THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1987 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 262, No. 25, Issue of September 5, pp. 12309-12315, 1987
Printed in U.S.A.

Identification and Characterization of the Dihydropyridine-binding Subunit of the Skeletal Muscle Dihydropyridine Receptor*

(Received for publication, March 23, 1987)

Alan H. Sharp, Toshiaki Imagawa‡, Albert T. Leung§, and Kevin P. Campbell¶

From the Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242

Photoaffinity labeling of isolated triads and purified dihydropyridine receptor with [^{3}H]azidopine and (+)-[^{3}H]PN200-110 has been used to identify and characterize the dihydropyridine-binding subunit of the 1,4-dihydropyridine receptor of rabbit skeletal muscle. The 1,4-dihydropyridine receptor purified from rabbit skeletal muscle triads contains four protein subunits of 175,000, 170,000, 52,000, and 32,000 Da (Leung, A., Imagawa, T., and Campbell, K. P. (1987) *J. Biol. Chem.* 262, 7943–7946). Photoaffinity labeling of isolated triads with [^{3}H]azidopine resulted in specific and covalent incorporation of [^{3}H]azidopine into only the 170,000-Da subunit of the dihydropyridine receptor and not into the 175,000-Da glycoprotein subunit of the receptor. The [^{3}H]azidopine-labeled 170,000-Da subunit was separated from the 175,000-Da glycoprotein subunit by sequential elution from a wheat germ agglutinin-Sepharose column with 1% sodium dodecyl sulfate followed by 200 mM *N*-acetylglucosamine. Photoaffinity labeling of purified dihydropyridine receptor with [^{3}H]azidopine or (+)-[^{3}H]PN200-110 also resulted in the specific and covalent incorporation of either ligand into only the 170,000-Da subunit. Therefore, our results show that the dihydropyridine-binding subunit of the skeletal muscle 1,4-dihydropyridine receptor is the 170,000-Da subunit and not the 175,000-Da glycoprotein subunit.

The 1,4-dihydropyridines are potent blockers of voltage-dependent Ca^{2+} channels present in smooth, cardiac, and skeletal muscles as well as in neuronal and other cells (1–7). High affinity receptors for the 1,4-dihydropyridines are enriched in the transverse tubular membrane of skeletal muscle (8), and the skeletal muscle receptor has been purified by a number of laboratories (9–12). The dihydropyridine-binding site of the skeletal muscle 1,4-dihydropyridine receptor has yet to be clearly identified. Ferry *et al.* (13, 14) have reported the photoaffinity labeling of proteins of M_r 240,000, 158,000, and 99,000 in guinea pig skeletal muscle transverse tubular membranes with [^{3}H]azidopine,[1] an aryl-azido derivative of the 1,4-dihydropyridine Ca^{2+} channel blockers. The apparent molecular mass of the 240,000-Da protein changed to 99,000 Da on reduction, whereas the 158,000- and 99,000-Da proteins remained unchanged in apparent molecular mass with reduction. Galizzi *et al.* (15) showed that direct photoaffinity labeling of skeletal muscle transverse tubule membranes with (+)-[^{3}H]PN200-110 resulted in covalent incorporation of label into a protein that migrated at 170,000 Da on SDS-polyacrylamide gels run in the presence of 2-mercaptoethanol. The significance of these results has been unclear because proteins of similar molecular weight under reducing conditions have not been observed previously in highly purified preparations of the dihydropyridine receptor (9–12). Recently, Striessnig *et al.* (16) reported the photoaffinity labeling of a protein of 155,000-Da in a purified preparation of the dihydropyridine receptor using enantiomerically pure (−)-[^{3}H]azidopine. The 155,000-Da protein was claimed to be a glycoprotein based on concanavalin A staining.

Recently, we have shown that the purified 1,4-dihydropyridine receptor contains two distinct high molecular mass subunits of 175,000 and 170,000 Da as well as subunits of 52,000 and 32,000 Da when analyzed on SDS-polyacrylamide gels under nonreducing conditions, and that the apparent molecular mass of the 175,000-Da subunit shifts to 150,000 Da upon reduction (17). Monoclonal antibodies capable of specifically immunoprecipitating (+)-[^{3}H]PN200-110-labeled dihydropyridine receptor stained the 170,000-Da subunit on immunoblots, whereas WGA-peroxidase stained the 175,000-Da glycoprotein subunit (17).

To identify the subunit that binds the dihydropyridine Ca^{2+} channel blockers, we have photolabeled isolated triads and the purified dihydropyridine receptor with [^{3}H]azidopine and (+)-[^{3}H]PN200-110. The 170,000-Da subunit of the dihydropyridine receptor covalently labeled with [^{3}H]azidopine or (+)-[^{3}H]PN200-110 has been resolved from the 175,000-Da glycoprotein subunit by SDS-PAGE and by WGA-Sepharose affinity chromatography. Our results demonstrate that the 170,000-Da subunit is the dihydropyridine-binding subunit of the skeletal muscle 1,4-dihydropyridine receptor.

EXPERIMENTAL PROCEDURES

Preparation of Membrane Vesicles—Triads were purified from adult rabbit skeletal muscle by a modification of the method of Mitchell *et al.* (18). Initial homogenization of muscle tissue was

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Muscular Dystrophy Association Postdoctoral Fellow.

§ American Heart Association Medical Student Research Fellow (1985–1986) and Iowa Graduate Fellow.

¶ Established Investigator of the American Heart Association and recipient of National Institutes of Health Grants HL-37187 and HL-14388. To whom correspondence and reprint requests should be addressed: Dept. of Physiology, Bowen Science Bldg., Rm. 6-530, Univ. of Iowa, Iowa City, IA 52242.

[1] The abbreviations and trivial names used are: [^{3}H]azidopine, 2,6-dimethyl-4-(2′-trifluoromethylphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (*N*-4′-azido[3′,5′-^{3}H]benzolyaminoethyl)-diester; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

performed in the presence of the following protease inhibitors: aprotinin (76.8 nM), iodoacetamide (1 mM), pepstatin A (0.7 μM), leupeptin (1.1 μM), benzamidine (0.83 mM), and PMSF (0.23 mM). All subsequent buffers contained PMSF (58 μM), benzamidine (0.83 mM), and iodoacetamide (1 mM). Microsomes prepared according to Mitchell *et al.* (18) (pyrophosphate variant) were layered onto sucrose step gradients consisting of 14% (4 ml), 25% (12 ml), 28% (7.5 ml), 36% (7.5 ml), and 50% (3.5 ml) sucrose in 20 mM sodium pyrophosphate, 20 mM sodium phosphate, and 1 mM $MgCl_2$ (pH 7.1) (pyrophosphate mix). The gradients were centrifuged for 90 min at 131,000 × *g* in a Beckman SW 28 rotor. A thick band at the 28–36% interface was collected, slowly diluted 2–4-fold with pyrophosphate mix, and pelleted by centrifugation at 235,000 × *g* for 40 min. Membranes were resuspended in a small volume of 0.3 M sucrose, 20 mM Tris maleate buffer (pH 7.0) containing PMSF (58 μM), benzamidine (0.83 mM), and iodoacetamide (1 mM), quick-frozen in liquid nitrogen, and stored at −135 °C. Protein was determined by the method of Lowry *et al.* (19) as modified by Peterson (20) using bovine serum albumin as the standard.

