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Structural Characterization of the 1,4-Dihydropyridine Receptor of the Voltage-dependent Ca^{2+} Channel from Rabbit Skeletal Muscle

EVIDENCE FOR TWO DISTINCT HIGH MOLECULAR WEIGHT SUBUNITS*

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The 1,4-dihydropyridine receptor purified from rabbit skeletal muscle triads was shown to contain four protein components of 175,000, 170,000, 52,000, and 32,000 Da when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. Monoclonal antibodies capable of specifically immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads recognized the 170,000-Da protein on nitrocellulose transfers of skeletal muscle triads, transverse tubular membranes, and purified dihydropyridine receptor. Wheat germ agglutinin peroxidase stained the 175,000-Da protein on similar nitrocellulose transfers, demonstrating that the 175,000-Da protein is the glycoprotein subunit of the purified dihydropyridine receptor. The apparent molecular weight of the M_r 170,000 protein remained unchanged with reduction, whereas the apparent molecular weight of the glycoprotein subunit shifted from 175,000 to 150,000 upon reduction. These results demonstrate that the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle contains two distinct high molecular weight subunits of 175,000 and 170,000.

Voltage-dependent Ca^{2+} channels are known to exist in cardiac, skeletal, and smooth muscle cells as well as excitable and secretory cells (1, 2). 1,4-Dihydropyridines are potent blockers of the voltage-dependent Ca^{2+} channel (3). The 1,4-dihydropyridine receptor has been found to be highly enriched in the transverse tubular membranes of skeletal muscle (4). Although the dihydropyridine receptor has been purified from transverse tubular membranes of rabbit skeletal muscle (5-7), its subunit composition remains to be elucidated completely. Curtis and Catterall (5) have proposed that it consists of three polypeptides of 160,000, 50,000, and 32,000 Da and that, under reducing conditions, the apparent molecular weight of the M_r 160,000 subunit shifted to 130,000. Borsotto *et al.* (6) have identified three putative subunits of 142,000, 33,000, and 32,000 Da in their preparation of the dihydropyridine receptor. Furthermore, they have shown by immunoblotting with polyclonal antibodies that the 142,000- and 32,000-Da subunits are produced by the reduction of a 170,000-Da polypeptide (8). Recently, Flockerzi *et al.* (7), using a modification of the procedure of Curtis and Catterall (5), have proposed that the dihydropyridine receptor contains three subunits of 142,000, 56,000, and 31,000 Da.

We have purified the 1,4-dihydropyridine receptor from rabbit skeletal muscle triads and have produced monoclonal antibodies that are capable of specifically immunoprecipitating dihydropyridine receptor labeled with [^{3}H]PN200-110,[1] a high affinity ligand for the dihydropyridine receptor with a K_d of 0.7 nM (9). In this report, we describe the identification and characterization of two distinct high molecular weight subunits of the 1,4-dihydropyridine receptor from rabbit skeletal muscle using these monoclonal antibodies and wheat germ agglutinin peroxidase.

EXPERIMENTAL PROCEDURES

Purification of Dihydropyridine Receptor from Skeletal Muscle Triads—Rabbit skeletal muscle triads (10) were labeled with [^{3}H]PN200-110 (Amersham Corp.) and solubilized with 1% digitonin in 0.5 M NaCl, 50 mM Tris-HCl (pH 7.4) (9). The [^{3}H]PN200-110-labeled receptor was purified from digitonin-solubilized triads using WGA-Sepharose affinity chromatography and DEAE-cellulose ion exchange chromatography by a modification of the procedure used by Curtis and Catterall on transverse tubular membranes (5). The partially purified dihydropyridine receptor from the initial WGA-Sepharose affinity column was referred to as the "GlcNAc-eluted dihydropyridine receptor" or "GlcNAc eluate." The purified dihydropyridine receptor was analyzed by SDS-PAGE (11) with Coomassie Blue staining and immunoblotting. The purified receptor and isolated membranes were run under reducing conditions with the inclusion of 10 mM dithiothreitol in the SDS sample buffer and nonreducing conditions with the inclusion of 20 mM *N*-ethylmaleimide.

