide were used to immunoprecipitate the phosphorylated dihydropyridine receptor. Experiments using mAb VD2$_1$ (anti-52,000-Da polypeptide) and mAb IIC12

(anti-170,000-Da subunit) to immunoprecipitate the ^{32}P-labeled receptor from solubilized triads have shown that immunoprecipitation of one phosphoprotein also co-immunoprecipitated the other phosphoprotein (Fig. 6). However, when the immunoprecipitates were washed with buffer containing 0.1% SDS, the 170,000-Da subunit and the 52,000-Da polypeptide were dissociated from each other, resulting in the immunoprecipitation of only one phosphoprotein by its specific antibody. These results demonstrate the association between the 170,000-Da subunit and the 52,000-Da polypeptide in digitonin-solubilized triads and, in addition, support the immunoblot data that the 170,000- and 52,000-Da polypeptides are distinct structural entities, each with its own epitope for the monoclonal antibodies. It could be seen that the 170,000-Da phosphoprotein band appeared more intense when the solubilized dihydropyridine receptor was immunoprecipitated by anti-170,000-Da subunit antibodies (Fig. 6), whereas the 52,000-Da phosphoprotein appeared more intense when the anti-52,000-Da protein antibody was used for the immunoprecipitation. This observation could possibly be explained by the different rates of dephosphorylation between the subunit bound to an antibody and the subunit not bound to an antibody. Alternatively, some of the oligomeric receptor complexes containing the four subunits of the dihydropyridine receptor may exist in equilibrium with monomeric subunits. A similar experiment has been reported recently by Takahashi and Catterall (21). Using a mouse polyclonal antiserum raised against the purified dihydropyridine receptor complex, they reported that immunoprecipitation of the high molecular weight subunits also resulted in the co-immunoprecipitation of the 52,000-Da polypeptide and that denaturation of the dihydropyridine receptor complex by boiling in 0.1% SDS resulted in the immunoprecipitation of the high molecular weight subunits only. However, the subunit specificity of their polyclonal antiserum has not been determined definitively, and it is not known whether the 52,000-Da polypeptide was not immunoprecipitated because the antiserum did not recognize the denatured 52,000-Da polypeptide. Furthermore, the converse experiment using antibodies to the 52,000-Da polypeptide has not been performed.

Densitometric scanning of the Coomassie Blue stained gels of the dihydropyridine receptor purified from either digitonin or CHAPS-solubilized triads provided further evidence that the 52,000-Da polypeptide is indeed an integral subunit of the dihydropyridine receptor (Fig. 7). The purified dihydropyridine receptor was analyzed on SDS-PAGE, stained with Coomassie Blue, and the density of the bands determined using a scanning densitometer. The areas under the various peaks of the density curve were normalized with respect to the apparent molecular weights of the corresponding bands. Under reducing conditions, in which the 175,000/150,000-Da glycoprotein and the 170,000-Da subunit were well resolved on the gel and the 175,000/150,000-Da glycoprotein migrated with an apparent molecular mass of 150,000, a stoichiometric ratio of 1.0:0.79:1.0:1.0 was obtained for the 150,000-, 175,000/170,000-, 52,000- and 32,000-Da proteins, respectively (Table I). The stoichiometric ratio of 1:1:1 among the 170,000-, 52,000-, and 32,000-Da polypeptides strongly suggests that the 52,000-Da polypeptide is an integral component of the dihydropyridine receptor. The anomalous ratio of 0.79:1 between the 175,000/150,000-Da glycoprotein and the other subunits of the dihydropyridine receptor can be explained by its glycoprotein nature. Barhanin *et al.* (22) have shown that the glycoprotein subunit of the dihydropyridine receptor is heavily glycosylated and that at least 20–22% of its mass could be removed by deglycosylation. Preliminary experiments in our laboratory with endoglycosidases have also indicated that carbohydrates contribute at least 25,000-Da to the apparent molecular mass of the 175,000/150,000-Da subunit. If one were to assume that the protein component of the 175,000/150,000-Da glycoprotein has a molecular mass of 120,000-Da, then the stoichiometric ratio would approach 1:1:1:1 for the four subunits of the dihydropyridine receptor.