Photoaffinity Labeling of Membranes—Membranes were photoaffinity labeled by a modification of the method of Ferry *et al.* (13). Isolated triads (2 mg/ml) in 50 mM Tris-HCl (pH 7.4), 1 μM pepstatin A, 0.1 mM PMSF were incubated for 90 min on ice with 50 nM [^{3}H]azidopine in the presence or absence of 20 μM unlabeled nitrendipine. The membranes were pelleted by centrifugation at 35,000 × *g* for 15 min, resuspended in 50 mM Tris-HCl (pH 7.4), 0.25% bovine serum albumin, 1 μM pepstatin A, 0.1 mM PMSF, and repelleted. Membranes were then resuspended at 1–2 mg/ml in 50 mM Tris-HCl (pH 7.4), 1 μM pepstatin A, 0.1 mM PMSF and irradiated in Petri dishes on ice with Mineralite UVSL-25 (Ultra-Violet Products, Inc.) and Spectroline ENF-24 (Spectronics Corp.) handheld UV lamps at 254 nm at a distance of 1 cm for 10 min. After irradiation, the membranes were again pelleted by centrifugation at 35,000 × *g* for 15 min and resuspended in a small volume of 50 mM Tris-HCl (pH 7.4). Protein assays (19, 20) were performed on the resuspended membranes. The membranes were quick-frozen in liquid nitrogen and stored at −70 °C until used. All steps were carried out in the dark or in reduced light.

Purification of 1,4-Dihydropyridine Receptor—Dihydropyridine receptor was purified by a modification of the method of Curtis and Catterall (9). In a typical purification procedure, isolated triads (20 mg of protein) were solubilized at a protein concentration of 1 mg/ml with 1% digitonin, 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.1 mM PMSF, and 1 μM pepstatin A or 0.75 mM benzamidine. After incubation on ice for 45 min, the solubilized membranes were centrifuged at 85,000 × *g* for 30 min and the supernatant was collected. All subsequent buffers contained 0.75 mM benzamidine and 0.1 mM PMSF. Solubilized triads were applied to a 2-ml column of WGA-Sepharose at a flow rate of 0.2 ml/min. The column was washed with 1% digitonin, 0.5 M NaCl, and 50 mM Tris-HCl (pH 7.4), followed by 0.3% digitonin, 50 mM Tris-HCl (pH 7.4) (Buffer A). The dihydropyridine receptor was eluted with 4 ml of 200 mM *N*-acetylglucosamine (GlcNAc) in Buffer A. The GlcNAc-eluted dihydropyridine receptor was applied to a DEAE-cellulose column (2 ml) and the column was washed with Buffer A. The column was eluted with a 50-ml linear gradient of 0–300 mM NaCl in Buffer A. Fractions of 2 ml were collected. Aliquots were counted (when receptor was prelabeled with [^{3}H]azidopine or (+)-[^{3}H]PN200-110) or assayed for (+)-[^{3}H]PN200-110-binding activity. To determine (+)-[^{3}H]PN200-110-binding activity, aliquots of column fractions were incubated with 10 nM (+)-[^{3}H]PN200-110 in 0.1% digitonin, 100 μM diltiazem, 0.2 mg/ml bovine serum albumin, 50 mM Tris-HCl (pH 7.4), 0.1 mM PMSF for 30 min at 37 °C. Bound (+)-[^{3}H]PN200-110 was determined by the polyethylene glycol precipitation assay of Glossmann and Ferry (21). Nonspecific binding was determined in the presence of 1 μM (+)-PN200-110.

Photoaffinity Labeling of Purified Dihydropyridine Receptor—Dihydropyridine receptor purified from isolated triads using WGA-Sepharose affinity chromatography or WGA-Sepharose affinity chromatography plus DEAE-cellulose ion-exchange chromatography was incubated with 10–20 nM [^{3}H]azidopine or (+)-[^{3}H]PN200-110 and 100 μM diltiazem in 0.1% digitonin, 100 mM NaCl, 0.1 mM PMSF, and 50 mM Tris-HCl (pH 7.4) for 30 min at 37 °C in the presence or absence of 1 μM (+)-PN200-110. Aliquots (0.25 ml) of receptor incubated with [^{3}H]azidopine were irradiated for 10 min in 12 × 75-mm test tubes with 254-nm UV light from a Mineralite UVSL-25 lamp positioned directly over the tubes. Aliquots of receptor incubated with (+)-[^{3}H]PN200-110 were exposed to light from a 1000-watt mercury arc lamp (Porta-Cure 1000, American Ultraviolet Co.) for 10 s at a distance of 25 cm from the lamp.

Separation of 170,000-Da Subunit and 175,000-Da Glycoprotein Subunit—Isolated triads (10 mg of protein) were subjected to photoaffinity labeling with [^{3}H]azidopine, solubilized, and applied to a 1-ml WGA-Sepharose column. Alternatively, purified dihydropyridine receptor (100 μg) was subjected to photoaffinity labeling with [^{3}H]PN200-110 and applied to a 1-ml WGA-Sepharose column. The column was washed with 1 M NaCl (isolated triads) or 0.5 M NaCl (purified receptor) in 0.3% digitonin, 50 mM Tris-HCl (pH 7.4), and 0.1 mM PMSF followed by 0.3% digitonin, 50 mM Tris-HCl (pH 7.4), and 0.1 mM PMSF. The column was further washed with 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and 0.1 mM PMSF. The 170,000-Da dihydropyridine receptor subunit was then eluted with 1% SDS in 50 mM Tris-HCl (pH 7.4), 0.1 mM PMSF. After another wash with 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, the glycoprotein subunit was eluted with 200 mM GlcNAc in 50 mM Tris-HCl (pH 7.4) and 0.1 mM PMSF. Fractions were collected at each step as indicated in the figure legends.

Preparation of Anti-dihydropyridine Receptor Monoclonal Antibodies—Monoclonal antibodies to the dihydropyridine receptor of rabbit skeletal muscle were prepared as described previously (17). Hybridoma cells were dilution-cloned and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Ascites fluid was produced in Pristane-primed BALB/c mice (25).

