

## Characterization of the 1,4-Dihydropyridine Receptor Using Subunit-specific Polyclonal Antibodies

EVIDENCE FOR A 32,000-Da SUBUNIT\*

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The purified receptor for the 1,4-dihydropyridine  $\text{Ca}^{2+}$  channel blockers from rabbit skeletal muscle contains protein components of 170,000 Da ( $\alpha_1$ ), 175,000 Da ( $\alpha_2$ ), 52,000 Da ( $\beta$ ), and 32,000 Da ( $\gamma$ ) when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. Subunit-specific polyclonal antibodies have now been prepared and used to characterize the association of the 32,000-Da polypeptide ( $\gamma$  subunit) with other subunits of the dihydropyridine receptor. Immunoblot analysis of fractions collected during purification of the dihydropyridine receptor shows that the 32,000-Da polypeptide copurified with  $\alpha_1$  and  $\alpha_2$  subunits at each step of the purification. In addition, monoclonal antibodies against the  $\alpha_1$  and  $\beta$  subunits immunoprecipitate the digitonin-solubilized dihydropyridine receptor as a multisubunit complex which includes the 32,000-Da polypeptide. Polyclonal antibodies generated against both the nonreduced and reduced forms of the  $\alpha_2$  subunit and the  $\gamma$  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  $\alpha_2$  subunit. In addition, polyclonal antibodies against the rabbit skeletal muscle 32,000-Da polypeptide specifically react with similar proteins in skeletal muscle of other species including avian and amphibian species. Thus, our results demonstrate that the 32,000-Da polypeptide ( $\gamma$  subunit) is an integral and distinct component of the dihydropyridine receptor.

Voltage-dependent  $\text{Ca}^{2+}$  channels play key roles in such important physiological processes as excitation-contraction coupling in muscle and excitation-secretion coupling in neuronal and secretory cells (1, 2). Three classes of voltage-dependent  $\text{Ca}^{2+}$  channels, termed L, N, and T types, have been identified by electrophysiological studies (3, 4). L type  $\text{Ca}^{2+}$  channels are highly sensitive to the 1,4-dihydropyridine class of  $\text{Ca}^{2+}$  channel blockers and are present in many excitable tissues (5, 6). Specific, high affinity receptors for radiolabeled dihydropyridines have also been identified in membranes of various excitable tissues (7-10) and are most enriched in skeletal muscle transverse tubular membranes (11, 12). Unlike other tissues, in skeletal muscle the function

of the dihydropyridine receptor as a  $\text{Ca}^{2+}$  channel is unclear (13). It has been proposed that the skeletal muscle dihydropyridine receptor acts primarily as the transverse tubular voltage sensor in excitation-contraction coupling (14).

The dihydropyridine receptor has been purified from skeletal muscle membranes and characterized in a number of laboratories (reviewed in Refs. 15-17). The purified dihydropyridine receptor from rabbit skeletal muscle triads prepared in our laboratory consists of proteins of 175, 170, 52, and 32 kDa when analyzed by SDS-PAGE<sup>1</sup> under nonreducing conditions (18). Similar proteins have been identified in purified preparations of the dihydropyridine receptor in a number of other laboratories (15-17) and have been termed  $\alpha_2$ ,  $\alpha_1$ ,  $\beta$ , and  $\gamma$ , respectively (19). The  $\alpha_1$  subunit (170 kDa) contains binding sites for the dihydropyridine and phenylalkylamine classes of  $\text{Ca}^{2+}$  channel blockers (19-22). This subunit was referred to as the  $\delta$  subunit in our previous publications (20, 23, 24). The  $\alpha_2$  subunit (175 kDa), which was originally thought to be the binding protein for the  $\text{Ca}^{2+}$  channel blockers, has an apparent molecular mass of 150 kDa on SDS-PAGE after reduction of disulfide bonds (18). Recently, we have used monoclonal antibodies against the  $\beta$  subunit (52 kDa) to show that this protein is tightly associated with the dihydropyridine receptor and is a distinct subunit of the receptor and not a proteolytic fragment of a larger subunit (23). Little data is currently available concerning the 32-kDa polypeptide. This protein has not been identified in preparations of dihydropyridine receptor from all laboratories and the relationship of the 32-kDa polypeptide to the larger subunits has not been determined. In addition to the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$  subunits, there is now some evidence for one or more smaller subunits of 24-32 kDa, collectively termed the  $\delta$  subunit (19), which are disulfide linked to the  $\alpha_2$  subunit (19, 25) and may account for the increase in mobility of  $\alpha_2$  observed on SDS-PAGE with reduction.

We have now produced subunit-specific polyclonal antibodies directed against the  $\alpha_2$ ,  $\gamma$ , and  $\delta$  subunits of the dihydropyridine receptor. In this report, we have used these antibodies to examine the association of the 32-kDa polypeptide ( $\gamma$  subunit) with the  $\alpha_1$ ,  $\alpha_2$ , and  $\beta$  subunits of the dihydropyridine receptor and to examine the relationship of the 32-kDa polypeptide ( $\gamma$  subunit) to the larger components of the dihydropyridine receptor. Our results show that the 32-kDa protein ( $\gamma$  subunit) is an integral and distinct component of the

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<sup>1</sup> The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; [<sup>3</sup>H]PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([<sup>3</sup>H]methoxycarbonyl)pyridine-3-carboxylate; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; mAb, monoclonal antibody.

dihydropyridine receptor. Polyclonal antibodies against the rabbit skeletal muscle  $\gamma$  subunit also specifically recognize proteins of similar size in skeletal muscle membranes from other species.

