Reprinted from **Calcium Channels: Structure and Function**
Volume 560 of the *Annals of the New York Academy of Sciences*
June 26, 1989

32,000-Dalton Subunit of the 1,4-Dihydropyridine Receptor[a]

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INTRODUCTION

The dihydropyridine receptor of the Ca^{2+} channel from skeletal muscle has been purified and characterized by several laboratories (for reviews, see Refs. 1-4). The purified dihydropyridine receptor from rabbit skeletal muscle triads prepared in our laboratory[5] consists of four polypeptides: 175,000 Da, 170,000 Da, 52,000 Da, and 32,000 Da, which have been termed α_2, α_1, β, and γ, respectively.[1] The 170,000-Da polypeptidc (α_1 subunit) contains binding sites for the dihydropyridine and phenylalkylamine classes of Ca^{2+}-channel blockers.[6-9] The 175,000-Da polypeptide (α_2 subunit) is the major glycoprotein of the dihydropyridine receptor and has an apparent molecular mass of 150,000 Da on SDS-PAGE after reduction of disulfide bonds.[5] The 52,000-Da polypeptide (β subunit) is tightly associated with the dihydropyridine receptor and is not a proteolytic fragment of a larger subunit.[10] Some evidence exists for one or more small subunits of 24,000-32,000 Da, collectively termed the δ subunit,[6] that are disulfide linked to the α_2 subunit[6,11] and may account for the increase in mobility of the α_2 subunit observed on SDS-PAGE with reduction. The association of the 32,000-Da polypeptide with the receptor has been previously demonstrated only by SDS-PAGE analysis of the purified receptor. However, the 32,000-Da polypeptide has not been identified in preparations of receptor from all laboratories and its relationship to the α_1 and α_2 subunits has not been determined.

RESULTS

Subunit-Specific Polyclonal Antibodies

Dihydropyridine receptor was purified from skeletal muscle triads by WGA-Sepharose affinity chromatography followed by DEAE-cellulose ion-exchange chro-

[a]KPC is an Established Investigator of the American Heart Association and recipient of National Institutes of Health Grants HL-37187, HL-14388, and HL-39265.

matography,[5] and individual components of the receptor were separated by SDS-polyacrylamide gel electrophoresis on 5-16% gradient gels under nonreducing or reducing conditions. Individual bands were cut from the gel, homogenized in Freund's adjuvant, and used for immunization of guinea pigs for the production of ascites containing the polyclonal antibodies.[12] Interfering antibodies to keratin, a common contaminant of SDS-PAGE, were removed by passing ascites fluid through an epidermal protein-Sepharose column. Alternatively, specific anti-dihydropyridine receptor antibodies were prepared by affinity purification.[12]