SDS-PAGE and Immunoblot Assay—SDS-PAGE was performed using the system of Laemmli (22) using 1.5-mm thick 3–12% gradient gels. Incorporation of tritium-labeled ligands was detected by fluorography and/or liquid scintillation counting of gel slices. Gels used for fluorography were stained with Coomassie Blue and destained with a solution of 10% acetic acid and 5% methanol, treated with Enlightning (New England Nuclear), and dried under vacuum. Kodak XAR-5 film was exposed to the dried gels at −70 °C. Gels for slice analysis were stained with Coomassie Blue or silver stain (Bio-Rad). Gel slices (1.5 mm) were digested overnight at 50 °C in 0.6 ml of 30% H_2O_2 before liquid scintillation fluid was added. Radioactivity was measured using a Beckman LS-5801 liquid scintillation counter. For immunoblots, protein was transferred electrophoretically onto nitrocellulose membrane according to Towbin *et al.* (23). Nitrocellulose blots were blocked with BLOTTO (Bovine Lacto Transfer Technique Optimizer: 50 mM NaH_2PO_4, 0.9% NaCl (pH 7.4) (PBS), 5% nonfat dry milk) (24) before incubation with ascites fluid or with PBS-Tween (PBS, 0.05% Tween-20) before incubation with WGA-peroxidase as described previously (17). Blots were developed using 4-chloro-1-naphthol as the substrate.

Materials—[^{3}H]Azidopine was generously supplied by Amersham Corp. (45 Ci/mmol) or was obtained from New England Nuclear (53 Ci/mmol). (+)-[^{3}H]PN200-110 (70–85 Ci/mmol) was obtained from Amersham Corp. (+)-PN200-110 was generously supplied by Sandoz Inc. Nitrendipine was generously supplied by Miles Laboratories. Bovine serum albumin (fraction V), WGA-Sepharose, and WGA-peroxidase were obtained from Sigma. Molecular weight standards were from Bethesda Research Laboratories or Sigma. Digitonin was obtained from Fisher or Sigma. Soluble digitonin was prepared by heating a 1.5% solution of digitonin to 95–100 °C for 10 min, allowing the solution to stand at room temperature for 2–3 days, and then removing the precipitate by filtration through a 0.22-μm filter. The filtrate was lyophylized and stored until used.

RESULTS

Photoaffinity Labeling of Triads with [^{3}H]Azidopine—Photoaffinity labeling with [^{3}H]azidopine, an aryl-azide derivative of the dihydropyridine Ca^{2+} channel blockers (13), was used to identify the dihydropyridine-binding component of the rabbit skeletal muscle dihydropyridine receptor. Isolated skeletal muscle triads containing high affinity (+)-[^{3}H]PN200-110-binding activity (20 pmol/mg of protein) were covalently labeled with [^{3}H]azidopine by irradiation with UV light. Labeled triads were solubilized with digitonin, and the dihydropyridine receptor was purified by affinity chromatography on WGA-Sepharose and subjected to SDS-PAGE. A fluorogram of the purified dihydropyridine receptor is shown in Fig. 1*A*. Covalent incorporation of [^{3}H]azidopine was observed in a single band of 170,000 Da under both reducing (1% 2-mercaptoethanol) and nonreducing (10 mM *N*-ethylmaleimide) con-

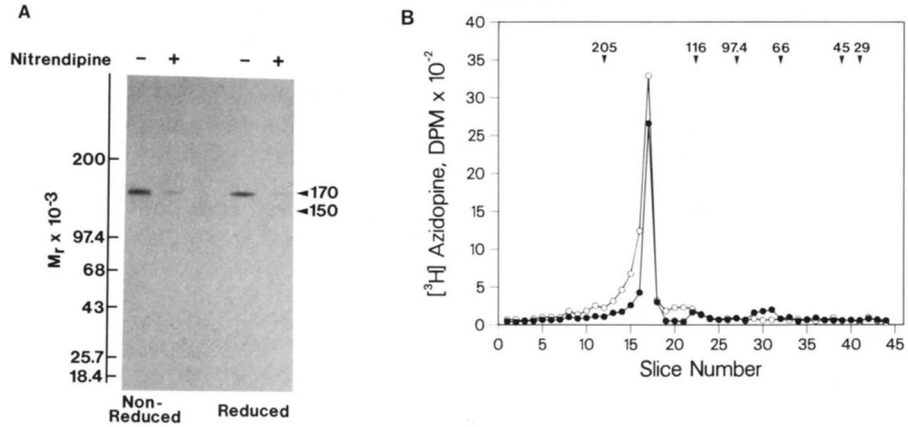

FIG. 1. **Photoaffinity labeling of dihydropyridine receptor in isolated triads with [^{3}H]azidopine.** Photoaffinity labeling of isolated rabbit skeletal muscle triads with [^{3}H]azidopine was performed as described under "Experimental Procedures" after incubation with [^{3}H]azidopine in the presence or absence of excess unlabeled nitrendipine. Isolated triads covalently labeled with [^{3}H]azidopine (20 mg each minus and plus nitrendipine) were solubilized as described under "Experimental Procedures" and the extracts were mixed with WGA-Sepharose (2.5 ml/20 mg triads) for 2 h at 4 °C. The WGA-Sepharose was washed with 15 ml of 1% digitonin, 0.5 M NaCl, 50 mM Tris-HCl (pH 7.4), and then poured into two identical columns. Each 2.5-ml column was washed with 4.5 ml of 0.3% digitonin, 50 mM Tris-HCl (pH 7.4), and then eluted with 300 mM GlcNAc, 0.3% digitonin, and 50 mM Tris-HCl (pH 7.4). The same peak fractions were pooled for each column, and aliquots of each pool (25 μl/lane) were subjected to SDS-PAGE on 3–12% gradient gels in the presence of 1% 2-mercaptoethanol (*Reduced*) or 10 mM *N*-ethylmaleimide (*Nonreduced*). *A*, the gel was stained with Coomassie Blue, treated with Enlightning, dried, and placed on Kodak XAR film for 12 days. The positions of the 170,000- and 150,000-Da subunits as determined by Coomassie Blue staining under reducing conditions are marked by the *arrowheads*. *B*, gels run under nonreducing conditions (○) or reducing conditions (●) were stained, sliced into 1.5-mm fractions, and solubilized with 30% H_2O_2. Solubilized slices were counted in a scintillation counter after addition of scintillation fluid. *Arrowheads* indicate the location of molecular weight standards. Numbers represent $M_r \times 10^{-3}$.

