Ca^{2+} Channel Antibodies: Subunit-Specific Antibodies as Probes for Structure and Function

K.P. Campbell, A.T. Leung, A.H. Sharp, T. Imagawa, and S.D. Kahl

Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA

Introduction

Voltage-dependent Ca^{2+} channels are known to exist in cardiac, skeletal, and smooth muscle cells as well as in neuronal and secretory cells [1,2]. 1,4-Dihydropyridines are potent blockers of voltage-dependent Ca^{2+} channels [3], and the receptor for 1,4-dihydropyridines has been found to be highly enriched in the transverse tubular system of skeletal muscle [4]. Curtis and Catterall [5] were the first to purify the dihydropyridine receptor from rabbit skeletal muscle T-system membranes. Analysis of their preparation of receptor by sodium dodecyi sulfate polyacrylamide gel electrophoresis (SDS-PAGE) suggested that the dihydropyridine receptor consisted of three subunits: an α subunit of 160000 Da, a β subunit of 50 000 Da, and a γ subunit of 32000 Da. The apparent molecular weight of the a subunit in their preparation shifted from 160000 to 130000 upon reduction, whereas the molecular weight of the β and γ subunits did not change upon reduction. The dihydropyridine receptor has also been purified from skeletal muscle membranes by Borsotto et al. [6] and Flockerzietal. [7]. These groups also identified three subunits in their preparations of dihydropyridine receptor but the exact composition of subunits and molecular weight of the subunits differ from the original report of Curtis and Catterall [5]. Our laboratory has shown that the purified 1,4-dihydropyridine receptor from rabbit skeletal muscle triads contains four protein components of 175 000 Da (α_2), 170 000 Da (α_1), 52000 Da (β) and 32000 Da (γ) and that the 170000 Da and 175000 Da components are distinct polypeptides [8]. The 170000 Da polypeptide (α 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 [9,10], and the 170000 Da (α_1 subunit) polypeptide and 52000 Da polypeptide (β subunit) have been shown to be substrates for various protein kinases [11-15]. Finally, the primary structure of the α_1 subunit shows considerable sequence and structural similarities to the α subunit of the sodium channel [16].

The structure or function of the lower molecular weight subunits of the dihydropyridine receptor has yet to be clearly identified. The smaller polypeptides (32000 and 52000 Da polypeptides) have been associated with the dihydropyridine receptor only by their presence on SDS polyacrylamide gels in the purified receptor preparations, and some of these smaller polypeptides have not been observed in certain preparations of purified receptor. In this report, we describe our work with monoclonal antibodies to the dihydropyridine receptor of the voltage-dependent Ca^{2+} channel.

The Calcium Channel:
Structure, Function and Implications
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Our results demonstrate that the 175000 Da (α_2), 170000 Da (α_1), and 52000 Da (β) polypeptides are distinct and integral subunits of the dihydropyridine receptor of the voltage-dependent Ca^{2+} channel.

Experimental Procedures

Heavy microsomes or isolated triads were purified from adult rabbit skeletal muscle in the presence of protease inhibitors as described by Sharp et al. [9]. Transverse tubular vesicles were isolated from rabbit skeletal muscle according to Rosemblatt et al. [17] in the presence of protease inhibitors. Light sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle in the presence of protease inhibitors by the method of Campbell et al. [18]. Protein was quantitated by the method of Lowry et al. [19] as modified by Peterson [20]. [^{3}H]PN200-110 binding to isolated membranes was determined as previously described by Leung et al. [8].

Dihydropyridine receptor was purified from triads using wheat-germ agglutinin (WGA) Sepharose affinity chromatography and diethylaminoethanol (DEAE) cellulose ion-exchange chromatography as described by Leung et al. [8,15]. 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 m*M*), and phenylmethylsulfonyl fluoride (PMSF; 0.1 m*M*). All other buffers contained 0.1 m*M* PMSF and 0.75 m*M* benzamidine. The partially purified dihydropyridine receptor from the initial WGA-Sepharose affinity column was referred to as the NAG *N*-acetyl-D-glucosamine) eluted dihydropyridine receptor, or NAG-eluate. Detergent-solubilized proteins were quantitated by the method of Lowry et al. [19] as modified by Peterson [20] after the proteins were precipitated with 5% trichloroacetic acid in the presence of 0.5 mg sodium deoxycholate. The purified dihydropyridine receptor was analyzed by SDS-PAGE on 5%-16% gradient gels according to the method of Laemmli [21] under both nonreducing (10 m*M* *N*-ethylmaleimide in sample buffer) and reducing (5m*M* dithiothreitol in sample buffer) conditions. Subunit stoichiometry of the purified dihydropyridine receptor was determined by densitometric scanning of 10 μg of the purified dihydropyridine receptor from nine preparations, isolated using either digitonin or 3-[(3-cholamidopropyl) dimethylammonio]-l-propanesulfonate (CHAPS) for solubilization. The Coomassie Blue stained gel was scanned with a Hoefer Model GS300 scanning densitometer. The relative densities of the various bands were determined using the GS350H Data System software from Hoefer.