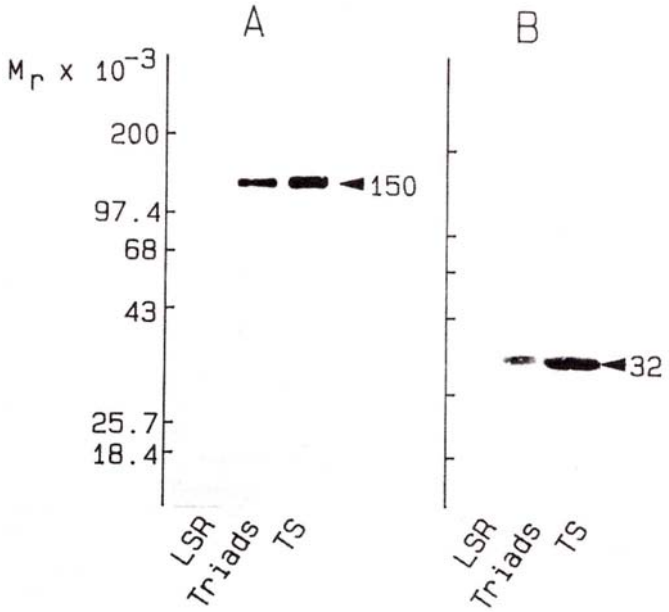

FIGURE 1. Iommunoblot staining of α_2 and γ submits in rabbit skeletal muscle membrane fractions. Isolated rabbit skeletal muscle membrane fractions, light sarcoplasmic reticulum (LSR), triads (Triads), and transverse tubular system (TS) (50 μg each) were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes Indirect immunoperoxidase staining of the nitrocellulose blots was performed using anti-α_2 subunit antiserum (GP5SA, 1:1000 dilution) (**A**) or affinity-purified anti-γ subunit antibody (GP16AP, 1:30) (**B**). Arrowheads indicate the Positions of the 150,000-Da α_2 subunit (150) (**A**) and the 32,000-Da γ subunit (32) (**B**). (From sharp & Campbell[12] with permission of the *Journal of Biological Chemistry.*)

Immunoblot Analysis of Fractions from the Purification of the Dihydropyridine Receptor

Characterization of the 32,000-Da protein of the dihydropyridine receptor in skeletal muscle membranes was performed using affinity-purified antibodies against the nonreduced 32,000-Da protein. The anti-32,000-Da antibodies reacted with a single band of the expected molecular weight in triads (FIG. 1). For comparison, the α_2

subunit was also examined using antiserum against the reduced 150,000-Da protein. Staining for both proteins was completely absent in light sarcoplasmic reticulum membranes and was more intense in transverse tubular membranes consistent with the distribution of [^{3}H]PN200-110 binding and our previous results on the α_1 subunit of the dihydropyridine receptor.[5] These results show the specificity of the polyclonal antibodies and demonstrate that the 32,000-Da protein is distinct from other protein components of the triad and transverse tubular membrane.

Fractions collected during a typical purification of the dihydropyridine receptor were subjected to immunoblot analysis to determine whether the 32,000-Da protein copurifies at each step with the 170,000-Da protein (α_1 subunit) and with the 150,000-Da (α_2 subunit) protein of the receptor. The 170,000-Da protein, which is known to be the dihydropyridine binding component of the receptor, was identified by staining with monoclonal antibody IIC12, which has been previously described.5 FIGURE 2 demonstrates that the 170,000-, 150,000-, and 32,000-Da proteins are each present in triads and are enriched in the GlcNAc-eluted fraction from the WGA-Sepharose. The elution profile of the 32,000-Da polypeptide from the DEAE-cellulose column was identical to and matched the elution profile of the 170,000-Da and 150,000-Da proteins and the elution profile of dihydropyridine receptor prelabeled with ['H1PN200-110 (not shown). Thus, the 32,000-Da protein copurifies with the dihydropyridine receptor at each step of the purification.

Immunoprecipitation of the 32,000-Da Protein of the Dihydropyridine Receptor by Anti- α_1 and Anti-β Subunit Monoclonal Antibodies

To demonstrate a tight association between the 170,000-, 150,000-, 52,000-, and 32,000-Da proteins, we examined the ability of antibodies directed against the 170,000- and 52,000-Da subunits to immunoprecipitate the 150,000- and 32,000-Da proteins (FIG. 3). Digitonin-solubilized triads containing approximately 20 pmol [^{3}H]PN200-110 binding sites per milligram protein were incubated with monoclonal antibody beads preformed as described.[5] After washing, the beads were extracted with SDS gel sample buffer and the immunoprecipitates were analyzed by immunoblot analysis (FIG. 3B). The 150,000-Da and 32,000-Da polypeptides were both present in the anti-170,000-Da and anti-52,000-Da immunoprecipitates (lanes 2 and 3, respectively). A negative control with an unrelated antibody (lane 1) and a positive control with WGA-Sepharose (lane 4) were also included. This data confirms that the 170,000-, 150,000-, 52,000-, and 32,000-Da polypeptides are tightly associated and suggests that the 150,000- and 32,000-Da proteins are subunits of the dihydropyridine receptor.