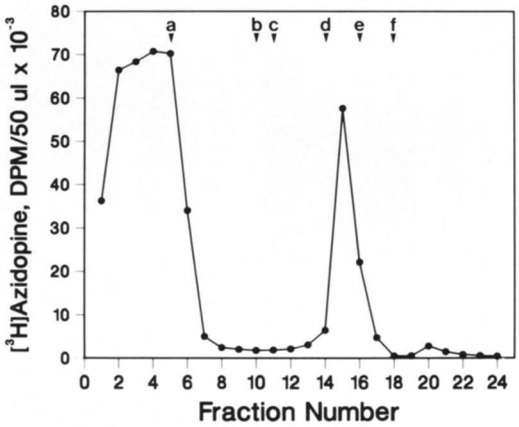

FIG. 2. **Elution profile of [^{3}H]azidopine from WGA-Sepharose column.** Isolated triads were covalently labeled with [^{3}H]azidopine as described under "Experimental Procedures." The [^{3}H]azidopine-labeled triads (10 mg of protein) were solubilized with digitonin as described under "Experimental Procedures" and applied to a 1-ml column of WGA-Sepharose. The column was washed successively with high salt buffer (1 M NaCl, 0.3% digitonin, 50 mM Tris-HCl (pH 7.4)) (*a*); low salt buffer (0.3% digitonin, 50 mM Tris-HCl (pH 7.4)) (*b*); and 0.1% SDS in 50 mM Tris-HCl (pH 7.4) (*c*). The column was then eluted with 1% SDS in 50 mM Tris-HCl (pH 7.4) (*d*). After washing again with 0.1% SDS in 50 mM Tris-HCl (pH 7.4) (*e*), the column was eluted with 200 mM GlcNAc in 0.1% SDS in 50 mM Tris-HCl (pH 7.4) (*f*). Aliquots (50 μl) of each fraction were counted in a scintillation counter. *Fractions 1–18* contained 2 ml each; *fractions 19–23*, 0.5 ml each; *fraction 24*, 2 ml.

ditions and was inhibited by the presence of 20 μM unlabeled nitrendipine during the incubation with 50 nM [^{3}H]azidopine. These results indicate that [^{3}H]azidopine specifically and covalently labels the 170,000-Da subunit of the dihydropyridine receptor. However, gel slice analysis (Fig. 1*B*) revealed that when electrophoresis of the covalently labeled receptor was performed under reducing conditions, a significant amount of label was lost. Because the WGA-positive glycoprotein subunit of the dihydropyridine receptor has almost the same mobility on SDS-polyacrylamide gels under nonreducing conditions as the 170,000-Da subunit, it was possible that a significant amount of label was incorporated into the WGA-positive glycoprotein but was subsequently lost on reduction. To exclude this possibility, it was necessary to resolve the two high molecular weight subunits in the absence of reducing agents.

Separation of [^{3}H]Azidopine-labeled 170,000-Da Subunit from the 175,000-Da Glycoprotein Subunit—Because the 170,000-Da subunit did not appear to be a glycoprotein (17), we attempted to elute the 170,000-Da subunit from the WGA-Sepharose column with 1% SDS before eluting the glycoprotein subunit with 200 mM GlcNAc. In Fig. 2, isolated triads were covalently labeled with [^{3}H]azidopine by irradiation with UV light, solubilized with digitonin, and applied to a WGA-Sepharose column. The column was washed with 1 M NaCl followed by 0.1% SDS to remove unbound [^{3}H]azidopine and [^{3}H]azidopine nonspecifically incorporated into lipids. The column was then eluted successively with 1% SDS buffer followed by 200 mM GlcNAc in 0.1% SDS buffer. Greater than 95% of the [^{3}H]azidopine remaining on the column after the washes was eluted with 1% SDS. Peak protein fractions eluted with 1% SDS and 200 mM GlcNAc (Fig. 2, *fractions 15* and *20*, respectively) were separated on SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The blots were then stained for either the 170,000-Da subunit of the receptor using monoclonal antibody IIF7 (Fig. 3*A*) or the 175,000-Da glycoprotein subunit of the receptor using WGA-peroxidase (Fig. 3*B*). Monoclonal antibody IIF7 is specific for the 170,000-Da subunit of the dihydropyridine receptor, whereas WGA-peroxidase has been shown to stain the 175,000-Da glycoprotein subunit of the dihydropyridine

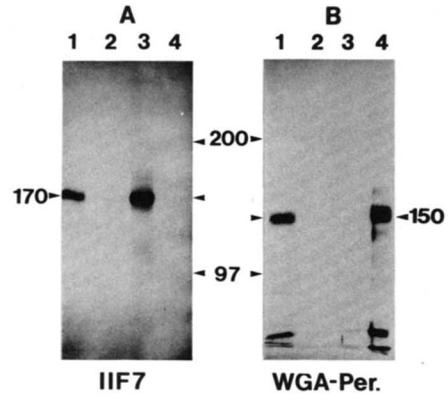

FIG. 3. **Immunoblot analysis of SDS and GlcNAc eluates from WGA-Sepharose column.** Aliquots of fractions from the experiment of Fig. 2 (50 μl) were subjected to SDS-PAGE on a 5% gel under reducing conditions. The proteins were then transferred electrophoretically to nitrocellulose paper (23). Indirect immunoperoxidase staining of nitrocellulose blots was performed as described under "Experimental Procedures." Nitrocellulose blots were stained with monoclonal antibody IIF7 (*A*) or WGA-peroxidase (*WGA-Per.*) (*B*). *Lane 1*, digitonin-solubilized triads; *lane 2*, void fraction of WGA-Sepharose; *lane 3*, peak fraction eluted with 1% SDS (Fig. 2, *fraction 15*); *lane 4*, peak fraction eluted with 200 mM GlcNAc (Fig. 2, *fraction 20*).

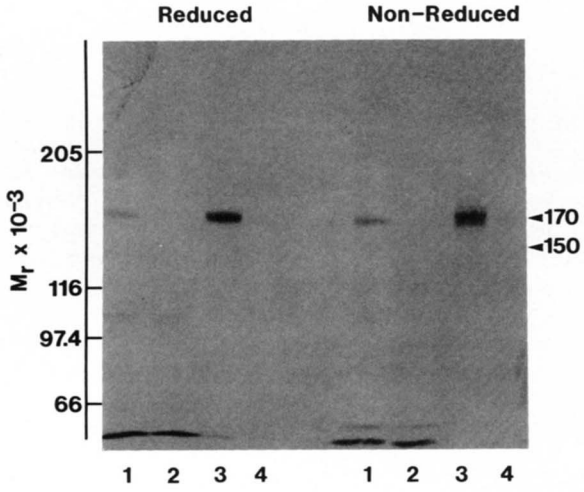

FIG. 4. **Covalent incorporation of [^{3}H]azidopine in SDS and GlcNAc eluates from WGA-Sepharose column.** Aliquots of fractions from the experiment in Fig. 2 (50 μl/lane) were subjected to SDS-PAGE on a 5% gel in the presence of 1% mercaptoethanol (*Reduced*) or 10 mM *N*-ethylmaleimide (*Nonreduced*). The gel was stained with Coomassie Brilliant Blue, soaked in Enlightning, and dried. Kodak XAR-5 film was then exposed to the gel for approximately 3 weeks. *Lane 1*, ditigonin-solubilized triads; *lane 2*, void fraction of WGA-Sepharose column; *lane 3*, peak fraction eluted with 1% SDS (Fig. 2, *fraction 15*); *lane 4*, peak fraction eluted with 200 mM GlcNAc (Fig. 2, *fraction 20*). *Arrowheads* mark the position of the 170,000- and 150,000-Da proteins under reducing conditions as identified by Coomassie Blue staining.