Preparation of Anti-dihydropyridine Receptor Monoclonal Antibodies—Five to 6-week-old female BALB/c mice were immunized intraperitoneally with 0.5 mg of rabbit skeletal muscle triads emulsified in Freund's complete adjuvant. After 4 weeks, the immunization was repeated three or four times at 2-week intervals with the same amount of triads in Freund's incomplete adjuvant. During the week prior to fusion, two intraperitoneal injections of GlcNAc-eluted dihydropyridine receptor (30 μg) were given followed by an intravenous injection of 40 μg of purified dihydropyridine receptor 2 days before fusion. Spleen cells from the mice were fused with NS-1 myeloma cells (12). Hybrid cells were grown and passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Immunoassays—Hybridoma supernatants were screened by an immunodot assay (13) against light sarcoplasmic reticulum vesicles (14), skeletal muscle triads, GlcNAc eluate, and the void fraction from the WGA-Sepharose column. The immunodot assay positive monoclonal antibodies were further screened for their ability to immunoprecipitate the [^{3}H]PN200-110-labeled, digitonin-solubilized receptor as described in the legend to Table I. The epitope of the anti-dihydropyr-

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[1] The abbreviations used are: [^{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; WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mAb, monoclonal antibody; GAM-IgG beads, goat anti-mouse IgG-Sepharose beads.

idine receptor monoclonal antibodies was determined by indirect immunoperoxidase staining of nitrocellulose transfers of skeletal muscle membranes and the purified receptor. Similar transfers were stained with WGA peroxidase to identify the WGA-positive glycoproteins.

RESULTS AND DISCUSSION

Monoclonal antibodies against the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle were produced by immunizing mice initially with rabbit skeletal muscle triads followed by booster immunizations with purified dihydropyridine receptor. An immunodot assay was used for the initial screening of the hybridoma supernatants (Fig. 1). A hybridoma supernatant was considered positive in the immunodot assay if it reacted with triads or the GlcNAc-eluted dihydropyridine receptor but showed no reactivity with light sarcoplasmic reticulum vesicles and the void fraction from the WGA-Sepharose column, two preparations that are devoid of the dihydropyridine receptor. The serum from the immunized mouse used for the fusion was used as a control in each screening and was shown to be strongly reactive against all the antigens.

The criterion for the specificity of an immunodot assay positive antibody toward the dihydropyridine receptor was its ability to immunoprecipitate the [^{3}H]PN200-110-labeled receptor from digitonin-solubilized triads. Monoclonal antibodies from hybridoma supernatants were preincubated with goat anti-mouse IgG-Sepharose beads (GAM-IgG beads) to form mAb-GAM-IgG beads. These monoclonal antibody beads were then tested in an immunoprecipitation assay. The radioactivity on the beads was counted to determine directly the amount of [^{3}H]PN200-110-labeled dihydropyridine receptor bound by the antibody (Table I). Three antibodies (IIF7, IIC12, and IIID5) were found to immunoprecipitate the [^{3}H]PN200-110-labeled dihydropyridine receptor from the assay mixture. The validity of the assay was established by three types of controls. 1) WGA-Sepharose was able to remove 98.1 ± 10.8% of the [^{3}H]PN200-110-labeled dihydropyridine receptor from the assay mixture, showing that the solubilized receptor had retained its dihydropyridine binding activity under the conditions of the assay. 2) Goat anti-mouse IgG-