#### EXPERIMENTAL PROCEDURES

**Preparation of Membrane Vesicles**—Heavy microsomes and triads were prepared from skeletal muscle of adult rabbit or other species (except porcine) by a modification of the method of Mitchell *et al.* (26) as previously described (20). Light sarcoplasmic reticulum vesicles were isolated from adult rabbit skeletal muscle in the presence of protease inhibitors by the method of Campbell *et al.* (27). Rabbit skeletal muscle transverse tubular membranes were isolated by the method of Roseblatt *et al.* (28). Rat brain synaptosomes were prepared by the method of Ray *et al.* (29) as modified by Dunn (30). Porcine heavy microsomes were prepared by the method of Meissner (31) as modified by Mickelson *et al.* (32) and were the generous gift of Drs. James R. Mickelson and Charles F. Louis, Department of Veterinary Biology, University of Minnesota. Membrane preparations were characterized for [ $^3$ H]PN200-110 binding (33) and [ $^3$ H]ryanodine binding (34) and stored frozen at  $-135^{\circ}\text{C}$ . Protein was determined by the method of Lowry *et al.* (35) as modified by Peterson (36) using bovine serum albumin as a standard. Detergent-solubilized protein samples were determined by the same method after addition of 50  $\mu\text{l}$  of 1% deoxycholate in 0.1 M NaOH and precipitation with 2.5 ml of 5% trichloroacetic acid.

**Production of Polyclonal Antibodies**—Dihydropyridine receptor was purified by WGA-Sepharose affinity chromatography followed by DEAE-cellulose ion exchange chromatography as previously described (20). Individual components of the receptor were separated by SDS-PAGE on 5–16% gradient gels in the absence (nonreducing condition) or presence of 1% 2-mercaptoethanol (reducing condition). The gels were stained for 5–10 min with Coomassie Blue stain in 10% acetic acid, 25% isopropanol and then destained in distilled water. Individual bands were cut from the gel, homogenized, and used for immunization of guinea pigs according to the method of Tung (37). Guinea pigs were injected intraperitoneally at weekly intervals for 4 weeks with protein bands derived from 10–25  $\mu\text{g}$  of dihydropyridine receptor in Freund's complete adjuvant. They were then injected intraperitoneally each week with Freund's complete adjuvant to induce promotion of ascites. The guinea pigs were boosted with homogenized gel bands from 25–50  $\mu\text{g}$  of dihydropyridine receptor in incomplete Freund's adjuvant or Ribi MPL + TDM adjuvant by subcutaneous and intraperitoneal injections at multiple sites on the back 6–7 weeks after the initial immunization and again 2 weeks later. Ascites were tapped whenever significant amounts accumulated. Total amounts of ascites collected from each animal ranged from 10 to 600 ml. Ascites was centrifuged at 500  $\times g$  for 10 min to remove cells and debris and clarified by treatment with Lipoclean (Behring Diagnostics). Ascites was mixed vigorously with one-half volume of Lipoclean and then centrifuged at 500  $\times g$  for 10 min. The upper layer was collected and centrifuged again at 20,000  $\times g$ . The clarified ascites was aliquoted and stored at  $-20^{\circ}\text{C}$ .

**Preparation of WGA-Void Column**—Digitonin-solubilized triads were depleted of dihydropyridine receptor by extensive adsorption with WGA-Sepharose followed by adsorption on anti-dihydropyridine receptor monoclonal antibody beads until immunoblot analysis failed to detect any remaining receptor. Antibody beads were prepared by overnight incubation of 100 ml of hybridoma culture supernatant containing monoclonal antibody VD2<sub>1</sub> (23) or IIF7 (18) with 1.5 ml of goat anti-mouse IgG Sepharose. Solubilized triads depleted of dihydropyridine receptor were then dialyzed against 0.1% digitonin in 100 mM HEPES (pH 7.5) to remove Tris buffer. Dialyzed, receptor-depleted triads (70 ml, 40 mg of protein) were covalently coupled to 10 ml of Affi-Gel-10 and remaining reactive groups were blocked with ethanolamine according to the manufacturer. Analysis of protein remaining in solution showed that approximately 15 mg of dihydropyridine receptor-depleted triads were bound.

**Preparation of Dihydropyridine Receptor Affinity Column**—Dihydropyridine receptor was purified by WGA-Sepharose affinity chromatography followed by DEAE-cellulose ion exchange chromatography as previously described (20). The purified receptor was exchanged into 0.3% (w/v) digitonin, 100 mM HEPES (pH 7.5), 0.5 M sucrose by gel filtration and  $\text{CaCl}_2$  was added to a concentration of 80 mM. Affi-Gel-10 (2.6 ml) was washed with distilled water, added to the receptor solution (1.2 mg, 14 ml), and allowed to react for 4 h at  $4^{\circ}\text{C}$

with gentle mixing. The gel was pelleted by centrifugation and the supernatant removed. The amount of receptor immobilized on the gel (265  $\mu\text{g}$ ) was determined by quantitation of protein remaining in solution. Remaining reactive groups were blocked by reaction with ethanolamine according to the manufacturer and the gel was then washed thoroughly in 0.3% digitonin, 0.5 M sucrose, 100 mM HEPES (pH 7.5) buffer.

**Preparation of Epidermal Protein-Sepharose Column**—Human epidermis (60 mg) was collected by scraping the surface of the skin with a pocket knife and solubilization buffer (1% Triton X-100 (v/v), 0.25% (v/v) 2-mercaptoethanol, 6 M urea, and 100 mM HEPES (pH 7.5)) (7.5 ml) was added. The solution was heated to boiling, mixed, and allowed to stand overnight. The sample was then heated to boiling again, mixed, and centrifuged at 1000  $\times g$  for 10 min to remove insoluble material. The solubilized epidermal protein (28 mg) was then exchanged into 10.5 ml of 1% Triton X-100, 6 M urea, 100 mM sodium acetate (pH 4.0) by gel filtration. Cyanogen bromide-activated Sepharose 6 MB (1 g) was preswollen in 1 mM HCl for 15 min, washed with distilled water, and added to the solubilized epidermal protein. The suspension was adjusted to pH 8.3 by addition of NaOH and allowed to react 2 h at room temperature with gentle mixing. The beads were then pelleted by brief centrifugation and the supernatant removed. Quantitation of protein still in solution established that 12 mg of epidermal protein was immobilized on the beads. To block remaining reactive sites, the beads were mixed with 10 ml of 1 M ethanolamine (pH 8.0) in 1% Triton X-100 and allowed to react for 2 h at room temperature. The beads were then washed with 1% Triton X-100, 6 M urea, 100 mM HEPES (pH 7.5) followed by exhaustive washing with TBS (20 mM Tris (pH 7.5), 200 mM NaCl) before use.