Hybridoma cells lines were prepared from mice which were initially immunized with isolated triads which are enriched in the dihydropyridine receptor. Tail bleeds from the immunized mice were screened using an immunoblot assay with purified dihydropyridine receptor, and positive mice were then boosted with two intraperitoneal injections of NAG-eluted dihydropyridine receptor followed by an intravenous injection of purified dihydropyridine receptor 2 days before fusion. Spleen cells from the mice were fused with NS-1 myeloma cells [22]. Hybrid cells were grown and passaged in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Hybridoma supernatants were screened by an immunodot assay [23] against light sarcoplasmic reticulum vesicles, skeletal muscle triads, NAG-eluted dihydropyridine receptor, and the void from the WGA-Sepharose (which is depleted of dihydropyridine receptor). The immunodot assay positive monoclonal antibodies were further screened for their ability to immunoprecipitate the [^{3}H]PN200-110-labeled digitonin-solubilized receptor. Monoclonal antibody beads were prepared by incubating 15 bed volumes of hybridoma supematants with goat anti-mouse IgG (GAM-IgG) Sepharose (Cooper, diluted to an IgG binding capacity of 1 mg/ml with Sepharose CL 4B) to form MAb-GAM-IgG beads. Triad vesicles were labeled with 10 nM [^{3}H]PN200-110 and solubilized with1% digitonin. 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 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 and assayed for dihydropyridine receptor activity using the PEG precipitation assay (8). The beads were washed twice with 1 ml buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.4) containing 0.1% digitonin and then counted in a scintillation counter.

Skeletal muscle membranes or purified dihydropyridine receptor were separated by SDS-PAGE on 5%-16% gradient gels and transferred to nitrocellulose membranes using a modification of the procedure of Towbin et al. [24]. BLOTTO (Bovine Lacto Transfer Technique Optimizer; 50 mM NaH_2PO_4, 0.9% NaCl, pH 7.4, 5% nonfat dry milk) [25] 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 GAM-IgG secondary antibody (Cooper, 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-PBS (50 mM 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-l-naphthol as the substrate.

Materials

[^{3}H]PN200-110 was obtained from Amersham. Electrophoretic reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Protease inhibitors and peroxidase-conjugated WGA were otained from Sigma. Digitonin was from Fisher and Sigma and prepared as previously described [9]. All other reagents were of reagent-grade quality.

Results

The purified dihydropyridine receptor has been shown by Coomassie Blue staining of SDS-polyacrylamide gels to contain four polypeptide components of molecular masses 175000,170000, 52000 and 32000 under nonreducing conditions and molecular masses 170000,150000,52000 and 32000 under reducing conditions (Fig. 1). The four polypeptide components of the dihydropyridine receptor have been referred to as the α_1 subunit (170 000 Da polypeptide), the α_2 subunit, formerly called α (175 000/150 000

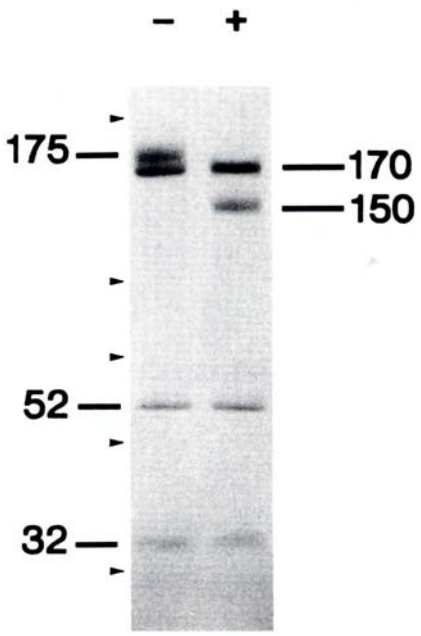

Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified dihydropyridine receptor of the voltage-dependent Ca^{2+} channel. The purified dihydropyridine receptor (10 μg per lane) was separated on a 5%-16% SDS-polyacrylainide gel under nonreducing (-) and reducing (+) conditions. The gels were stained with Coomassie blue and destained. The α_1 subunit (170), α_2 subunit (175; 150), β subunit (52), and γ subunit (32) of the dihydropyridine receptor are indicated. *Arrowheads* indicate the positions of the molecular weight standards (from left to right): 200000,97400,68000,43000,25700,18400, and 14300 Da

Da polypeptide), the β subunit (52000 Da polypeptide), and the γ subunit (32000 Da polypeptide). In our previous publications [9,11,15] the 170000 Da polypeptide was referred to as the δ subunit. 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 polypeptides (Fig. 2). 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 polypeptides. The purified dihydropyridine receptors from nine different preparations were analyzed; the results are summarized in Table 1. The 175 000/ 150000 Da, 170000 Da, 52000 Da and 32000 Da polypeptides exhibited a stoichiometric ratio of 1.0:0.79:1.0:1.0. No difference in the stoichiometric ratio of the polypeptides was seen between the preparations using CHAPS and those using

Table 1. Subunit stoichiometry of the purified dihydropyridine receptor of the voltage-dependent Ca^{2+} channel

Subunit	M_r	Relative Intensity % ± SE[a]	Stoichiometric ratio
α_1	170000	28.1 ± 1.0	1.00
α_2	175000	19.6 ± 0.8	0.79
	150000		
β	52000	8.8 ± 0.3	1.03
γ	32000	5.5 ± 0.3	1.04

[a] Standard error of the mean, $n = 18$

The purified dihydropyridine receptor was subjected to SDS-PAGE on 5% -16% gradient gels (10 μg per lane), stained with Coomassie Blue and scanned with a Hoefer Model GS 300 scanning densitometer. The data are compiled from two scans of each of nine different preparations

digitonin for the solubilization of the dihydropyridine receptor. Therefore, it appears from SDS-PAGE analysis of the purified dihydropyridine receptor that the receptor consists of four subunits.