The 175,000/150,000-Da and the 32,000-Da Proteins Are Immunologically Distinct

A 32,000-Da protein that appears only after reduction of the dihydropyridine receptor and that is apparently disulfide linked to the α_2 subunit has been reported by Schmid *et al.*[11] To investigate the relationship of the 32,000-Da protein of our preparation to the protein reported by Schmid *et al.*[11] as well as to the α_1 and α_2 subunits of the dihydropyridine receptor, we have produced polyclonal antibodies

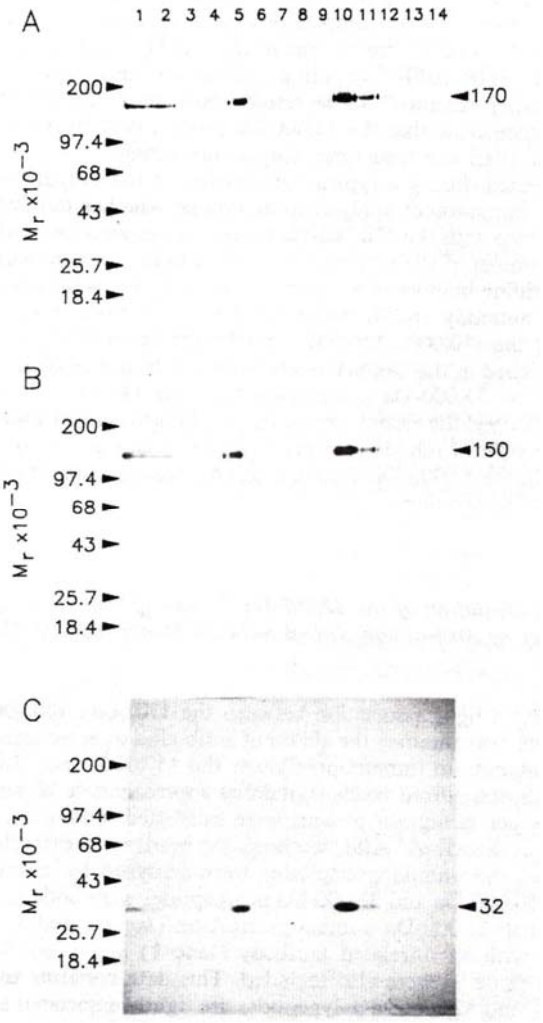

FIGURE 2. Copurification of α_1 (170,000 Da), α_2 (150,000 Da), and γ (32,000 Da) subunits of the dihydropyridine receptor. Dihydropyridine receptor was purified from rabbit skeletal muscle triads as described previously[5] by wheat germ agglutinin affinity chromatography followed by DEAE-cellulose ion-exchange chromatography. Aliquots of various fractions from the purification (50 µl each) were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The blots were stained by the indirect immunoperoxidase method using monoclonal antibody IIC12, anti-α_1 subunit (**A**); GP5SA, polyclonal anti-α_2 subunit antiserum (**B**); or GP16AP, affinity-purified polyclonal anti-γ subunit antibody (**C**). The samples on the transfers are: lane 1, triads; lane 2, digitonin-solubilized triads; lane 3, void fraction from WGA-Sepharose column; lane 4, wash of WGA-Sepharose column; lane 5, GlcNAc eluate from WGA-Sepharose; lane 6, void fraction from DEAE-cellulose column; lane 7, wash of DEAE-cellulose column; lanes 8-14, fractions 3, 5, 7, 9, 11, 13, and 15, respectively, from a linear 0 to 300 m*M* NaCl gradient elution of DEAE-cellulose column (fraction 1, no NaCl, fraction 22, 300 mM NaCl). Arrowheads indicate the positions of the 170,000-Da a, subunit (170) (**A**), the 150,000-Da a; subunit (150) (**B**), and the 32,000-Da γ subunit (32) (**C**). (From Sharp & Campbell[12] with permission of the *Journal of Biological Chemistry*.)