**Adsorption of Nonspecific Antibodies**—Ascites containing anti-dihydropyridine receptor antibodies to be used without affinity purification was preadsorbed using an epidermal protein-Sepharose column, prepared as described above, to remove interfering anti-keratin antibodies. Ascites was mixed with the epidermal protein-Sepharose overnight at  $4^{\circ}\text{C}$  in the presence of 0.02%  $\text{NaN}_3$ . The epidermal protein-Sepharose was then poured into a column and the ascites was collected. Ascites from guinea pig 5, against the reduced  $\alpha_2$  subunit was always treated in this manner and used without further treatment. Epidermal protein-column adsorbed guinea pig 5 ascites was designated GP5SA. The epidermal protein-Sepharose was regenerated by washing with 1% Triton X-100, 6 M urea, 100 mM sodium acetate (pH 4.0) followed by exhaustive washing with TBS before use. Ascites from guinea pig 13 against the nonreduced  $\alpha_2$  subunit was diluted 20-fold in 50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.4), 0.9% NaCl and adsorbed on epidermal protein-Sepharose as described above, followed by adsorption on a WGA-void column prepared from triads depleted of dihydropyridine receptor as described above. After this treatment, the guinea pig 13 ascites were designated GP13VSA. Adsorbed ascites was stored at  $4^{\circ}\text{C}$  after addition of 0.02–0.05%  $\text{NaN}_3$  or frozen at  $-20^{\circ}\text{C}$ . GP5SA and GP13VSA ascites was used in immunoblot assays at a final dilution of 1:500–1:1000. Alternatively, guinea pig 13 antibodies against the nonreduced  $\alpha_2$  subunit were affinity purified as described below. All other antibodies were affinity purified.

**Affinity Purification of Subunit-specific Polyclonal Antibodies**—Polyclonal antibodies against the nonreduced form of the  $\alpha_2$  subunit and the nonreduced and reduced forms of the  $\gamma$  subunit were affinity purified using a dihydropyridine receptor affinity column. Dihydropyridine receptor-Affi-Gel (2.6 ml) was packed into a column and preequilibrated with 100 mM NaCl in Tris buffer (50 mM Tris, pH 7.4). Guinea pig ascites was thawed and sodium azide (0.02%), PMSF (0.2 mM), and benzamide (0.8 mM) were added. Ascites was then cycled through the dihydropyridine receptor-Affi-Gel column overnight at  $4^{\circ}\text{C}$ . The column was washed with 20 ml of 100 mM NaCl in Tris buffer by 40 ml of 500 mM NaCl in Tris buffer and a final wash of 100 mM NaCl in Tris buffer before elution. To elute bound antibodies, 1.5 ml of elution buffer (50 mM glycine HCl (pH 2.5), 300 mM NaCl) was applied and allowed to stand on the column for 10 min before flow of the same buffer was resumed. Fractions of 1.5 ml were collected and the pH adjusted immediately to pH 7.5 with 2 M Tris (pH 8.0). Protein was estimated by absorbance at 280 nm and peak fractions were pooled. BSA (fatty acid free grade) (1–2 mg/ml) and sodium azide (0.02%) were added and the antibody solution was stored at  $4^{\circ}\text{C}$ . Affinity-purified antibody was used for staining of immunoblots at 30- to 50-fold dilution in 3% (w/v) BSA in TBS (200 mM NaCl, 20 mM Tris (pH 7.5)).

Antibodies against the 24- and 20-kDa  $\delta$  subunits were affinity

purified using blots of individual proteins separated by SDS-PAGE essentially according to Olmsted (38). Purified dihydropyridine receptor (370  $\mu$ g) was subjected to SDS-PAGE on gels with single wells of nearly the same width as the gel. The separated proteins were then transferred electrophoretically to Immobilon-P membranes. Vertical strips were cut from the blots and stained with antibodies against the nonreduced form of the  $\alpha_2$  subunit (guinea pig 13) to identify the  $\delta$  subunit bands. Blot strips containing the  $\delta$  subunit bands were cut from the transfer and blocked with TBS-BSA. Ascites from guinea pig 13 (0.5 ml) was diluted 4-fold in 150 mM NaCl, 15 mM Tris (pH 7.4) and incubated with the blot strips overnight at 4 °C. The strips were then washed three times for 10–15 min in 500 mM NaCl, 50 mM Tris (pH 7.4) followed by a final 10-min wash in 100 mM NaCl, 50 mM Tris (pH 7.4). Bound antibodies were eluted by incubation of the strips in 50 mM glycine HCl (pH 2.5). After a 10-min incubation with gentle mixing, the eluate was removed from the blot strips and adjusted to pH 7.5 by addition of 2 M Tris (pH 8.0). Protein was estimated by absorbance at 280 nm and BSA and sodium azide were then added to final concentrations of 2 mg/ml and 0.02%, respectively. Antibodies were stored at 4 °C. Antibodies, affinity purified using blot strips of the 24-kDa protein, were designated GP13 $\delta$ L, and those affinity purified using blot strips of the 20-kDa protein were designated GP13 $\delta$ S.

**Immunoprecipitation of the Dihydropyridine Receptor**—Monoclonal antibodies against the dihydropyridine receptor have been described previously (18, 23). Monoclonal antibody beads were produced by incubation of 50 ml of hybridoma culture supernatant overnight with 1 ml of goat anti-mouse-Sepharose beads that had been diluted to a binding capacity of 1 mg of IgG/ml of beads with Sepharose CL-4B. The beads were then precipitated by centrifugation, the supernatant removed, and the incubation repeated with another 50-ml portion of culture supernatant. The beads were washed three times with 50 ml of PBS (0.9% NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4)). Rabbit skeletal muscle triads were solubilized with 1% digitonin, 0.5 M NaCl, 50 mM Tris (pH 7.4) as described previously (15) in the presence of 0.1 mM PMSF, 0.8 mM benzamide, and 1  $\mu$ M pepstatin A. Solubilized triads were diluted 5-fold in 50 mM Tris (pH 7.4) and 1 ml of diluted triad solution was mixed with 100  $\mu$ l (packed volume) of monoclonal antibody beads or WGA-Sepharose beads at 4 °C for 3 h. After precipitation of beads by centrifugation (5s at 13,000  $\times$  g), the supernatants were aspirated and the beads were washed three times with ice-cold TBS plus 0.1% digitonin. SDS gel sample buffer was then added to the beads, the suspension was boiled for 2 min, and the beads were precipitated by centrifugation. Supernatants were then subjected to SDS-PAGE on 5–16% gradient gels and the separated proteins were transferred to nitrocellulose membranes. Nitrocellulose blots were then subjected to immunoblot analysis.

