Monoclonal Antibody Characterization of the 1,4-Dihydropyridine Receptor of Rabbit Skeletal Muscle

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The 1,4-dihydropyridine receptor (DHPR) of the voltage-dependent Ca^{2+} channel has been purified from transverse tubular membranes of skeletal muscle.[1,2] Curtis and Catterall have shown that it consists of three polypeptides of 160,000 daltons, 50,000 Da, and 32,000 Da and that under reducing conditions the apparent molecular weight of the 160,000 Da subunit shifted to 130,000.[1] Borsotto *et al.* have identified three polypeptides of 142,000 Da, 33,000 Da, and 32,000 Da in their preparation of the dihydropyridine receptor.[2] Furthermore, they have shown by immunoblotting with polyclonal antibodies that the 142,000 Da and 32,000 Da subunits are produced by the reduction of a 170,000 Da polypeptide.[3] We report here the identification and characterization with monoclonal antibodies (MAb) of an additional high molecular weight subunit of the DHPR which is distinct from that described by Curtis and Catterrall[1] and Borsotto *et al.*[3]

Monoclonal antibodies capable of specifically immunoprecipitating the [^{3}H]PN200-110-labeled DHPR of rabbit skeletal muscle were produced by immunizing BALB/c mice initially with skeletal muscle triad vesicles followed by booster immunizations with purified DHPR from skeletal muscle triads. Hybridoma supernatants were screened for the production of anti-DHPR antibodies with an immunodot assay. Supernatants that reacted positively against the partially purified DHPR were then tested for their ability to immunoprecipitate the [^{3}H]PN200-110-labeled receptor from digitonin-solubilized triads (see TABLE 1).

All three MAbs (IIC12, IIF7, IIID5) that were capable of immunoprecipitating the DHPR recognized a protein with an M_r of 170,000 on nitrocellulose transfers of skeletal muscle triads and transverse tubular membranes separated on sodium dodecyi sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (FIGURE la). Wheat-germ agglutinin (WGA) peroxidase stained a 175,000 Da protein on similar nitrocellulose transfers. Neither the 170,000 Da polypeptide nor the 175,000 Da polypeptide was detected in light sarcoplasmic reticulum vesicles, a preparation devoid of DHPR. Under reducing conditions, the M_r of the 170,000 Da

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polypeptide on SDS-PAGE remained unchanged whereas the M_r of the glycoprotein shifted from 175,000 to 150,000.

The DHPR was purified from skeletal muscle triads using a modification of the procedure according to Curtis and Catterall.[1] The purified DHPR was shown to consist of four subunits of M_r 175,000, 170,000, 50,000, and 30.000 on a 5-16% polyacryl. amide gel under nonreducing conditions (FIGURE 1b). Under reducing conditions, the M_r of the 175,000 Da subunit shifted to 150,000. On nitrocellulose transfers, the 170,000 Da subunit was stained by the anti-DHPR MAbs and its M_r did not change with reduction. The 175,000 Da subunit was stained by WGA-peroxidase and the M_r

TABLE 1. Immunoprecipitation of [^{3}H]PN200-110-Labeled Receptor Using Various Monoclonal Antibody Beads

	Amount of [^{3}H]PN200-110-Labeled Receptor (fmol)[a]
Controls	
GAM-IgG beads	2.1 ± 0.3
GAM-IgG beads preincubated with RPMI-1640	3.8 ± 0.8
MAb IIID5-GAM-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
WGA-Sepharose	106.0 ± 6.8
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

[a]Monoclonal antibody beads were prepared by incubating 15 bed volumes of hybridoma supernatants with goat-anti-mouse IgG Sepharose (Cooper, diluted to an IgG binding capacity of 1 ing/ml with Sepharose CL 4B) to form MAb-GAM-IgG beads. Triad vesicles 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 were incubated with 50 μl of MAb-GAM-IgG Sepharose at 4°C for 2 hours with gentle mixing. The mixture was then centrifuged in an Eppendorf centrifuge, and the supernatants were removed and assayed for dihydropyridine receptor activity using the PEG precipitation assay. 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 scintillation counter.

of this glycoprotein shifted to 150,000 Da upon reduction. These results are consistent with the results of anti-DHPR MAb and WGA-peroxidase staining of immunoblots of skeletal muscle transverse tubular membranes and triads. Results from our laboratory have further demonstrated that the M_r 170,000 polypeptide and not the M_r 175,000 glycoprotein is a phosphoprotein[4] and binds dihydropyridines.[5]

In summary, our 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, a glycoprotein of M_r 175,000 and a polypeptide of M_r 170,000.