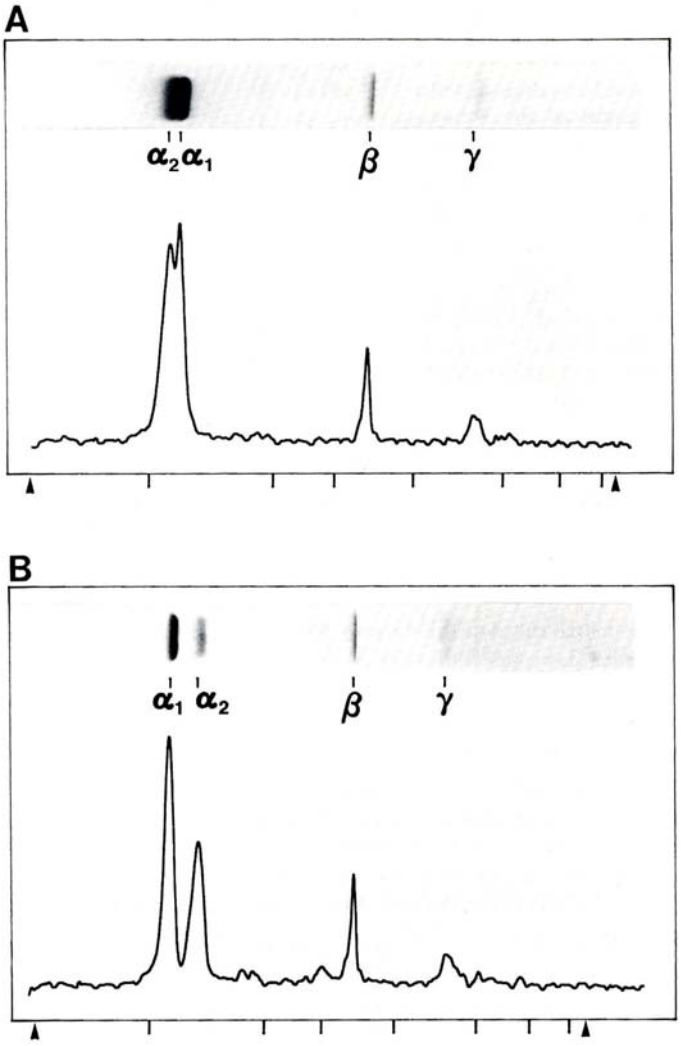

Fig. 2A, B. Densitometric scans of SDS-polyacrylamide gels of the purified dihydropyridine receptor of the voltage-dependent Ca^{2+} channel. The purified dihydropyridine receptor was separated on a 5% -16% SDS-polyacrylamide gel under nonreducing **(A)** and reducing **(B)** conditions. The gels were stained with Coomassie blue, and following destaining the densitometric scans were obtained with a Hoefer GS300 scanning densitometer. The top and dye front of the gels are indicated by the *left* and *right arrowheads*, respectively. *Hash marks* indicate the positions of the molecular weight standards (from left to right) M_r of 200000, 97400, 68000, 43000, 25700,18400, and 14300

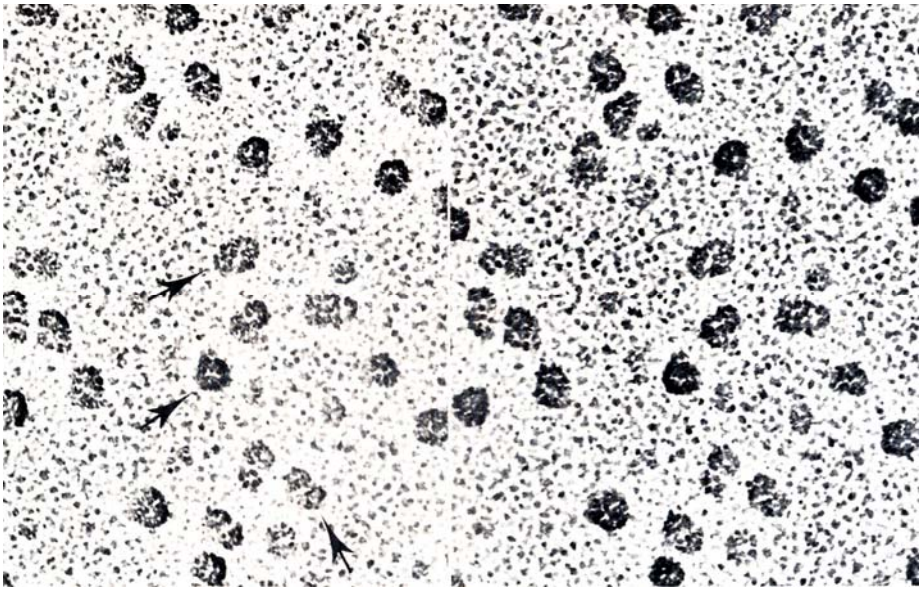

Fig. 3. Stereomicrographs offreeze-dried, rotary-shadowed dihydropyridine receptor of the voltage-dependent Ca^{2+} channel. The purified dihydropyridine receptor was freeze-dried, rotary-shadowed with carbon-platinum, and imaged in an electron microscope. Note variations in shape from round to elongated of the globular molecule and separation into two halves *(arrows)*. A stereo viewer with a magnification of two to three fold should be used to fuse the micrographs, (× 300000) (With permission of the Journal of Biological Chemistry)

The purified dihydropyridine receptors are globular, with a round or slightly elongated profile, depending on the orientation of the receptor on the mica (Fig. 3). The round profiles have a heavier platinum shadow, indicating that they are taller. The average diameter of the receptor is 16 ± 0.9 nm. The elongated profiles are less heavily shadowed and have a length of up to 22 nm. The receptor also appears to be primarily composed of two components of similar size, separated by a small central gap (Fig. 3). Thus, the dihydropyridine receptor has an ovoidal shape with long and short diameters of 16 and 22 nm.