**Preparation of Partially Purified Dihydropyridine Receptor from Membranes of Various Species**—Membrane (10-mg) samples were solubilized at a protein concentration of 4 mg/ml with 1% digitonin in 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.4 M sucrose and the following protease inhibitors: pepstatin A (0.6  $\mu$ g/ml), aprotinin (0.5  $\mu$ g/ml), iodoacetamide (18.5  $\mu$ g/ml), leupeptin (0.5  $\mu$ g/ml), benzamide (0.75 mM), and PMSF (0.1 mM). After incubation in the digitonin solution at 4 °C for 1 h, the membranes were centrifuged at 85,000  $\times$  g for 30 min to remove insoluble material. The solubilized membranes were added to 0.5 ml (packed volume) of WGA-Sepharose 6MB and the suspension was mixed at 4 °C overnight. The WGA-Sepharose was then washed three times with 1 ml of 0.1% digitonin in 50 mM Tris-HCl (pH 7.4), 0.5 M sucrose, 0.5 M NaCl, 0.75 mM benzamide, and 0.1 mM PMSF followed by three additional washes using the same buffer without NaCl. Bound proteins were then eluted from the washed beads by mixing for 1 h at 4 °C with 1 ml of 300 mM GlcNAc in 0.1% digitonin, 50 mM Tris-HCl (pH 7.4), 0.5 M sucrose, 0.75 mM benzamide, and 0.1 mM PMSF. The beads were removed by brief centrifugation and the supernatant was collected.

**SDS-PAGE and Immunoblot Analysis**—SDS-PAGE was performed using the system of Laemmli (39) on 1.5-mm thick 5–16% gradient polyacrylamide gels. Gels were either stained with Coomassie Blue, transferred to nitrocellulose, or transferred to Immobilon-P membranes according to Towbin *et al.* (40). Blots were blocked with 3% BSA (fraction V) in TBS (TBS-BSA) before overnight incubation at 4 °C with guinea pig antibodies in the same solution. Blots were then washed with TBS-BSA twice for 10 min each and incubated with horseradish peroxidase-linked rabbit anti-guinea pig secondary antibody for 1 h at room temperature. Blots were washed twice again and then developed using 4-chloro-1-naphthol as the substrate. Alternatively, <sup>125</sup>I-labeled protein A was used in place of secondary

antibody at a concentration of 250,000 dpm/ml. Following incubation, blots were washed three times in TBS, dried, and placed on Kodak XAR-5 film to identify antibody-reactive bands.

**Materials**—<sup>3</sup>H]PN200-110 was obtained from Amersham Corp. and <sup>3</sup>H]ryanodine was from Du Pont-New England Nuclear. <sup>125</sup>I-labeled protein A was obtained from ICN Radiochemicals. Electrophoretic reagents were from Bio-Rad and molecular weight standards were from Bethesda Research Laboratories. Nitrocellulose and Immobilon-P transfer membranes were from Millipore. Peroxidase-conjugated secondary antibodies were from Boehringer Mannheim. Lipoclean was from Behring Diagnostics. Ribi MLP + TDM adjuvant was from Ribi ImmunoChem Research. Bovine serum albumin (fatty acid-free grade and fraction V grade), protease inhibitors, and WGA-Sepharose were from Sigma. Gel filtration columns were prepacked PD-10 columns from Pharmacia LKB Biotechnology Inc. Digitonin was from Fisher or Sigma and soluble digitonin was prepared as described previously (20). All other chemicals were of reagent grade quality.

## RESULTS

**Production of Subunit-specific Polyclonal Antibodies**—Dihydropyridine receptor was purified by WGA-Sepharose affinity chromatography followed by DEAE-cellulose ion exchange chromatography as described previously (20). Individual components of the receptor were separated by SDS-polyacrylamide gel electrophoresis on 5–16% gradient gels under non-reducing or reducing conditions. Under nonreducing conditions the 175-kDa ( $\alpha_2$  subunit) and 170-kDa ( $\alpha_1$  subunit) bands were fully resolved by running the dye front and the lower molecular mass standards off the gel. Individual bands were cut from the gel, homogenized in Freund's adjuvant, and used for immunization of guinea pigs according to the method of Tung (37). This method results in the production of ascites containing the polyclonal antibodies. Antibodies were raised against the nonreduced (175-kDa) and reduced (150-kDa) forms of the  $\alpha_2$  subunit and the nonreduced and reduced forms of the 32-kDa  $\gamma$  subunit. Initial immunoblot testing of the polyclonal ascites revealed the presence of interfering antibodies, especially antibodies against keratin, a common contaminant of SDS-polyacrylamide gels (41). To remove interfering antibodies, ascites was passed through an epidermal protein-Sepharose column, prepared as described under "Experimental Procedures." Alternatively, specific anti-dihydropyridine receptor antibodies were prepared by affinity purification as described under "Experimental Procedures."

**Immunoblot Characterization of 32-kDa Protein in Rabbit Skeletal Muscle Membranes**—Initial characterization of the 32-kDa protein of the dihydropyridine receptor in skeletal muscle membranes was performed using affinity-purified antibodies against the nonreduced 32-kDa protein. For comparison, the  $\alpha_2$  subunit was also examined using epidermal protein-column adsorbed antiserum against the reduced 150-kDa protein. In Fig. 1, these antibodies each reacted with a single band of the expected molecular weight in triads. Staining was completely absent in light sarcoplasmic reticulum membranes and was more intense in transverse tubular membranes consistent with the distribution of <sup>3</sup>H]PN200-110 binding and our previous results on the  $\alpha_1$  subunit of the dihydropyridine receptor (18). These results exemplify the specificity of the antibodies used and demonstrate that the 32-kDa protein is distinct from other protein components of the dihydropyridine receptor.