receptor in isolated triads or in the purified dihydropyridine receptor (17). The 170,000-Da subunit was found only in the 1% SDS eluate from the WGA-Sepharose column (Fig. 2, *fraction 15*; Fig. 3*A*, *lane 3*), whereas the 175,000-Da glycoprotein subunit of the receptor was found only in the GlcNAc eluate from the column (Fig. 2, *fraction 20*; Fig. 3*B*, *lane 4*). These results demonstrated that the two high molecular mass subunits were completely resolved by this elution procedure. Fig. 4 shows the electrophoretic profile of covalently bound [^{3}H]azidopine in the two fractions under reducing and nonreducing conditions. [^{3}H]Azidopine was detected only in the 170,000-Da subunit eluted with 1% SDS and not in the GlcNAc-eluted glycoprotein subunit. Therefore, the site(s) of covalent attachment of [^{3}H]azidopine resides on only the 170,000-Da subunit of the dihydropyridine receptor.

Photoaffinity Labeling of Purified Dihydropyridine Receptor with [^{3}H]Azidopine and (+)-[^{3}H]PN200-110—Photoaffinity labeling of the dihydropyridine receptor after purification was also examined using [^{3}H]azidopine and (+)-[^{3}H]PN200-110. Figs. 5 and 6 compare the results of photoaffinity labeling of purified dihydropyridine receptor with [^{3}H]azidopine and (+)-[^{3}H]PN200-110. Dihydropyridine receptor was purified from isolated triads using WGA-Sepharose affinity chromatography and DEAE-cellulose ion-exchange chromatography before photoaffinity labeling. Covalent labeling by both ligands was observed exclusively in a 170,000-Da protein and was inhibited by the presence of unlabeled (+)-PN200-110 (Figs. 5 and 6). The apparent molecular mass of the 170,000-Da protein was unaffected by the presence or absence of disulfide reducing agents (Fig. 5, compare *left* and *right panels*). Thus, photoaffinity labeling with both [^{3}H]azidopine and (+)-[^{3}H]PN200-110 resulted in the specific and covalent labeling of the same 170,000-Da protein in purified receptor as labeled by [^{3}H]azidopine in isolated triads. These results provide strong evidence that the 170,000-Da subunit of the dihydropyridine receptor binds the dihydropyridine Ca^{2+} channel blockers. However, quantitation of tritium label incorporated into the (+)-[^{3}H]PN200-110-labeled dihydropyridine receptor

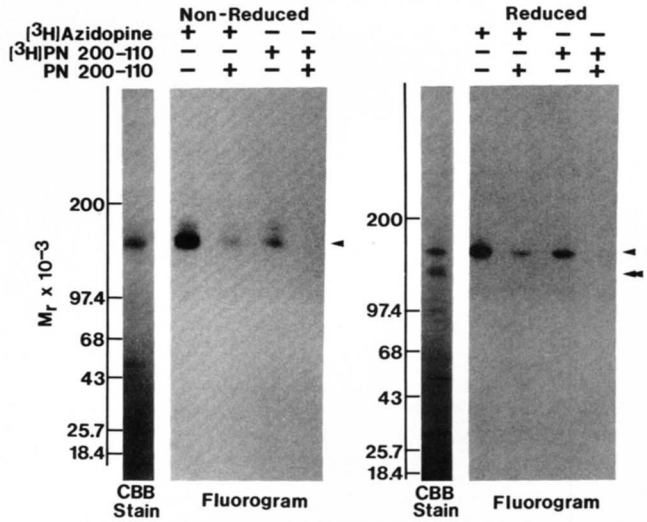

FIG. 5. **Photoaffinity labeling of purified dihydropyridine receptor with [^{3}H]azidopine and (+)-[^{3}H]PN200-110 (fluorogram).** Dihydropyridine receptor was purified by WGA-Sepharose affinity chromatography followed by DEAE-cellulose ion-exchange chromatography in the absence of dihydropyridine ligands as described under "Experimental Procedures." Purified receptor was incubated with 20 nM [^{3}H]azidopine or 10 nM (+)-[^{3}H]PN200-110 in the presence of 100 μM diltiazem for 30 min at 37 °C. Nonspecific photoaffinity labeling was determined by competition of label with 1 μM unlabeled (+)-PN200-110. After incubation, purified receptor was irradiated as described under "Experimental Procedures." The photolabeled receptor (2 μg/lane) was subjected to SDS-PAGE on a 3–12% gradient gel in the presence of 10 mM *N*-ethylmaleimide (*Nonreduced*) or 1% 2-mercaptoethanol (*Reduced*), stained with Coomassie Blue, soaked in Enlightning, and placed on Kodak XAR film at −70 °C for 4 weeks. Representative Coomassie Blue-stained gel lanes (*CBB Stain*) are shown next to fluorograms of the same gels. The *minus* and *plus signs* refer to the absence and presence of [^{3}H]azidopine, (+)-[^{3}H]PN200-110, or (+)-PN200-110 as indicated. The position of the 170,000-Da subunit is indicated by the *arrowhead*, and the position of the 150,000-Da subunit under reducing conditions is indicated by the *double arrowhead*.

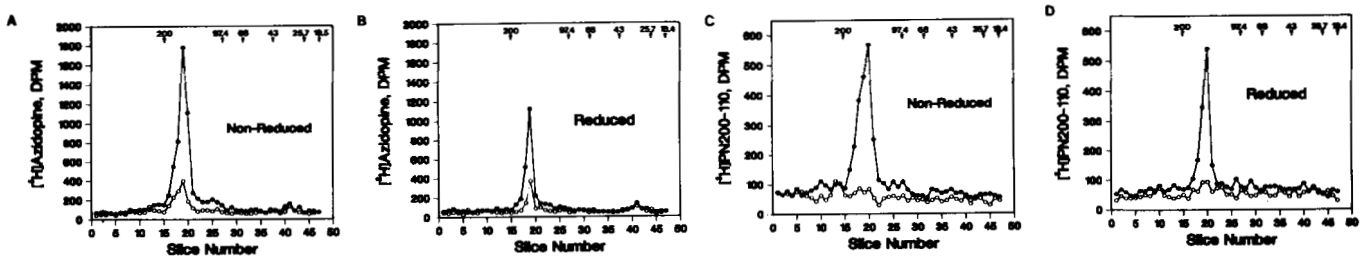

FIG. 6. **Photoaffinity labeling of purified dihydropyridine receptor with [^{3}H]azidopine and (+)-[^{3}H] PN200-110 (electrophoretic profile).** Dihydropyridine receptor was purified and photolabeled with [^{3}H] azidopine (*A, B*) or (+)-[^{3}H]PN200-110 (*C, D*) in the absence (●) or presence (○) of (+)-PN200-110 as described in the legend of Fig. 5 and under "Experimental Procedures." Photolabeled receptor (2 μg/lane) was subjected to SDS-PAGE on a 3–12% gradient gel in the presence of 10 mM *N*-ethylmaleimide (*Nonreduced, A, C*) or 1% 2-mercaptoethanol (*Reduced, B, D*). Gels were visualized by silver staining, and lanes were sliced into 1.5-mm fractions, dissolved with 30% H_2O_2, and counted in a scintillation counter. *Arrowheads* indicate the positions of molecular weight standards ($M_r \times 10^{-3}$).