Monoclonal antibodies against the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel were produced by immunizing mice with rabbit skeletal muscle triads followed by booster immunizations with purified dihydropyridine receptor. An immunodot assay was used for screening of the hybridoma supernatants, and antiserum from the mouse used for the fusion was used as a control in each screening. Preparations containing different amounts of dihydropyridine receptor were used to differentiate among the antibodies against the dihydropyridine receptor and those that react with other proteins in triads and sarcoplasmic reticulum (Fig. 4). A hybridoma supernatant was considered positive in the immunodot assay if it reacted with dihydropyridine receptor and/or membranes enriched in the dihydropyridine receptor but showed no reactivity with preparations that are devoid of the dihydropyridine receptor.

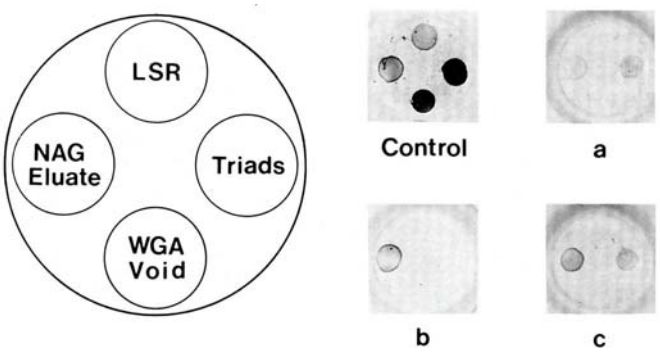

Fig. 4. Immunodot assay for anti-dihydropyridine receptor monoclonal antibodies. Light sarcoplasmic reticulum vesicles *(LSR)*; rabbit skeletal muscle triads *(Triads)*; the void of the WGA-Sepharose column after incubation with digitonin-solubilized triads *(WGA-void)*; dihydropyridine receptor eluted 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) and allowed to dry as diagrammed. Specific [^{3}H]PN200-110 binding activity for the preparations are: LSR, 0.5 fmol/μl; Triads, 21.7 fmol/μl; WGA-void, 0.1 fmol/μl; and NAG eluate, 21.8 fmol/μl. The plates were blocked with 3% BSA-TBS (20 m*M* Tris-HQ, 200 m*M* NaCl, pH 7.5) and allowed to react with hybridoma supematants. A peroxidase-conjugated goat anti-mouse IgG secondary antibody (Cooper) at 1:1000 dilution in 3% BSA-TBS was used, and the plates were developed using 4-chloro-1-naphthol as the substrate. *Control,* Serum from an immunized mouse used for the fusion, diluted 1:500 in 3% BSA-TBS; *a, b, and c,* results for the immunodot assay using 50 μl positive hybridoma supematants IIC12, IIF7, and IDD5, respectively (With permission of the Journal of Biological Chemistry)

Immunodot assay positive antibodies were next tested for their ability to immunoprecipitate the [^{3}H]PN200-110-labeled receptor from solubilized triads. Monoclonal antibodies from hybridoma supematants were preincubated with GAM-IgG Sepharose beads to form MAb-GAM-IgG beads which were then used to immunoprecipitate the digitonin-solubilized [^{3}H]PN200-110-labeled dihydropyridine receptor. The radioactivity on the beads was counted to determine directly the amount of labeled dihydropyridine receptor bound by the antibody. Figure 5 shows the results of an immunoprecipitation assay with a monoclonal antibody (MAb $VD2_1$) to the β subunit (52000 Da polypeptide) of the receptor in comparison with an unrelated T-system monoclonal antibody (MAb $IXE12_2$) and WGA-Sepharose. Monoclonal antibody to the β subunit was found to specifically immunoprecipitate the [^{3}H]PN200-110-labeled dihydropyridine receptor from the assay mixture and was equally efficient as WGA Sepharose in binding the receptor. Similar results were obtained with monoclonal antibodies to the α_1 subunit (170000 Da polypeptide). The anti-dihydropyridine receptor antibodies were also shown to bind saturably to the [^{3}H]PN200-110-labeled dihydropyridine receptor, and a close inverse correlation was found between the amount of dihydropyridine receptor immunoprecipitated by the antibody and the amount of [^{3}H]PN200-110-labeled dihydropyridine receptor remaining in the supernatant (Fig. 6). The highest level of dihydropyridine receptor immunoprecipitated by a monoclonal antibody to the α_1 subunit (170 000 Da polypeptide) ranged from 80% to 95% of the total amount present in the assay mixture. The results show that this assay was able to select those antibodies that bind to the digitonin-solubilized