**Immunoblot Analysis of Fractions from the Purification of the Dihydropyridine Receptor**—Various fractions collected during a typical purification of the dihydropyridine receptor were subjected to immunoblot analysis to determine whether the 32-kDa protein copurifies at each step with the 170-kDa protein ( $\alpha_1$  subunit), which is known to be the dihydropyridine binding component of the receptor, and with the 150-kDa

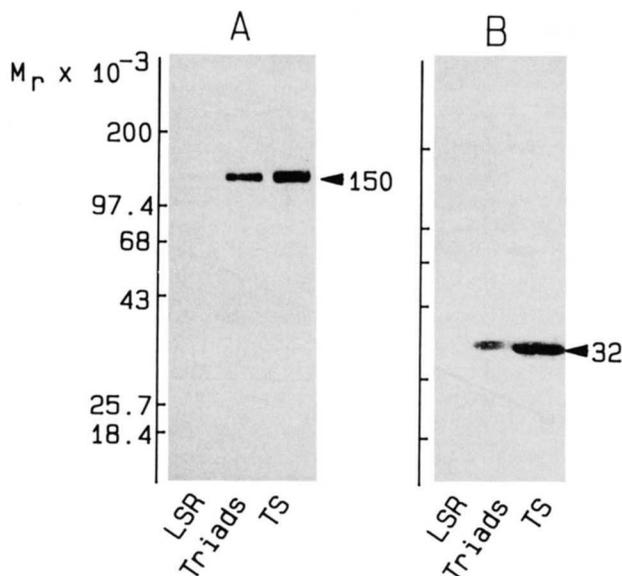


FIG. 1. Immunoblot staining of  $\alpha_2$  and  $\gamma$  subunits in rabbit skeletal muscle membrane fractions. Isolated rabbit skeletal muscle membrane fractions, light sarcoplasmic reticulum (LSR), triads (Triads), and transverse tubular system (TS) (50  $\mu$ g each) were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Indirect immunoperoxidase staining of the nitrocellulose blots was performed as described under "Experimental Procedures" using anti- $\alpha_2$  subunit antiserum (GP5SA, 1:1000 dilution) (A) or affinity-purified anti- $\gamma$  subunit antibody (GP16AP, 1:30 dilution) (B). Arrowheads indicate the position of the 150-kDa  $\alpha_2$  subunit (150) (A) and the 32-kDa  $\gamma$  subunit (32) (B).

protein ( $\alpha_2$  subunit) of the receptor. The 170-kDa protein was identified by staining with monoclonal antibody IIC12, which has been previously described (18). Fig. 2 shows that the 170-, 150-, and 32-kDa proteins are each present in triads and are enriched in the GlcNAc-eluted fraction from the WGA-Sepharose. The elution profile of the 32-kDa protein from the DEAE-cellulose column was identical and matched the elution profile of the 170- and 150-kDa proteins. The elution profiles were also consistent with the elution profile of dihydropyridine receptor labeled with [ $^3$ H]PN200-110 (not shown). Therefore, the 32-kDa protein copurifies with the dihydropyridine receptor at each step of the purification.

**Immunoprecipitation of the 32-kDa Protein of the Dihydropyridine Receptor by Anti- $\alpha_1$  and Anti- $\beta$  Subunit Monoclonal Antibodies**—To confirm a tight association between the 170-, 150-, 52-, and 32-kDa proteins, we examined the ability of antibodies directed against the 170- and 52-kDa subunits to immunoprecipitate the 150- and 32-kDa proteins (Fig. 3). Solubilized triads containing approximately 20 pmol of [ $^3$ H]PN200-110 binding sites/mg of protein were incubated with monoclonal antibody beads preformed as described under "Experimental Procedures." After washing, the beads were extracted with SDS gel sample buffer and the immunoprecipitates were analyzed by immunoblot analysis (Fig. 3B). The 150- and 32-kDa proteins were both present in the anti-170- and anti-52-kDa immunoprecipitates (lanes 2 and 3, respectively) and absent from the immunoprecipitates obtained with an unrelated antibody (lane 1). As a positive control, WGA-Sepharose precipitates were also included (lane 4). This data confirms that the 170-, 150-, 52-, and 32-kDa proteins are tightly associated and suggests that the 150- and 32-kDa proteins are subunits of the dihydropyridine receptor.

**The 175/150-kDa and the 32-kDa Proteins Are Immunologically Distinct**—Schmid *et al.* (25) reported a 32-kDa protein which appears only after reduction of the dihydropyridine