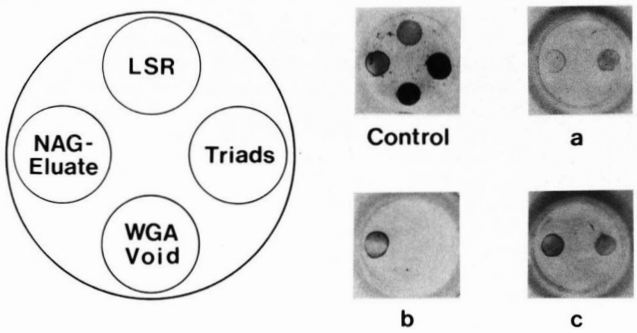

FIG. 1. **Immunodot assay for anti-dihydropyridine receptor monoclonal antibodies.** Light sarcoplasmic reticulum vesicles (*LSR*), rabbit skeletal muscle triads (*Triads*), the void of the WGA-Sepharose column after incubation with digitonin-solubilized triads (*WGA Void*), and dihydropyridine receptor eluated from the WGA-Sepharose column with *N*-acetylglucosamine (*NAG-Eluate*) were dotted (~0.5 μl) onto the nitrocellulose at the four quadrants of each well of a Millititer plate (Millipore) as diagramed and allowed to dry. Specific [^{3}H]PN200-110 binding activity for the preparations is light sarcoplasmic reticulum, 0.5 fmol/μl; rabbit skeletal muscle triads, 21.7 fmol/μl; WGA void, 0.1 fmol/μl; and GlcNAc eluate, 21.8 fmol/μl. The plates were blocked with 3% bovine serum albumin (BSA)-Tris-buffered saline (TBS) (20 mM Tris-HCl, 200 mM NaCl, pH 7.5) and allowed to react with hybridoma supernatants. A peroxidase-conjugated goat anti-mouse IgG secondary antibody (Cooper Biomedical) at 1:1000 dilution in 3% BSA-TBS was used and the plates were developed using 4-chloro-1-naphthol as the substrate. *Control* was the serum from an immunized mouse used for the fusion, diluted 1:500 in 3% BSA-TBS. *a*, *b*, and *c* are the results for the immunodot assay using 50 μl of the positive hybridoma supernatants IIC12, IIF7, and IIID5, respectively.

TABLE I

Immunoprecipitation of [^{3}H]PN200-110-labeled receptor using various monoclonal antibody beads

Monoclonal antibody beads were prepared by incubating 15 bed volumes of hybridoma supernatants with goat anti-mouse IgG-Sepharose (Cooper Biomedical, diluted to an IgG binding capacity of 1 mg/ml with Sepharose CL-4B) to form mAb-GAM-IgG beads. Triads were labeled with 10 nM [^{3}H]PN200-110 and solubilized with 1% digitonin in 0.5 M NaCl and 50 mM Tris-HCl (pH 7.4). The solubilized membranes were then diluted 1:10 with 50 mM Tris-HCl (pH 7.4). Five hundred μl of this mixture was incubated with 50 μl of mAb-GAM-IgG-Sepharose at 4 °C for 2 h with gentle mixing. The mixture was then centrifuged in an Eppendorf centrifuge and the supernatants were removed. The beads were washed twice with 1 ml of buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.1% digitonin and then counted in a liquid scintillation counter.

	[^{3}H]PN200-110-labeled receptor
	fmol ± S.E.
Monoclonal antibody beads	
mAb IIC12-GAM-IgG beads	63.8 ± 8.8
mAb IIF7-GAM-IgG beads	52.9 ± 1.7
mAb IIID5-GAM-IgG beads	48.8 ± 1.6
WGA-Sepharose	106.0 ± 6.8
Controls	
GAM-IgG beads	2.1 ± 0.3
GAM-IgG beads preincubated with RPMI 1640 medium	3.8 ± 0.8
mAb IIID5GAM-IgG beads incubated with [^{3}H]PN200-110 in the absence of solubilized triads	3.4 ± 0.8
GAM-IgG beads preincubated with preimmune serum	6.7 ± 2.0
GAM-IgG beads preincubated with immunized mouse serum	17.6 ± 1.2