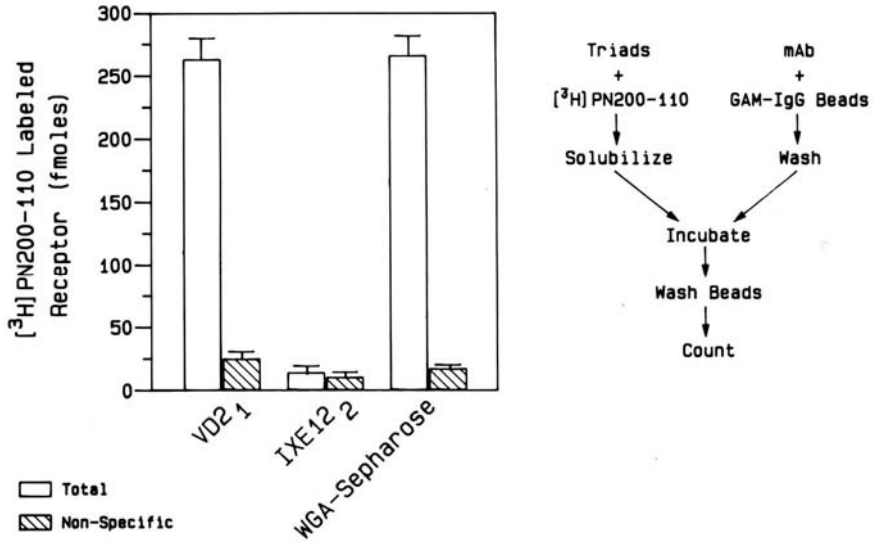

Fig. 5. Immunopredpitation of the dihydropyridine receptor with monoclonal antibody to the β subunit. Hybridoma supematants were tested for their ability to immunopredpitate the [^{3}H]PN200-110-labeled receptor from digitonin-solubilized membranes as shown at right. IXE12$_2$ is an unrelated antibody used as a control and WGA-Sepharose is used as a positive control. The nonspedfically labeled receptor was determined in the presence of 10 μ*M* nitrendipine. *Error bars* represent the standard error of the mean from three independent repeats of the experiments

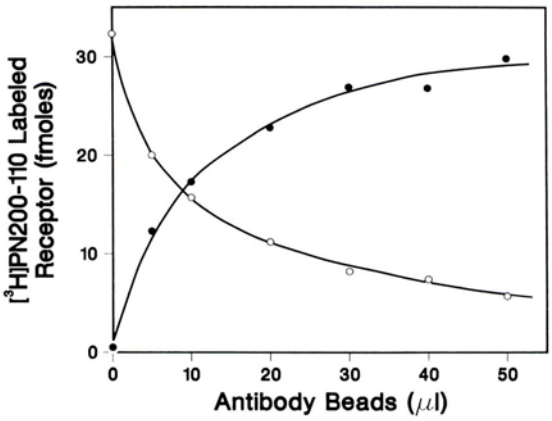

Fig. 6. Immunopredpitation of [^{3}H]PN200-110-labeled dihydropyridine receptor with monoclonal antibody to the α_1 subunit. Immunopredpitation assays were carried out as described in "Experimental Procedures." Various amounts of MAb IIC12-GAM-IgG Sepharose were used with the volume of the beads kept constant using Sepharose CL 4B. The maximum amount of [^{3}H]PN200-110-labeled receptor immunopredpitated 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 immunopredpitation of nonspecific binding activity determined in the presence of 10 μ*M* nitrendipine was less than 6% of the total binding (With permission of the Journal of Biological Chemistry

[^{3}H]PN200-110-labeled dihydropyridine receptor and do not compete directly with the dihydropyridine binding site on the receptor.

The molecular components of the skeletal muscle dihydropyridine receptor were characterized by immunoblot assays using monoclonal antibodies capable of immunoprecipitating the dihydropyridine receptor from digitonin-solubilized mem-