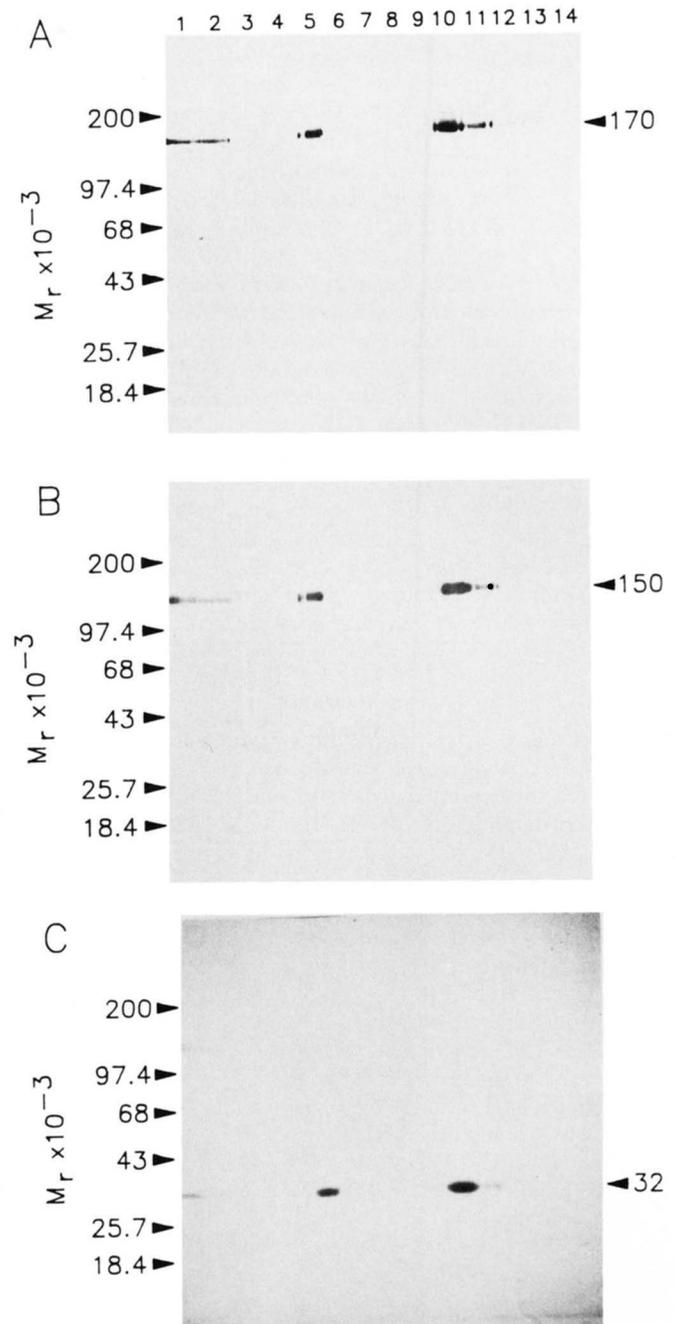
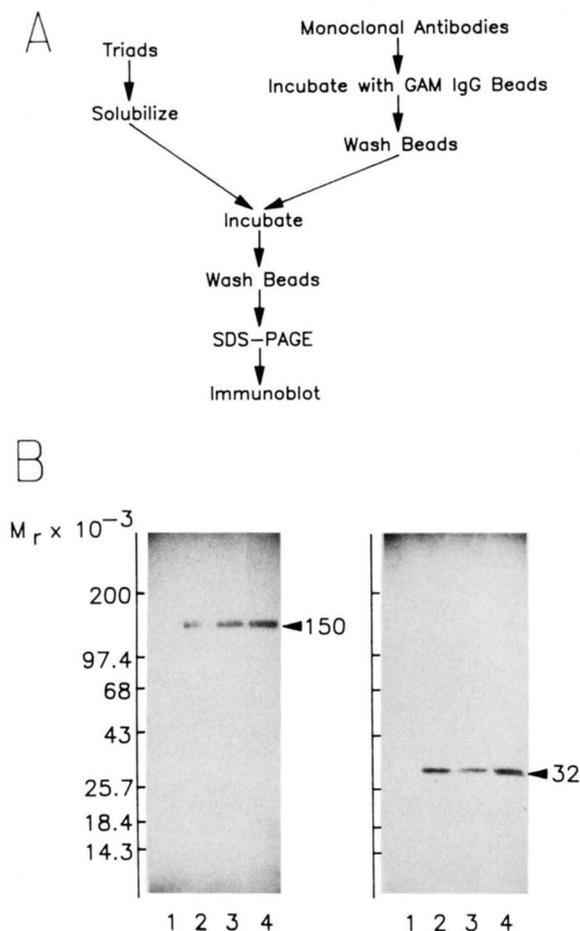


FIG. 2. Copurification of  $\alpha_1$  (170-kDa),  $\alpha_2$  (150-kDa), and  $\gamma$  (32-kDa) subunits of the dihydropyridine receptor. Dihydropyridine receptor was purified from rabbit skeletal muscle triads as previously described (20) by wheat germ agglutinin affinity chromatography followed by DEAE-cellulose ion exchange chromatography. Aliquots of various fractions from the purification (50  $\mu$ l each) were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The blots were stained by the indirect immunoperoxidase method as described under "Experimental Procedures" using monoclonal antibody IIC12, anti- $\alpha_1$  subunit (A); GP5SA, polyclonal anti- $\alpha_2$  subunit antiserum (B); or GP16AP, affinity-purified polyclonal anti- $\gamma$  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 0-300 mM NaCl gradient elution of DEAE-cellulose column (fraction 1, no NaCl; fraction 22, 300 mM NaCl). Arrowheads indicate the positions of the 170-kDa  $\alpha_1$  subunit (170) (A), the 150-kDa  $\alpha_2$  subunit (150) (B), and the 32-kDa  $\gamma$  subunit (32) (C).



**FIG. 3. Coimmunoprecipitation of the  $\alpha_2$  and  $\gamma$  subunits by anti- $\alpha_1$  and anti- $\beta$  subunit antibodies.** Isolated triads were solubilized with 1% digitonin and incubated with mAb goat anti-mouse (GAM) IgG-Sepharose or WGA-Sepharose as described under "Experimental Procedures." *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 mAb IID8 (anti-cardiac  $\text{Ca}^{2+}$  +  $\text{Mg}^{2+}$ -ATPase) (lane 1); a mixture of mAbs IID5 and IIC12 (anti- $\alpha_1$  dihydropyridine receptor subunit) (lane 2); and mAb VD<sub>21</sub> (anti- $\beta$  subunit) (lane 3). As a positive control, precipitation was also performed using WGA-Sepharose 6MB (lane 4). The nitrocellulose blots were stained using the indirect immunoperoxidase method as described under "Experimental Procedures" using polyclonal antiserum GP5SA against the  $\alpha_2$  subunit (left panel) or GP16AP affinity-purified antibody against the  $\gamma$  subunit (right panel). Arrowheads on far right show the positions of the 150-kDa  $\alpha_2$  subunit and the 32-kDa  $\gamma$  subunit.