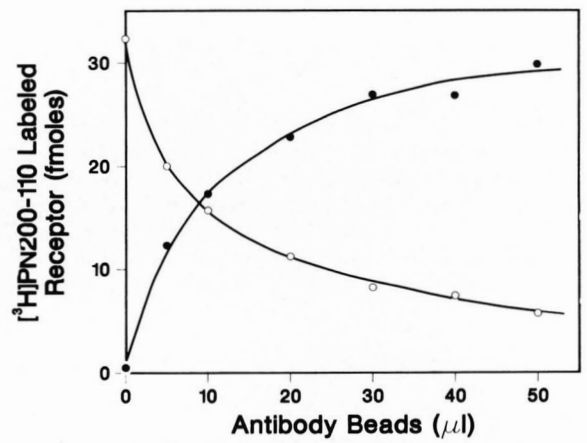

FIG. 2. **Immunoprecipitation of [^{3}H]PN200-110-labeled dihydropyridine receptor.** Immunoprecipitation assays were carried out as described in the legend to Table I except that various amounts of mAb IIC12-GAM-IgG-Sepharose were used and the volume of the beads was kept constant with Sepharose CL-4B. The amount of [^{3}H]PN200-110-labeled receptor bound to the beads is represented by ●. The amount remaining in the supernatant was determined by a polyethylene glycol precipitation assay (9) and is represented by ○. The maximum amount of [^{3}H]PN200-110-labeled receptor immunoprecipitated in this experiment corresponds to 29.8 ± 1.9 fmol (83.9 ± 5.2%). This correlates with the amount remaining in the supernatant of 5.7 ± 0.5 fmol (16.1 ± 1.4%). The immunoprecipitation of nonspecific binding activity determined in the presence of 10 μM nitrendipine was less than 6% of the total binding.

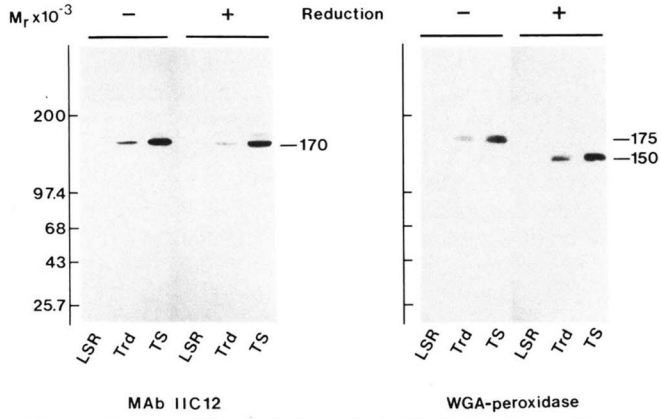

FIG. 3. **Immunoblot staining of rabbit skeletal muscle membrane fractions.** Light sarcoplasmic reticulum (*LSR*), skeletal muscle triads (*Trd*), and transverse tubular membranes (*TS*) (15) (30–50 μg) were separated on SDS-PAGE (11) under nonreducing and reducing conditions and transferred to nitrocellulose membranes using a modification of the procedure of Towbin *et al.* (16). Five percent nonfat dry milk in 50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4 (17), was used for blocking of the nitrocellulose transfers and dilution of the antibodies. Nitrocellulose transfers were first incubated with hybridoma supernatants (1:10 or 1:20 dilution) and then with peroxidase-conjugated goat anti-mouse IgG secondary antibody (Cooper Biomedical, 1:1000 dilution). WGA peroxidase (Sigma) was used to stain WGA-positive glycoproteins on nitrocellulose blots. The nitrocellulose blots were blocked with 0.05% Tween in phosphate-buffered saline (50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4) and incubated with WGA peroxidase (1:2000) in 0.05% Tween in phosphate-buffered saline. The color was developed in both cases using 4-chloro-1-naphthol as the substrate. The *left panel* shows nitrocellulose transfers of the gel stained with the anti-dihydropyridine receptor monoclonal antibody IIC12. The *right panel* shows nitrocellulose transfers of the gel stained with WGA peroxidase.

Sepharose, by itself or preincubated with RPMI 1640 medium supplemented with 10% fetal bovine serum or with preimmune serum, and monoclonal antibody beads incubated with [^{3}H]PN200-110 in the absence of solubilized triads all failed to immunoprecipitate [^{3}H]PN200-110 binding activity beyond a background level. 3) The serum from an immunized mouse was also shown to contain antibodies capable of immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor.

The anti-dihydropyridine receptor antibodies were shown to quantitatively remove the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads in a dose-dependent manner (Fig. 2). In addition, a close inverse correlation was found between the amount of [^{3}H]PN200-110-labeled dihydropyridine receptor immunoprecipitated by the antibody and the amount of [^{3}H]PN200-110-labeled dihydropyridine receptor remaining in the supernatant of the mAb-GAM-IgG beads (Fig. 2). The maximum amount of [^{3}H]PN200-110-labeled dihydropyridine receptor immunoprecipitated by the anti-dihydropyridine receptor monoclonal antibodies ranged from 80 to 95% of the total amount present in the assay mixture, depending upon the preparation of triads and antibody used. Less than 6% of the total [^{3}H]PN200-110 binding activity in the assay mixture was immunoprecipitated by the mAb-GAM-IgG beads when immunoprecipitation experiments were performed using triads prelabeled with [^{3}H]PN200-110 in the presence of 10 μM nitrendipine (not shown). Thus, our results demonstrate that the anti-dihydropyridine receptor monoclonal antibodies are capable of quantitatively and specifically immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads.