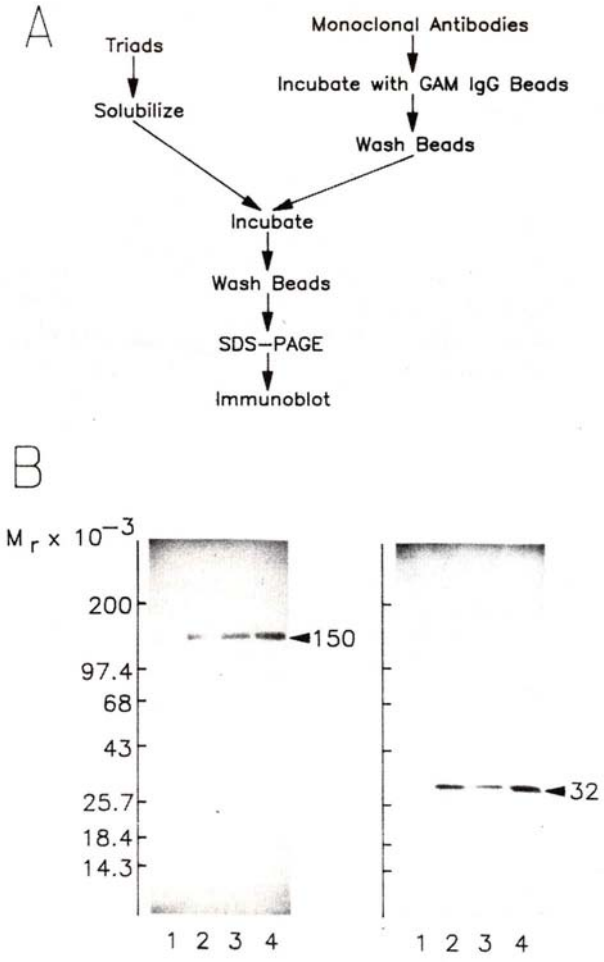

FIGURE 3. Coimmunoprecipitation of the α_2 and γ subunits by anti-α_1 and anti-β subunit antibodies. Isolated triads were solubilized with 1% digitonin and incubated with mAb-goat anti-mouse-IgG-Sepharose or WGA-Sepharose as described.[5] (**A**) Schematic diagram of immunoprecipitation procedure. (**B**) Immunoprecipitates or WGA-Sepharose precipitates were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Immunoprecipitation was performed using: lane 1, mAb IID8 (anti-cardiac Ca^{2+} + Mg^{2+} ATPase); lane 2, a mixture of mAbs IIID5 and IIC12 (anti-α_1 dihydropyridine receptor subunit); and lane 3, mAb $VD2_1$ (anti-β subunit). As a positive control (lane 4) precipitation was also performed using WGA-Sepharose 6MB. The nitrocellulose blots were stained using polyclonal antiserum GP5SA against the α_2 subunit (left panel) or GP16AP affinity-purified antibody against the γ subunit (right panel). Arrowheads on far right show the positions of the 150,000-Da α_2 subunit and the 32,000-Da γ subunit. (From Sharp & Campbell[12] with permission of the *Journal of Biological Chemistry*.)

against both the nonreduced and reduced forms of the 175,000/150,000-Da protein and the 32,000-Da protein. FIGURE 4 shows immunoblot analysis of the dihydropyridine receptor using these antibodies. Purified dihydropyridine receptor was subjected to SDS-PAGE under either nonreducing (20 m*M* *N*-ethylmaleimide) or reducing (10 mM dithiothreitol) conditions and transferred to Immobilon-P transfer membranes. The transfer membranes were then probed with antibodies against the nonreduced