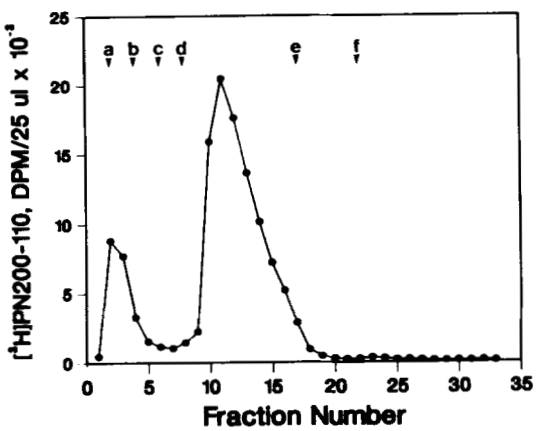

FIG. 7. **Elution profile of (+)-[^{3}H]PN200-110 from WGA-Sepharose column.** Isolated triads were solubilized with 1% digitonin, and the dihydropyridine receptor was purified by WGA-Sepharose affinity chromatography as described under "Experimental Procedures." The purified dihydropyridine receptor was incubated with 10 nM (+)-[^{3}H]PN200-110. The receptor-(+)-[^{3}H]PN200-110 complexes were further purified by DEAE-cellulose ion-exchange chromatography as described under "Experimental Procedures." the purified receptor-(+)-[^{3}H]PN200-110 complexes (100 μg) were then exposed to a 1000-watt UV lamp for 10 s and applied to a 1-ml WGA-Sepharose column. The column was washed with 0.5 M NaCl buffer containing 0.3% digitonin, 0.1 mM PMSF, and 50 mM Tris-HCl (pH 7.4) (*a*). Subsequent washes and elutions were performed as described in the legend of Fig. 2. *Fractions 1–8* contained 1 ml each; *fractions 9–16*, 0.5 ml each; *fractions 17–21*, 1 ml each; *fractions 22–33*, 0.5 ml each.

by slice analysis of SDS-polyacrylamide gels revealed loss of label under reducing conditions as for [^{3}H]azidopine (Fig. 6). Therefore, to conclusively determine whether only the 170,000-Da subunit was labeled by (+)-[^{3}H]PN200-110, it was necessary to resolve the two high molecular mass subunits in the absence of reducing agents as described previously for [^{3}H]azidopine-labeled receptors.

Separation of (+)-[^{3}H]PN200-110-labeled 170,000-Da Subunit from 175,000-Da Glycoprotein Subunit—In Fig. 7, dihydropyridine receptor was purified from solubilized triads using WGA-Sepharose affinity chromatography and then incubated with (+)-[^{3}H]PN200-110. (+)-[^{3}H]PN200-110-receptor complexes were further purified using DEAE-cellulose ion-exchange chromatography and then photolyzed by irradiation with a 1000-watt UV light for 10 s. The labeled dihydropyridine receptor was then applied to a WGA-Sepharose column, washed, and eluted as described previously for the [^{3}H]azidopine-labeled receptor. Nearly all (+)-[^{3}H]PN200-110 bound to the column eluted with 1% SDS, and only background amounts of (+)-[^{3}H]PN200-110 were present in the GlcNAc-eluted fraction. In Fig. 8, column fractions from the WGA-Sepharose column of Fig. 7 were run on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The immunoblots were then stained with monoclonal antibody IIF7 (Fig. 8*A*) or WGA-peroxidase (Fig. 8*B*), revealing the elution profiles of the 170,000-Da subunit and the WGA-positive glycoprotein subunit. The 170,000-Da subunit was present only in the SDS-eluted fractions that corresponded with the eluted peak of (+)-[^{3}H]PN200-110. Covalent labeling was detected only in the 170,000-Da subunit eluted with 1% SDS and not in the 175,000-Da glycoprotein subunit eluted with 200 mM GlcNAc (Fig. 9). These results also demonstrate that the binding site for the 1,4-dihydropyridine Ca^{2+} channel blockers resides on the 170,000-Da subunit of the 1,4-dihydropyridine receptor and not on the 175,000-Da glycoprotein subunit.

DISCUSSION

We have used photoaffinity labeling with [^{3}H]azidopine to identify components of the dihydropyridine receptor involved in binding of the 1,4-dihydropyridine Ca^{2+} channel blockers. A protein of 170,000 Da in isolated rabbit skeletal muscle triads was covalently labeled by a modification of the method of Ferry *et al.* (13) with [^{3}H]azidopine (Fig. 1). In this experiment, isolated triads were incubated with 50 nM [^{3}H]azidopine to obtain a high level of labeling of the dihydropyridine receptor. Because isolated triads contain about 20 pmol of dihydropyridine-binding sites/mg of protein and the protein concentration in the incubation mixture is 2 mg/ml, the concentration of dihydropyridine receptor is estimated to be 40 nM. Therefore, the free concentration of [^{3}H]azidopine in the incubation mixture was probably very low. In addition, much of the free [^{3}H]azidopine is removed by washing the membranes before photolysis. Incorporation of [^{3}H]azidopine into the 170,000-Da protein was inhibited by the presence of excess nitrendipine during the incubation, suggesting specific labeling by [^{3}H]azidopine. Incorporation of [^{3}H]azidopine was stereoselectively inhibited by (+)-PN200-110 in preference to (−)-PN200-110 (not shown). These results suggested that the 170,000-Da protein was the dihydropyridine-binding subunit of the dihydropyridine receptor.