receptor and which is apparently disulfide linked to the  $\alpha_2$  subunit. To investigate the relationship of the 32-kDa protein of our preparation to the protein reported by Schmid *et al.* as well as to the larger protein components of the dihydropyridine receptor, we have produced polyclonal antibodies against both the nonreduced and reduced forms of the 175/150-kDa protein and the 32-kDa protein. Fig. 4 shows immunoblot analysis of the dihydropyridine receptor using these antibodies. Purified dihydropyridine receptor was subjected to SDS-PAGE under either nonreducing (20 mM NEM) or reducing (20 mM DTT) conditions and transferred to Immobilon-P transfer membranes. The transfer membranes were then probed with antibodies against the nonreduced 32-kDa  $\gamma$  subunit, the reduced 32-kDa  $\gamma$  subunit, the reduced 150-kDa  $\alpha_2$  subunit, and the nonreduced 175-kDa form of the  $\alpha_2$  subunit (Fig. 4, A–D, respectively). Antibodies against either

the nonreduced or reduced form of the 32-kDa protein did not stain other components of the dihydropyridine receptor on immunoblots prepared under either nonreducing or reducing conditions (Fig. 4, A and B). Antibodies against the reduced  $\alpha_2$  subunit (150-kDa protein) stained only the nonreduced 175-kDa form and the reduced 150-kDa form of the  $\alpha_2$  subunit (Fig. 4C) and no other components of the receptor. Antibodies against the nonreduced 175-kDa  $\alpha_2$  subunit stained the nonreduced 175-kDa and the reduced 150-kDa forms of the  $\alpha_2$  subunit, but did not stain the 32-kDa protein even after reduction of the receptor (Fig. 4D). These results show that the 32-kDa  $\gamma$  subunit and 175/150-kDa subunit are distinct components of the dihydropyridine receptor and that the 32-kDa 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.

Antibodies against the nonreduced 175-kDa form of the  $\alpha_2$  subunit stained proteins of 24 and 20 kDa in addition to the 150-kDa form of the  $\alpha_2$  subunit after reduction of disulfide bonds (Fig. 4D). To further investigate the relationship of the 24- and 20-kDa proteins to the  $\alpha_2$  subunit and to each other, antibodies against the 24- and 20-kDa proteins were affinity purified from whole ascites against the nonreduced 175-kDa  $\alpha_2$  subunit using transfer membrane strips containing either the isolated 24-kDa protein or the 20-kDa protein. Affinity-purified antibodies against the 24-kDa protein stained only the 175-kDa band on the nonreduced portion of an immunoblot (Fig. 4E). After reduction of the dihydropyridine receptor with DTT, the antibodies recognized both the 24- and 20-kDa proteins. A third, faintly stained band at 16 kDa was observed when larger amounts of the dihydropyridine receptor were present on the blots (not shown). The reduced 150-kDa form of the  $\alpha_2$  subunit was not recognized by these antibodies. Affinity-purified antibodies against the 20-kDa protein produced exactly the same results as affinity-purified antibodies against the 24-kDa protein (Fig. 4F). These results suggest that the 24- and 20-kDa proteins are closely related in structure and are disulfide linked to the 150-kDa  $\alpha_2$  subunit.

**Immunoblot Detection of  $\gamma$  and  $\alpha_2$  Subunits of Various Species**—To determine whether dihydropyridine receptors of other species have  $\gamma$  subunits similar to the rabbit  $\gamma$  subunit, triads were prepared from guinea pig and chicken skeletal muscle and tested for cross-reactivity with affinity-purified polyclonal antibodies against the rabbit skeletal muscle  $\gamma$  subunit by immunoblot analysis. Fig. 5 shows that proteins similar to the rabbit  $\gamma$  subunit (lane 1) were labeled by the anti- $\gamma$  subunit antibodies in both guinea pig and chicken triads (lanes 2 and 3). Reaction was blocked by preincubation of the antibodies with purified rabbit skeletal muscle dihydropyridine receptor in each case (lanes 4–6, respectively) indicating that the proteins detected are labeled by specific immunoreaction with the antibodies.

To increase the sensitivity of detection of dihydropyridine receptor components in other species, the receptor was partially purified from microsomal membranes or triads by WGA-Sepharose affinity chromatography after solubilization with digitonin. Partially purified receptor was then subjected to SDS-PAGE, transferred to Immobilon-P transfer membranes, and stained with affinity-purified antibodies against the  $\gamma$  subunit. By using this method, immunoreactive skeletal muscle proteins were detected in a wide variety of species including guinea pig, dog (Fig. 6A), pig (Fig. 6B), chicken (Fig. 7A), and frog (Fig. 7B). The immunoreaction was blocked in each case by preincubation of the antibody with purified rabbit skeletal muscle dihydropyridine receptor, in-