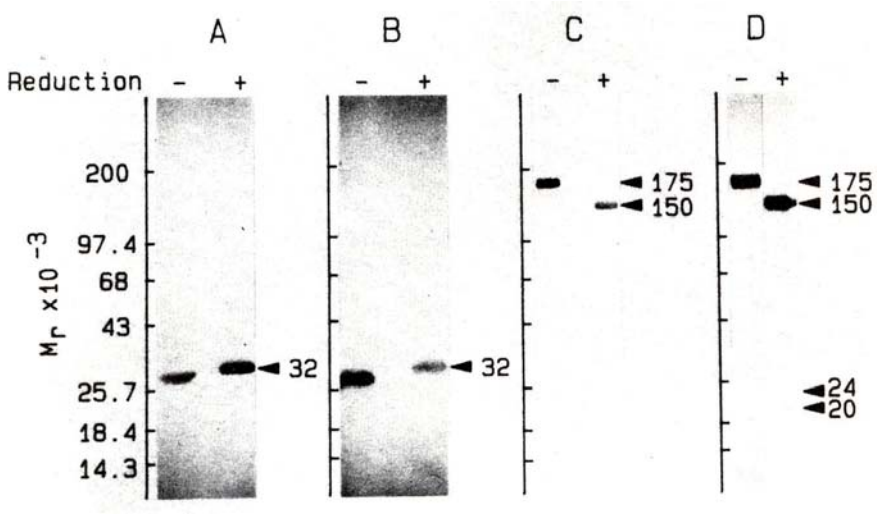

FIGURE 4. Immunoblot analysis of purified dihydropyridine receptor. Purified dihydropyridine receptor was subjected to SDS-PAOE under nonreducing (10 m*M* NEM) or reducing (10 m*M* DTT) conditions. Each lane contained 3 µg dihydropyridine receptor except B (6 µg/lane) and D (2 µg/lane). Proteins were then transferred to Immobilon-P membranes and stained by the indirect immunoperoxidase method. Blots were stained with (**A**) GP16AP, affinity-purified antibodies against the nonreduced 32,000-Da γ subunit; (**B**) GP11AP affinity-purified antibodies against the reduced 32,000-Da γ subunit; (**C**) GP5SA epidermal protein-column-adsorbed antiserum against the reduced 150,000-Da α_2 subunit; (**D**) GP13AP affinity-purified antibody against the nonreduced 175,000-Da form of the α_2 subunit. (From Sharp & Campbell[12] with permission of the *Journal of Biological Chemistry*.)

32,000-Da γ subunit, the reduced 32,000-Da γ subunit, the reduced 150,000-Da α_2 subunit, and the nonreduced 175,000-Da form of the α_2 subunit (FIG. 4, A-D, respectively). Antibodies against either the nonreduced or reduced form of the 32,000-Da protein did not stain other components of the dihydropyridine receptor on immunoblots prepared under either nonreducing or reducing conditions (FIG. 4A, B). Antibodies against the reduced α_2 subunit (150,000-Da protein) stained only the

nonreduced 175,000-Da form and the reduced 150,000-Da form of the α_2 subunit (FIG. 4C) and no other components of the receptor. Antibodies against the nonreduced 175,000-Da α_2 subunit stained the nonreduced 175,000-Da and the reduced 150,000-Da forms of the α_2 subunit, but did not stain the 32,000-Da protein even after reduction of the receptor (FIG. 4D). These results show that the 32,000-Da γ subunit and 175.000/150,000-Da α_2 subunit are distinct components of the dihydropyridine receptor and that the 32,000-Da protein is not a proteolytic fragment of another component of the dihydropyridine receptor or linked by disulfide bonds to another component of the dihydropyridine receptor.

SUMMARY

Polyclonal antibodies to the 32,000-Da polypeptide of the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel have been produced and used to characterize the association of the 32,000-Da polypeptide (γ subunit) with other subunits of the dihydropyridine receptor. The 32,000-Da polypeptide was found to copurify with α_1 and α_2 subunits at each step of the purification of the dihydropyridine receptor. Monoclonal antibodies against the α_1 and β subunits immunoprecipitate the digitonin-solubilized dihydropyridine receptor as a multisubunit complex that includes the 32,000-Da polypeptide. Polyclonal antibodies generated against both the nonreduced and reduced forms of the α_2 subunit and the γ subunit have been used to show that the 32,000-Da polypeptide is not a proteolytic fragment of a larger component of the dihydropyridine receptor and not disulfide linked to the α_2 subunit. Our results demonstrate that the 32,000-Da polypeptide (γ subunit) is an integral and distinct component of the dihydropyridine receptor.

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