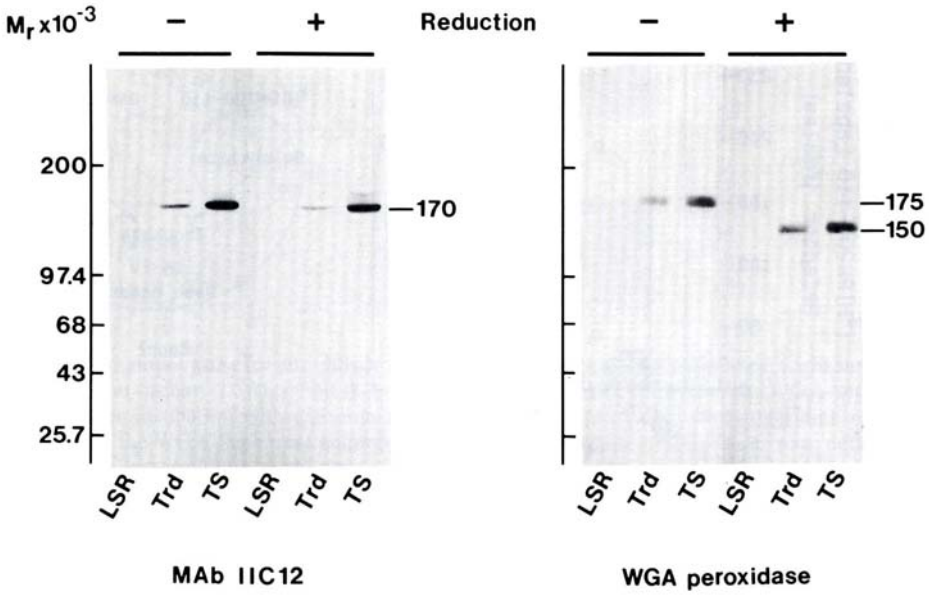

Fig. 7. Immunoblot staining of rabbit skeletal muscle membrane fractions with monoclonal antibodies to the dihydropyridine receptor. Indirect immunoperoxidase staining of membrane fractions with anti-dihydropyridine receptor antibodies was performed as described in the "Experimental Procedures." Light sarcoplasmic reticulum *(LSR)*, triads *(Trd)*, and transverse tubular *(TS)* membranes were subjected to SDS-PAGE on a 3%-12% gradient gel under nonreducing (+ 10 m*M* *N*-ethylmaleimide) and reducing (+ 5 m*M* dithiothreitol) conditions. *Left panel* shows nitrocellulose transfers of the gel stained with monoclonal antibody IIC12 (anti- α_1); *Right panel* shows nitrocellulose transfers of the gel stained with WGA-peroxidase. The α_1 subunit (170 kd) and the α_2 subunit (175,150 kd) are indicated (With permission of the Journal of Biological Chemistry)

branes. A monoclonal antibody to the α_1 subunit stained a polypeptide of 170000Da on nitrocellulose transfers of transverse tubular membranes, and isolated triads separated on SDS-PAGE (Fig. 7). Identical results were obtained for two other monoclonal antibodies to the α_1 subunit. WGA-peroxidase staining of the same membrane fractions has demonstrated that the 175000 Da polypeptide (α_2 subunit) is the major WGA-positive glycoprotein in these membranes. The apparent molecular weight of the α_1 subunit remained unchanged with reduction, while the apparent molecular weight of the α_2 subunit shifted from 175 000 to 150 000 upon reduction. The 170 000 Da polypeptide (α_1 subunit) and the 175 000 Da glycoprotein (α_2 subunit) were not detected in light sarcoplasmic reticulum membranes, a preparation devoid of dihydropyridine receptor.

Figure 8 shows the immunoblot staining of the various fractions from the purification of the dihydropyridine receptor. The α_1 and β subunits of the dihydropyridine receptor were detected by immunoblot staining with mAb IIC12 (Fig. 8 A) and mAb $VD2_1$ (Fig. 8B), respectively. The β subunit copurified with the α_1 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 in the peak fractions from the

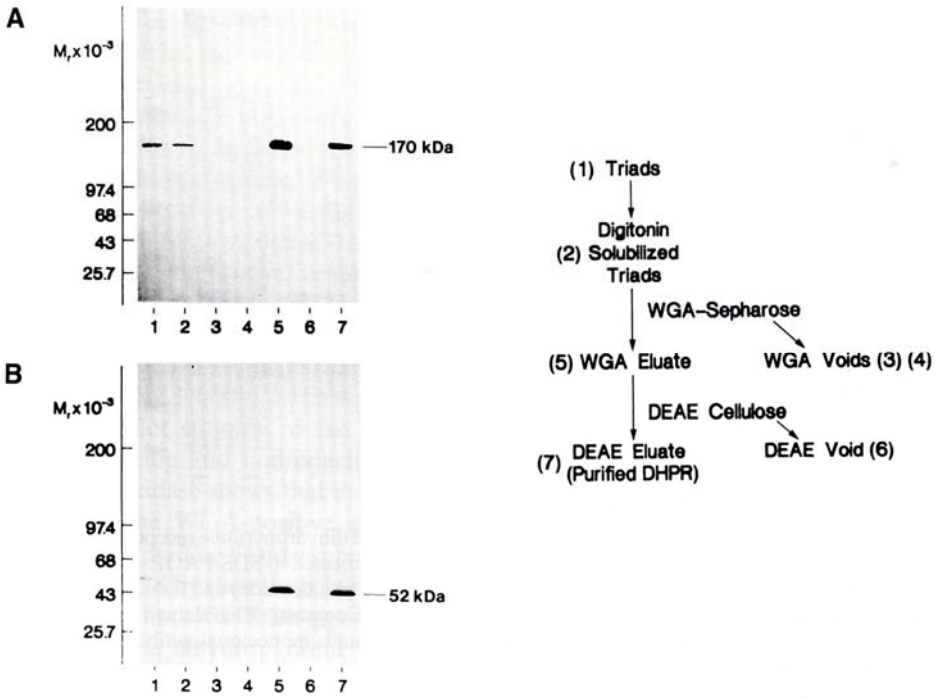

Fig. 8A, B. Immunoblot staining of fractions in purification of the dihydropyridine receptor from skeletal muscle triads. The various fractions from the purification of the dihydropyridine receptor from triads were subjected to SDS-PAGE on a 5%-16% gradient gel and transferred to nitrocellulose. The immunoblots were stained with mAb IIC12, anti-α_1 **(A)** and mAb VD2$_1$, anti-β **(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)*. The α_1 subunit (170 kd) and β subunit (52 kda) are indicated

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 β subunit in the CHAPS-purified dihydropyridine receptor by immunoblot staining with mAb VD2$_1$. Figure 9 shows Coomassie Blue staining and immunoblot staining of the purified dihydropyridine receptor. The α_1 subunit was the only protein stained by anti-α_1 monoclonal antibodies, and its molecular weight remains unchanged with reduction. The α_2 subunit was the only protein stained by WGA-peroxidase, and its molecular weight shifted upon reduction. Finally, the β subunit antibody stained a 52-kDa polypeptide under both reducing and nonreducing conditions and did not stain the α_1, α_2, or γ subunits of the receptor.