Although [^{3}H]azidopine clearly labels only the 170,000-Da subunit (Figs. 1–5), it has been suggested that this aryl-azido photoaffinity ligand may be capable of reacting with sites distant from the dihydropyridine-binding site because the aryl-azido side chain extends approximately 15 Å from the

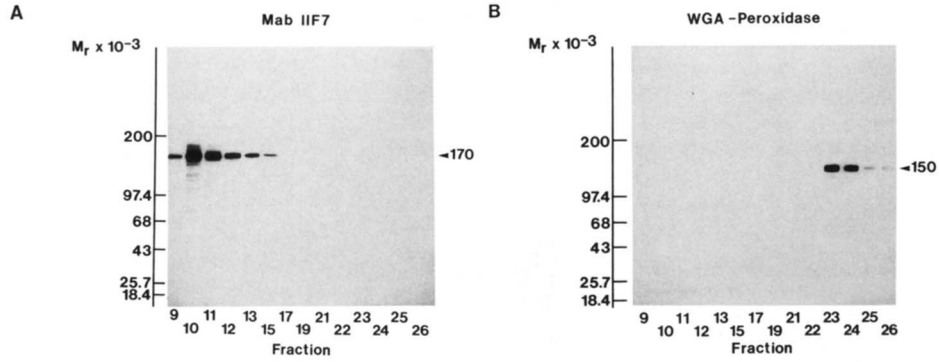

FIG. 8. **Elution profiles of 170,000-Da and glycoprotein subunits from WGA-Sepharose column.** Aliquots (50 μl each) of the fractions from Fig. 7 were subjected to SDS-PAGE on a 3–12% gradient gel used in the presence of 1% 2-mercaptoethanol, and the proteins were transferred electrophoretically to nitrocellulose membranes. The nitrocellulose blots were then stained with IIF7 monoclonal antibody against the 170,000-Da subunit (*A*) or WGA-peroxidase (*B*) as described under "Experimental Procedures." The positions of the 170,000-Da subunit and the glycoprotein subunit (150,000 Da after reduction) are indicated by the *arrowheads*.

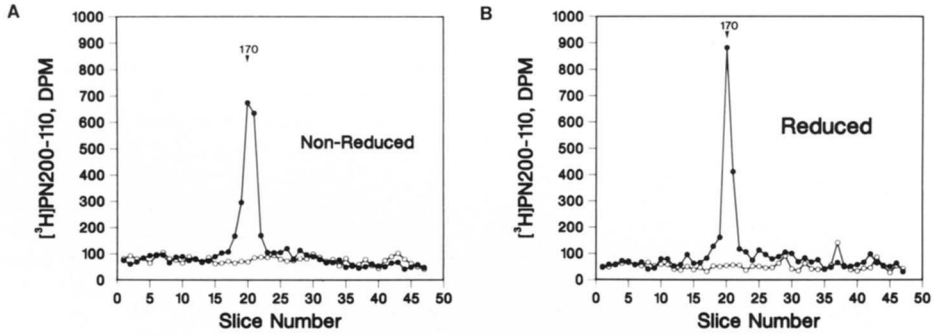

FIG. 9. **Covalent incorporation of (+)-[^{3}H]PN200-110 in SDS and GlcNAc eluates from WGA-Sepharose column.** Aliquots (50 μl each) of the 1% SDS-eluted pool (●) (*fractions 10* and *11*, Fig. 7) and the GlcNAc-eluted pool (○) (*fractions 23* and *24*, Fig. 7) were subjected to SDS-PAGE on a 3–12% gel in the presence of 10 mM *N*-ethylmaleimide (*A*) or 1% 2-mercaptoethanol (*B*). Gels were visualized by silver staining and lanes were sliced into 1.5-mm fractions, dissolved with 30% H_2O_2, and counted in a scintillation counter. The position of the 170,000-Da subunit in the 1% SDS eluate pool under nonreducing and reducing conditions are indicated by the *arrowheads*.

center of the dihydropyridine ring (13). An alternative to the use of ligands modified with aryl-azido side chains for photoaffinity labeling is direct photoaffinity labeling using underivatized ligands. Direct photoaffinity labeling with (+)-[^{3}H]PN200-110 has been demonstrated previously (15). Therefore, photoaffinity labeling with (+)-[^{3}H]PN200-110 was chosen as an independent technique to test our conclusion based on [^{3}H]azidopine photoaffinity labeling that the 170,000-Da protein is the dihydropyridine-binding subunit of the 1,4-dihydropyridine receptor. Essentially identical results were obtained by photoaffinity labeling of purified receptor with (+)-[^{3}H]PN200-110 as were obtained with [^{3}H]azidopine (Figs. 5–9).

Our purified preparation of the dihydropyridine receptor contains four protein subunits (Ref. 17 and Table I), including two distinct high molecular mass subunits (175,000 Da and 170,000 Da) and subunits of 52,000 and 32,000 Da. The two high molecular mass subunits run as a single band of 170,000 Da on nonreducing 3–12% gradient gels and are only resolved in this gel system under reducing conditions. The high molecular weight subunits are resolved on 5–16% gradient gels even under nonreducing conditions. The 175,000-Da subunit has been identified as a glycoprotein based on staining with WGA-peroxidase and concanavalin A-peroxidase on nitrocellulose blots (17). The apparent molecular mass of this glycoprotein shifts to 150,000 Da upon reduction. The 170,000-Da subunit protein is stained by several monoclonal antibodies that are

TABLE I

Characterization of the subunit structure of the purified dihydropyridine receptor from rabbit skeletal muscle

Dihydropyridine receptor was purified as described (17) and analyzed on 5–16% SDS-PAGE using Coomassie Blue staining. The apparent molecular weights ($M_r \times 10^{-3}$) were determined under nonreducing conditions. Value in parentheses is the apparent molecular weight under reducing conditions. The 175,000-Da subunit (α subunit) was identified as the WGA-positive glycoprotein using WGA peroxidase (17). The 170,000-Da subunit (δ subunit) and the 52,000-Da subunit (β subunit) have been identified as substrates for an intrinsic protein kinase in isolated triads (26). The 170,000-Da subunit (δ subunit) has been identified as the dihydropyridine-binding subunit of the receptor (this paper). Nomenclature of the subunits of the dihydropyridine receptor is the same as that of Curtis and Catterall (9) with the addition of δ subunit that was not identified in their publications.

Subunit	M_r	WGA staining	Phosphorylation	Dihydropyridine binding
α	175 (150)	+	−	−
δ	170	−	+	+
β	52	−	+	−
γ	32	−	−	−

able to specifically immunoprecipitate the (+)-[^{3}H]PN200-110-labeled receptor (17). We have also recently shown that the 170,000- and 52,000-Da subunits are phosphorylated by an intrinsic protein kinase in isolated triads but the 175,000- and 32,000-Da subunits are not (26). The apparent molecular

mass of the 170,000-Da subunit remains unchanged with reduction. These results suggest that the 175,000-Da glycoprotein is the α subunit described by Curtis and Catterall (9) because they reported the apparent molecular weight change of the α subunit upon reduction. The two lower molecular weight subunits of our preparation are similar to the β and γ subunits observed by Curtis and Catterall (9). The 170,000-Da subunit that we observed in our preparation of the purified dihydropyridine receptor was not identified by Curtis and Catterall (9) in their preparation of the receptor. In this report, we clearly demonstrate that the binding site for the 1,4-dihydropyridines resides on the 170,000-Da subunit of the receptor; hence, this subunit has been termed the δ subunit.