The present study represents the first report of the subunit stoichiometry of the purified dihydropyridine receptor from skeletal muscle. Curtis and Catterall (11) have reported the densitometric scanning of silver-stained SDS-polyacrylamide gels of the purified dihydropyridine receptor and that the molar ratio of the 52,000-Da polypeptides to the dihydropyridine binding activity was less than 1:1. However, the densities of the various protein bands have not been normalized with respect to the apparent molecular masses of the corresponding bands to determine the stoichiometry. Furthermore, the 170,000-Da dihydropyridine-binding subunit was not reported by Curtis and Catterall (11), although the high molecular mass (~160,000 Da) band on the gels appeared very broad and could very possibly represent the superposition of the 175,000- and 170,000-Da bands. Flockerzi *et al.* (12) also have previously reported the density ratios of the various protein bands in a purified preparation of the dihydropyridine receptor separated by SDS-PAGE and stained by the silver method. However, a small amount of protein (100 ng/lane) was used in these experiments, and the densities had not been normalized to determine the subunit stoichiometry. We have observed that the staining of the purified dihydropyridine receptor by the silver method resulted in a highly anomalous staining pattern. The color of the 175,000/150,000-Da glycoprotein was a dark brown that was distinctly different from the dark gray color of the other bands, and the staining of the 52,000-Da polypeptide was much less intense than that of the 32,000-Da protein.

The 52,000-Da polypeptide had not been reported to copurify with the dihydropyridine receptor when certain procedures were used for the purification. Lazdunski and co-workers (13), using CHAPS to solubilize the receptor, have reported a single polypeptide of 170,000 Da under nonreducing conditions which is converted to a polypeptide of 140,000 Da and several small polypeptides upon reduction. We have shown in this report that, with our purification procedure, the substitution of digitonin with CHAPS for the solubilization of the receptor produced the same four protein components in the purified receptor, and the 52,000-Da polypeptide was present in the purified receptor as determined by staining with mAb $VD2_1$. In another procedure using freeze-thawed muscle, a single 191,000-Da polypeptide was reported (14). It is likely that the protease activity present during the overnight thawing process had destroyed the 170,000-, 52,000-, and 32,000-Da subunits observed in our preparation.

We have demonstrated in this report that the 52,000-Da polypeptide is an integral subunit of the dihydropyridine receptor from rabbit skeletal muscle and that it exists in a 1:1 stoichiometric ratio with the 170,000-, 175,000/150,000-, and 32,000-Da subunits of the purified dihydropyridine receptor. While dihydropyridines are well known as a blocker of the voltage-dependent Ca^{2+} channel, the physiological role of the dihydropyridine receptor and whether it functions as a Ca^{2+} channel in skeletal muscle remain to be elucidated. The entry of Ca^{2+} through the sarcolemma has been postulated to be unimportant for the contraction of skeletal muscle (3), and it has been shown that the number of functional Ca^{2+} channels represents less than 5% of the dihydropyridine receptors in skeletal muscle (2). It is therefore likely that the dihydropyr-

idine receptor does not function exclusively as a Ca^{2+} channel. It has been suggested that the dihydropyridine receptor acts as a voltage sensor in excitation-contraction coupling to release Ca^{2+} from the sarcoplasmic reticulum (4). This hypothesis is supported by the observation that dihydropyridines inhibit charge movement in the transverse-tubular membranes and the release of Ca^{2+} from the sarcoplasmic reticulum with a similar dose dependence (4). Our data on isolated skeletal muscle triads have shown that they are enriched in both dihydropyridine (5–7) and ryanodine binding activity (23), suggesting that the dihydropyridine receptor might be in close proximity to and capable of communicating with the ryanodine-sensitive Ca^{2+} release channel of the sarcoplasmic reticulum (24).

Further characterization of the receptor comes from structural information demonstrating that the components of the purified dihydropyridine receptor exist as a single complex. The two subunits of the complex may represent the two larger components in association with the two smaller polypeptides. Our data are consistent with the hydrodynamic studies of the cardiac dihydropyridine receptor reported recently by Horne et al. (25), in which the dihydropyridine receptor was reported to be a large ellipsoidal transmembrane protein with a molecular weight of ~370,000.

The dihydropyridine receptor is a major component of the transverse tubular membrane. We propose that the receptor is represented by a distinctive set of intramembranous particles which are only found on the junctional surfaces of transverse tubules and plasmalemma in freeze-fracture images (26, 27). These particles are clustered in regular arrays with a spacing that is directly related to that of the junctional feet (28). This morphological correspondence between the particles (possibly the dihydropyridine receptor) and the junctional feet strengthens the hypothesis of a direct interaction between transverse tubular and sarcoplasmic reticulum junctional proteins.

Acknowledgments—We acknowledge the expert technical assistance of Steven D. Kahl, Linda K. Madson, Mitchell G. Gaver, and Denah Appelt of our laboratories and Charles Lovig and Douglas Purtle of the University of Iowa Cancer Center Hybridoma Tissue Culture Facilities. We also wish to thank Alan H. Sharp for helpful discussions and Dr. Alexander Scriabine of Miles Laboratories for the nitrendipine.

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