The immunoreactive molecular component of the skeletal muscle dihydropyridine receptor was identified and characterized by immunoblot assays using the anti-dihydropyridine receptor monoclonal antibodies. As shown in Fig. 3, monoclonal antibody IIC12 stained a single protein of M_r 170,000 on nitrocellulose transfers of isolated triads and transverse tubular membranes separated on SDS-PAGE under both reduc-

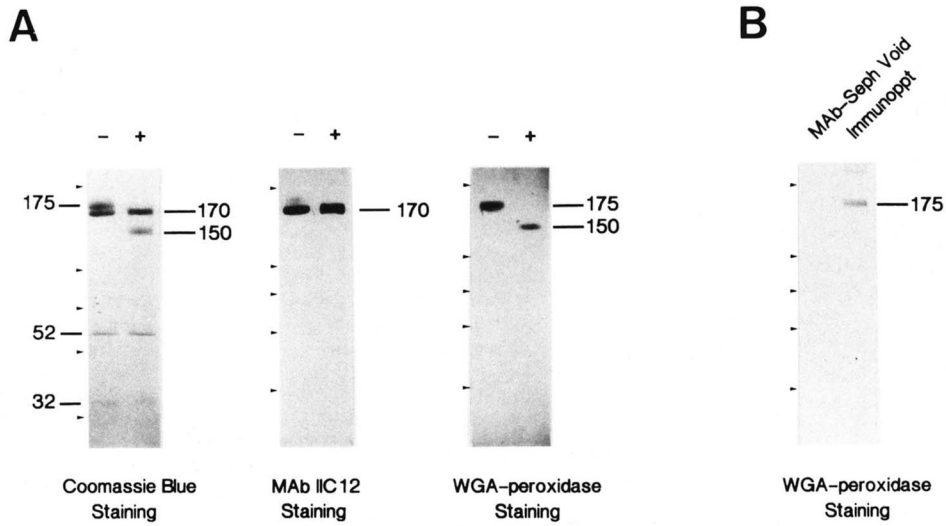

FIG. 4. **Immunoblot staining of the purified dihydropyridine receptor and immunoprecipitates from rabbit skeletal muscle triads.** In *A*, the purified dihydropyridine receptor (20 μg per lane) was subjected to SDS-PAGE on a 5–16% gradient gel under nonreducing (−) and reducing (+) conditions. This *panel* shows the Coomassie Blue stained gel and nitrocellulose transfers of similar gels stained with anti-dihydropyridine receptor mAb IIC12 and WGA peroxidase, respectively, as described in the legend to Fig. 3. In *B*, the void from the anti-dihydropyridine receptor mAb IIF7-Sepharose column after immunoprecipitation of the dihydropyridine receptor (*MAb-Seph Void*) and the immunoprecipitated dihydropyridine receptor (*Immunoppt*) were separated on a 5–16% gradient gel under nonreducing conditions. This *panel* shows a nitrocellulose transfer of the gel stained with WGA peroxidase as described in the legend to Fig. 3. The apparent molecular weights of the subunits of the dihydropyridine receptor are given as $M_r \times 10^{-3}$. The location of the molecular weight standards are marked by the *arrowheads* and their M_r values are, from top to bottom, 200,000; 97,400; 68,000; 43,000; and 25,700.

ing and nonreducing conditions. Identical results were obtained for monoclonal antibodies IIID5 and IIF7. When similar nitrocellulose transfers were stained for WGA-positive glycoproteins with WGA peroxidase, a single band of M_r 175,000 was stained in isolated triads and transverse tubular membranes (Fig. 3). The molecular weight of the M_r 175,000 WGA-positive glycoprotein shifted to 150,000 under reducing conditions. The 170,000-Da protein and the 175,000-Da glycoprotein were not detected in light sarcoplasmic reticulum membranes, a preparation devoid of dihydropyridine receptor.