The 170,000-Da protein may not have been identified earlier in purified preparations of the dihydropyridine receptor due to its sensitivity to proteolytic degradation. In particular, we have identified with monoclonal antibodies a proteolytic fragment of the 170,000-Da subunit that migrates with the mobility of intact receptor on SDS-polyacrylamide gels under nonreducing conditions and shifts to a lower apparent M_r ~150,000 with reduction.[2] This fragment could easily be confused with the high molecular weight glycoprotein subunit.

Ferry *et al.* (13, 14) previously reported that photoaffinity labeling with [^{3}H]azidopine resulted in covalent incorporation of label into proteins of 240,000, 158,000, and 99,000 Da in guinea pig skeletal muscle transverse tubular membranes. Recovery of [^{3}H]azidopine in SDS-polyacrylamide gels was greatly decreased when electrophoresis was performed using reducing conditions compared with recovery in gels performed under nonreducing conditions. The 158,000-Da protein observed by Ferry *et al.* (14) may be identical to the 170,000-Da protein identified as the dihydropyridine-binding subunit in our purified preparation of dihydropyridine receptor because the molecular mass of the 158,000-Da protein did not change with reduction. The significance of the other photoaffinity labeled proteins is as yet unknown because similar proteins have not been observed in purified preparations of the dihydropyridine receptor. Recently, the same group published results indicating that (−)-[^{3}H]azidopine specifically photoaffinity labels only a protein of 155,000 Da in purified dihydropyridine receptor. Based on concanavalin A staining, it was claimed that the 155,000-Da protein was a glycoprotein. However, analysis was performed by SDS-PAGE only under nonreducing conditions. Because our results have shown that the dihydropyridine-binding subunit and the glycoprotein subunit are usually not well resolved on SDS-polyacrylamide gels under nonreducing conditions, it is likely that the protein labeled by this group is actually identical to our 170,000-Da protein. Galizzi *et al.* (15) performed direct photoaffinity labeling of rabbit skeletal muscle transverse tubular membranes with [^{3}H]diltiazem, [^{3}H]bepridil, and (+)-[^{3}H]PN200-110. Electrophoresis of photoaffinity labeled membranes on SDS-polyacrylamide gels under reducing conditions showed that each ligand specifically incorporated into a band of approximately 170,000 Da. Although it is difficult to evaluate the relationship of this protein to the 170,000-Da protein identified in our preparation because no gels run under nonreducing conditions were shown, it seems possible that the proteins are identical. If so, this would place binding sites for the dihydropyridine, phenylalkylamine, and benzothiazipine classes of Ca^{2+} channel blockers all in the 170,000-Da subunit. Further work will be required to prove this hypothesis.

In conclusion, our results demonstrate that the 170,000-Da subunit of the 1,4-dihydropyridine receptor is the dihydropyridine-binding subunit of the receptor. In addition, we have developed a method for resolution of the 170,000-Da dihydropyridine-binding subunit from the 175,000-Da glycoprotein subunit in the absence of reducing agents. This was necessary to establish that only the 170,000-Da subunit was photoaffinity labeled. This method may also be useful in large-scale purification of individual subunits for further biochemical analysis.

Acknowledgments—We acknowledge the expert technical assistance of Alyson Fletcher, Mitchell Gaver, Steven D. Kahl, and Linda Madson of our laboratory. We are grateful to Tamara Pretz for typing this manuscript and to Charles Lovig and Doug Purtle of the University of Iowa Cancer Center Hybridoma Tissue Culture Facilities. We also wish to thank Dr. Alexander Scriabine for the nitrendipine.

[2] A. H. Sharp, T. Imagawa, A. T. Leung, and K. P. Campbell, unpublished observations.

REFERENCES

1. Reuter, H. (1983) *Nature* **301,** 569–574
2. Tsien, R. W. (1983) *Annu. Rev. Physiol.* **45,** 341–358
3. Miller, R. J. (1987) *Science* **235,** 46–52
4. Triggle, D. J. (1981) in *New Perspectives on Calcium Antagonists* (Weiss, G. B., ed) pp. 1–18, American Physiological Society, Bethesda
5. Fleckenstein, A. (1983) *Calcium Antagonism in Heart and Smooth Muscle* pp. 34–108, John Wiley & Sons, New York
6. Janis, R. A., and Scriabine, A. (1983) *Biochem. Pharmacol.* **32,** 3499–3507
7. Janis, R. A., and Triggle, D. J. (1984) *Drug Dev. Res.* **4,** 257–274
8. Fosset, M., Jaimovich, E., Delpont, E., and Lazdunski, M. (1983) *J. Biol. Chem.* **258,** 6086–6092
9. Curtis, B. M., and Catterall, W. A. (1984) *Biochemistry* **23,** 2113–2118
10. Borsotto, M., Barhanin, J., Fosset, M., and Lazdunski, M. (1985) *J. Biol. Chem.* **260,** 14255–14263
11. Flockerzi, V., Oeken, H.-J., Hofmann, F., Pelzer, D., Cavalie, A., and Trautwein, W. (1986) *Nature* **323,** 66–68
12. Flockerzi, V., Oeken, H.-J., and Hofmann, F. (1986) *Eur. J. Biochem.* **161,** 217–224
13. Ferry, D. R., Rombush, M., Goll, A., and Glossmann, H. (1984) *FEBS Lett.* **169,** 112–118
14. Ferry, D. R., Kampf, K., Goll, A., and Glossmann, H. (1985) *EMBO J.* **4,** 1933–1940
15. Galizzi, J.-P., Borsotto, M., Barhanin, J., Fosset, M., and Lazdunski, M. (1986) *J. Biol. Chem.* **261,** 1393–1397
16. Striessnig, J., Moosburger, K., Goll, A., Ferry, D. R., and Glossmann, H. (1986) *Eur. J. Biochem.* **161,** 603–609
17. Leung, A. T., Imagawa, T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 7943–7946
18. Mitchell, R. D., Palade, P., and Fleisher, S. (1983) *J. Cell Biol.* **96,** 1008–1016
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
20. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
21. Glossmann, H., and Ferry, D. (1985) *Methods Enzymol.* **109,** 513–550
22. Laemmli, U. K. (1970) *Nature* **227,** 680–685
23. Towbin, H., Staehlin, T., and Bordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
24. Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H. (1984) *Gene Anal. Techn.* **1,** 3–8
25. McKearn, T. J. (1980) in *Monoclonal Antibodies* (Kennett, R. H., McKearn, T. J., and Bechtol, K. B., eds) pp. 403–404, Plenum Press, New York
26. Imagawa, T., Leung, A. T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 8333–8339