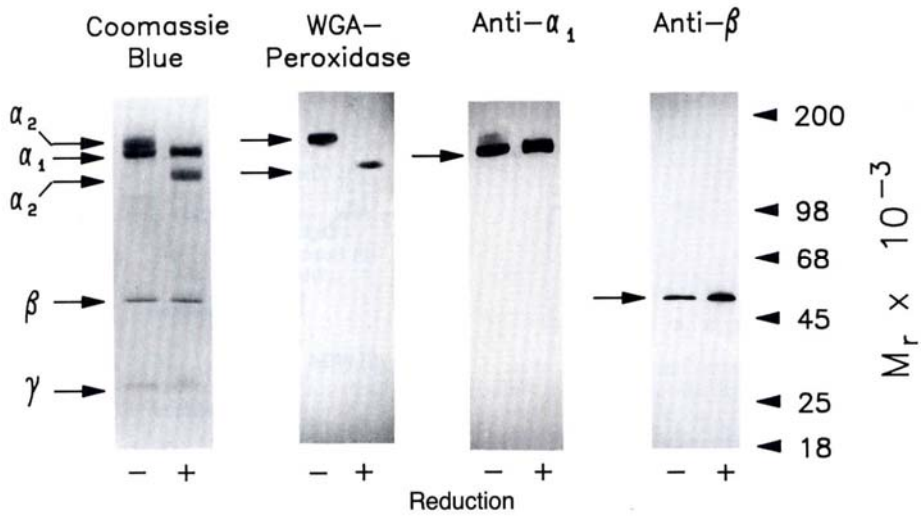

Fig. 9. Coomassie blue and immunoblot staining of the purified dihydropyridine receptor from rabbit skeletal muscle. The purified dihydropyridine receptor was subjected to SDS-PAGE on a 5%-16% gradient gel under nonreducing (-)conditions (+ 20 m*M* *n*-ethylmaleimide) and reducing (+)conditions (+ 10 m*M* dithiothreitol). *Left panel* is a photograph of a Coomassie Blue stained gel; *right three* panels are immunoblots with WGA peroridase to the α_2 subunit, monoclonal antibodies to the α_1 subunit, and monoclonal antibodies to the β subunit. The α_1, α_2 and β submits are indicated by arrows. Molecular weight standards are indicated on the right

Discussion

The 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel has been purified in our laboratory from rabbit skeletal muscle triads. We have found skeletal muscle triads to be the best starting material for the purification of the 1,4-dihydropyridine receptor because skeletal muscle triads contain one major WGA-positive glycoprotein (see Fig. 7). In addition, skeletal muscle triads are enriched in [^{3}H]PN200-110 binding activity (10-40 pmol/mg) and the yield of skeletal muscle triads ranges from 600 to 1000 mg/kg tissue as compared to 10-30 mg/kg for transverse tubular membranes.

The purified dihydropyridine receptor was characterized by SDS-PAGE with Coomassie Blue staining and immunoblotting with monoclonal antibodies and WGA peroxidase. Densitometric scanning of the Coomassie Blue stained gels of the purified dihydropyridine receptor provided evidence that each of the four polypeptides are indeed integral subunits of the dihydropyridine receptor (Fig. 3). Under reducing conditions, in which the α_1 and α_2 subunits were well resolved on the gel, and the α_2 subunit migrated with an apparent molecular mass of 150000, a stoichiometric ratio of 1.0:0.79:1.0:1.0 was obtained for the α_1, α_2, β, and γ subunits, respectively (Table 1). The stoichiometric ratio of 1:1:1 among the α_1, β, γ subunits strongly suggests that the β subunit is an integral component of the dihydropyridine receptor. The anomalous ratio of 0.79:1 between the α_2 subunit and the other subunits of the dihydropyridine receptor can be explained by its glycoprotein nature. The α_2 subunit has been shown to be heavily glycosylated, and if one were to assume that the protein component of the

α_2 subunit has a molecular mass of 120000 Da then the stoichiometric ratio would approach 1:1:1:1 for the four subunits of the dihydropyridine receptor.

Electron-microscopic characterization of the purified dihydropyridine receptor provided additional structural information on the dihydropyridine receptor. Rotary-shadowed stereographs of the freeze-dried dihydropyridine receptor revealed a homogeneous preparation of ovoidal particles 16 × 22 nm in size, demonstrating that the protein components of the purified dihydropyridine receptor exist as a single complex with two symmetrical halves. The two halves 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 by Home and Oswald [26], in which the dihydropyridine receptor was reported to be a large ellipsoidal transmembrane protein with a molecular weight of approximately 370000.