We have found skeletal triads to be the best starting material for the purification of the 1,4-dihydropyridine receptor for the following reasons: 1) skeletal triads are enriched in [^{3}H]PN200-110 binding activity (10–40 pmol/mg), 2) skeletal triads contain only one WGA-positive protein (Fig. 3), and 3) the yield of skeletal triads ranges from 600 to 1,000 mg/kg of tissue as compared to 10–30 mg/kg for transverse tubular membranes, resulting in a high yield of purified dihydropyridine receptor with a specific activity that was comparable to the previously reported values (5, 6).

Our preparation of purified dihydropyridine receptor was characterized by SDS-PAGE with Coomassie Blue staining and immunoblotting (Fig. 4*A*). The purified receptor was shown to contain four proteins of M_r 175,000, 170,000, 52,000, and 32,000 when analyzed on a 5–16% polyacrylamide gel under nonreducing conditions (Fig. 4*A*). Upon reduction, however, the molecular weight of the M_r 175,000 protein shifted to 150,000. The M_r 170,000 protein was the only protein stained by the anti-dihydropyridine receptor monoclonal antibodies and its molecular weight remained unchanged with reduction (Fig. 4*A*). The M_r 175,000 glycoprotein, on the other hand, was the only protein stained by WGA peroxidase and its molecular weight shifted to 150,000 upon reduction (Fig. 4*A*). The electrophoretic mobility of the 175,000-Da glycoprotein was also very sensitive to the concentration of acrylamide in the gel (*i.e.* on a 3–12% gradient gel the 175,000-Da glycoprotein ran with a mobility similar to the 170,000-Da protein). The results of experiments with endoglycosidase H and concanavalin A peroxidase have further demonstrated that the 175,000-Da subunit is a glycoprotein and the 170,000-Da subunit is not a glycoprotein (not shown). The 52,000 and 32,000-Da subunits were not stained by the anti-dihydropyridine receptor monoclonal antibodies (IIC12, IIF7, and IIID5) or WGA peroxidase (Fig. 4*A*).

The 170,000-Da protein and the 175,000-Da glycoprotein co-purified with the [^{3}H]PN200-110-labeled dihydropyridine receptor at all stages of the purification. The void fraction of the WGA-Sepharose column, which was depleted of dihydropyridine receptor, did not contain the 170,000-Da protein or the 175,000-Da glycoprotein. Since the 170,000-Da protein does not bind WGA, these results demonstrate that the 170,000-Da protein is retained by the WGA-Sepharose column because it is tightly associated with the 175,000-Da glycoprotein. The 170,000-Da protein and the 175,000-Da glycoprotein were also found to elute from DEAE-cellulose in the same single peak as that of the dihydropyridine binding activity. Analogously, when the immunoprecipitated dihydropyridine receptor and the void of the mAb-Sepharose beads were separated on SDS-PAGE, transferred to nitrocellulose, and stained with WGA peroxidase (Fig. 4*B*), the 175,000-Da glycoprotein was the only WGA-positive protein in the immunoprecipitate, and the void of the mAb-Sepharose did not contain the 175,000-Da glycoprotein. Since the anti-dihydropyridine receptor monoclonal antibodies bind only the 170,000-Da protein, the co-immunoprecipitation of the WGA-positive glycoprotein from digitonin-solubilized triads by the antibodies and its concomitant absence in the void also demonstrate that both high molecular weight proteins are tightly associated. Thus, the results of these experiments demonstrate that both high molecular weight proteins are subunits of the dihydropyridine receptor.

The 170,000- and the 175,000-Da subunits of the dihydropyridine receptor are distinct in several major respects: 1) the 175,000-Da subunit is a glycoprotein, whereas the 170,000-Da subunit is not a glycoprotein; 2) the molecular weight of the M_r 175,000 subunit shifts to 150,000 upon reduction, whereas the molecular weight of the M_r 170,000 subunit remains unchanged with reduction; 3) monoclonal antibodies against the 170,000-Da subunit do not recognize the 175,000-Da subunit; 4) only the 170,000-Da subunit and not the 175,000-Da subunit is phosphorylated in isolated triads[2]; and 5) the 170,000-Da subunit and not the 175,000-Da subunit is photoaffinity-labeled by dihydropyridine ligands.[3]

In conclusion, our data demonstrate that the purified dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle contains two distinct high molecular weight subunits—an M_r 170,000 protein and an M_r 175,000 glycoprotein.

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