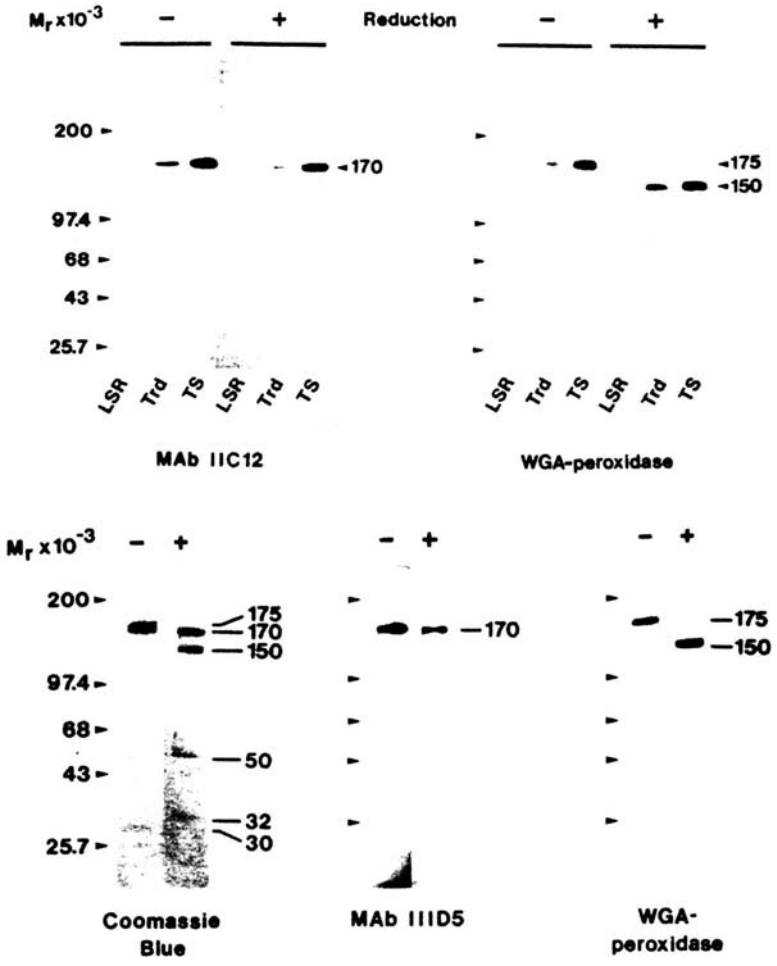

FIGURE 1. Coomassie Blue staining and immunoblot staining of rabbit skeletal muscle membrane fractions and purified dihydropyridine receptor. Light sarcoplasmic reticulum (LSR) triads (Trd), and transverse tubular (TS) membrane (30-50 μg) or the purified dihydropyridine receptor 20 μg) was subjected to SDS-PAGE on a 3-12% polyacrylamide gradient gel under nonreducing (+ 20 mN *N*-ethylmaleimide) and reducing (+10 mM dithiotleitol) conditions and stained with Coomassie Blue or transferred to nitrocellulose membranes. BLOTTO-Bovine Lacto Transfer Technique Optimizer (50 mM NaH_2PO_4, 0.9% NaCl, 5% nonfat dry milk, pH 7.4)–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 immunoglobulin G (IgG) secondary antibody (Cooper, 1:1000 dilution). WGA-peroxidase (Sigma) was used to stain WGA-positive glycoproteins on nitrocellulose transfers. The nitrocellulose transfers were blocked with 0.05% Tween-PBS (50mM NaH_2PO_4, 0.9% NaCl, pH 7.4) and incubated with WGA-peroxidase (1:2000) in 0.05% Tween-PBS. The color was developed in both cases using 4-chloro-1-naphthol as the substrate. (a-top) The left panel shows nitrocelluose transfers of the gel stained with monoclonal antibody IIC12. The right panel shows nitrocellulose transfers of the gel stained with WGA-peroxidase. (b-bottom) The purified dihydropyridine receptor was subjected to SDS-PAGE on a 5-16% polyacrylamide gradient gel. The left panel is a photograph of a Coomassie Blue stained gel. The center panel shows a nitrocellulose transfer of the gel stained with monoclonal antibody IIID5, illustrating the insensitivity of the relative mobility of the HO W Da subunit to reduction. The right panel shows a nitrocellulose transfer of the gel stained with WGA-peroxidase, illustrating the shift of the relative mobility of the 175,000 Da subunit to 150,000 Da upon reduction.

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