Immunoblot staining of the purified dihydropyridine receptor on SDS-PAGE under reducing and nonreducing conditions with the monoclonal antibodies and WGA-peroxidase shows that the α_1 and α_2 subunits are distinct proteins, and the α_2 subunit is the WGA-positive glycoprotein component of the purified dihydropyridine receptor. The β and γ subunits are not related to the α_1 or α_2 subunits since they are not stained by the monoclonal antibodies to the α_1 subunit or WGA-peroxidase. The α_2 subunit (175000 Da glycoprotein) appears to be equivalent to the 160000 Da subunit of the dihydropyridine receptor described by Curtis and Catterall [5] and Borsotto et al. [6]. It undergoes a decrease in its apparent molecular mass upon reduction and is stained by WGA-peroxidase on nitrocellulose blots. The α_1 subunit of the dihydropyridine receptor appears to be equivalent to the 142000 Da subunit described by Flockerzi et al. [7] since it has been found to be a phosphoprotein [11].

The association of the four subunits of the dihydropyridine receptor had been shown previously only by protein staining of polyacrylamide gels of the purified dihydropyridine receptor. Work from our laboratory has further demonstrated their association by other approaches. A monoclonal antibody specific for the β subunit of the dihydropyridine receptor from rabbit skeletal muscle is capable of immunoprecipitating the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized triads. Since the 170000 Da subunit of the dihydropyridine receptor contains the dihydropyridine binding site [9, 10], the immunopredpitation of dihydropyridine binding activity by an antibody specific to the 52000 Da polypeptide demonstrates that the 52000 Da and 170000 Da polypeptides are associated in digitonin-solubilized triads. It also shows that the 52000 polypeptide is not an unrelated polypeptide that copurifies nonspecifically with the dihydropyridine receptor. Immunoblot staining of the various fractions from the chromatographic procedures used in the purification of the dihydropyridine receptor revealed that this 52000 Da polypeptide copurifies with the 170000 Da subunit throughout the entire purification process (Fig. 3), demonstrating the close association between these two proteins. Immunoprecipitation of the α_1 subunit also resulted in the coprecipitation of the α_2 subunit [8], and when the digitonin-solubilized dihydropyridine receptor was bound to WGA Sepharose, a buffer containing 1% SDS was required to separate the α_1 subunit from the α_2 subunit [9]. Considered together, these results demonstrate the close association between the α_2 subunit and the α_1 subunit (dihydropyridine binding subunit) of the receptor.

The 52000 Da polypeptide had not been reported to copurify with the dihydropyridine receptor when certain procedures were used for the purification. Lazdunski and coworkers, using CHAPS to solubilize the receptor, have reported a single polypeptide of 170000 Da under nonreducing conditions that is converted to a polypeptide of 140 000 Da and several small polypeptides upon reduction [6]. We have shown 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 52000 Da polypeptide was present in the purified receptor, as determined by staining with mAb $VD2_1$.

In summary, work from our laboratory has demonstrated that the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel purified from rabbit skeletal muscle contains four distinct subunits that are closely associated in a 1:1:1:1 stoichiometric ratio. The α_1 subunit is the dihydropyridine binding subunit of the receptor [9, 10] and probably also contains the ion-conducting pore of the Ca^{2+} channel complex [16]. The function of the α_2 subunit and lower molecular weight subunits (β, γ) remains to be elucidated. Preliminary results from Coronado's laboratory [27] have shown that the monoclonal antibody (VD2i) to the 52000 Da subunit (β) described in this report is capable of activating the Ca^{2+} channel whereas polyclonal antibodies to the 32000 Da subunit (γ) are capable of inhibiting the Ca^{2+} channel, suggesting that these lower molecular weight subunits might have regulatory roles in the function of the dihydropyridine-receptor-Ca^{2+} complex.

Summary

Monoclonal antibodies to the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel have been produced and used in immunoprecipitation and immunoblotting experiments to probe the structure and function of the Ca^{2+} channel. The purified 1,4-dihydropyridine receptor from rabbit skeletal muscle contains four polypeptide components of 175 000 Da (α_2), 170000 Da (α_1), 52000 Da (β), and 32000 Da (y) when analyzed by SDS-PAGE under nonreducing conditions. Densitometric scanning of Coomassie Blue stained SDS-polyacrylamide gels of the purified dihydropyridine receptor has shown that the four polypeptide components exist in a 1:1:1:1 stoichiometric ratio. 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 receptor.

Monoclonal antibodies to the 170000 Da polypeptide (α_1 subunit) and monoclonal antibodies to the 52000 Da polypeptide (β subunit) were able to specifically imunoprecipitate the [^{3}H]PN200-110-labeled dihydropyridine receptor from digitonin-solubilized membranes. Immunoblot staining of the purified dihydropyridine receptor using monoclonal antibodies to the α_1 subunit and WGA peroxidase have demonstrated that the α_1 and the β_2 subunits are distinct proteins, and that the α_2 subunit is the glycoprotein component of the dihydropyridine receptor. The apparent molecular weight of the α_1 subunit on SDS-PAGE remained unchanged with reduction while the apparent molecular weight of the α_2 subunit shifted from 175000 to 150000 upon reduction. Immunoblotting experiments with a monoclonal antibody to the β subunit.

have shown that the 52000 Da polypeptide copurifies with the 175000 Da and 170000 Da polypeptides at all stages of the purification, and that the higher molecular weight subunits of the receptor were not labeled by the monoclonal antibody to the β subunit. In conclusion, we have demonstrated that the 175000 Da (α_2), 170000 Da (α_1) and 52000 Da (β) polypeptides are integral and distinct subunits of the purified dihydropyridine receptor of the voltage-dependent Ca^{2+} channel.

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