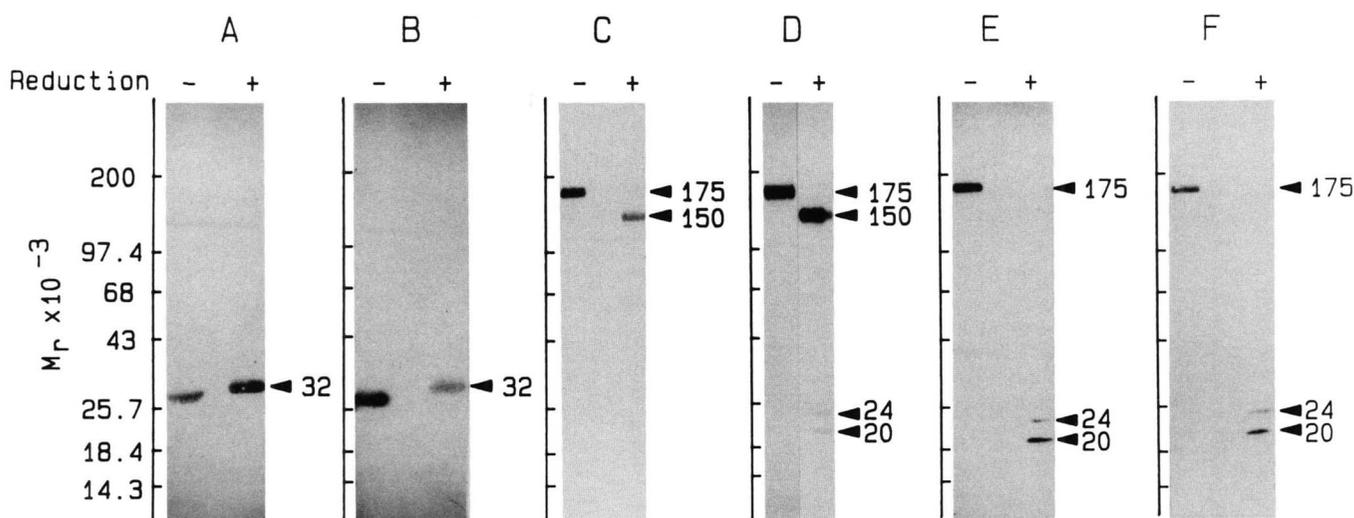


FIG. 4. Immunoblot analysis of purified dihydropyridine receptor. Dihydropyridine receptor was purified as described previously (20) and subjected to SDS-PAGE under nonreducing (20 mM NEM) or reducing (20 mM DTT) conditions. Each lane contained 3  $\mu$ g of dihydropyridine receptor except lanes B (6  $\mu$ g/lane) and D (2  $\mu$ g/lane). Proteins were then transferred to Immobilon-P membranes and stained by the indirect immunoperoxidase method as described under "Experimental Procedures." Blots were stained with: A, GP16AP, affinity-purified antibodies against the nonreduced 32-kDa  $\gamma$  subunit; B, GP11AP affinity-purified antibodies against the reduced 32-kDa  $\gamma$  subunit; C, GP5SA epidermal protein-column adsorbed antiserum against the reduced 150-kDa  $\alpha_2$  subunit; D, GP13AP affinity-purified antibody against the nonreduced 175-kDa form of the  $\alpha_2$  subunit; E, GP13 $\delta$ L antibody against the 24-kDa  $\delta$  subunit affinity purified from whole GP13 antiserum against the nonreduced 175-kDa  $\alpha_2$  subunit; F, GP13 $\delta$ S antibody against the 20-kDa  $\delta$  subunit affinity purified from whole GP13 antiserum.

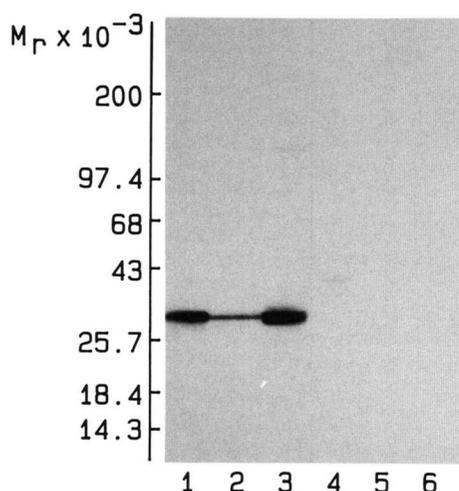


FIG. 5. Identification of  $\gamma$  subunit in guinea pig and chicken skeletal muscle triads. Isolated triads were subjected to SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes. The membranes were stained by incubation with GP16AP, affinity-purified anti- $\gamma$  subunit antibodies (lanes 1-3) or with an identical aliquot of antibody to which was previously added excess purified rabbit skeletal muscle dihydropyridine receptor (lanes 4-6). After washing, the blots were incubated with  $^{125}$ I-protein A, washed again, dried, and placed on film as described under "Experimental Procedures." Membrane samples were: lanes 1 and 4, rabbit skeletal muscle triads (100  $\mu$ g); lanes 2 and 5, guinea pig skeletal muscle triads (250  $\mu$ g); and lanes 3 and 6, chicken skeletal muscle triads (250  $\mu$ g). Film was exposed for 5 days.

dicating specificity of the reactions. In pig,  $\gamma$  subunits slightly smaller than the rabbit skeletal muscle  $\gamma$  subunit were detected in partially purified dihydropyridine receptor from both vastus intermedius muscle, composed of approximately 75% type I (slow) muscle fibers (42) and longissimus dorsi muscle, composed of approximately 80% type II (fast) muscle fibers (42) (Fig. 5B, lanes 2 and 3, respectively). No staining by anti-

$\gamma$  subunit antibodies was observed in WGA concentrated proteins prepared from digitonin extracts of brain and cardiac membranes.

Antibodies against the rabbit skeletal muscle  $\alpha_2$  subunit were also used to detect  $\alpha_2$  subunits in membranes from a variety of sources. Proteins similar in size to the rabbit  $\alpha_2$  subunit were detected in chicken and frog triads (Fig. 8, A and B, respectively) and rat brain synaptosomes (Fig. 8C). In each case, the stained bands shifted to a lower apparent molecular mass after reduction of disulfide bonds, analogous to the rabbit skeletal muscle  $\alpha_2$  subunit. Lanes containing rabbit skeletal muscle triads are included for comparison.

#### DISCUSSION

The skeletal muscle dihydropyridine receptor from a number of laboratories has been shown to consist of at least four components of about 155-220, 130-150, 52-65, and 30-32 kDa on SDS-PAGE after reducing conditions (15-17). These proteins have been termed the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$  subunits, respectively (19). The 170-kDa protein ( $\alpha_1$  subunit) has been shown to contain the binding site for the dihydropyridines (20). The  $\alpha_2$  subunit and the  $\gamma$  subunit have been shown to be heavily glycosylated (19). Monoclonal antibodies against the 170-kDa  $\alpha_1$  subunit and the 52-kDa  $\beta$  subunit have been shown to immunoprecipitate the [ $^3$ H]PN200-110-labeled dihydropyridine receptor. In addition, these antibodies have been used to show that the  $\beta$  subunit copurifies with the dihydropyridine receptor at each step of purification (23). Since the  $\alpha_1$  subunit has been shown to have significant sequence homology to the sodium channel  $\alpha$  subunit (43), this subunit may form a  $\text{Ca}^{2+}$  channel pore. Our laboratory and others have shown that the  $\alpha_1$  and  $\beta$  subunits are substrates for a variety of protein kinases (reviewed in Refs. 15-17), thus providing a possible mechanism for regulation of channel activity. In contrast, no likely function has been identified for the  $\alpha_2$  and  $\gamma$  subunits. Sieber *et al.* (22, 44) reported that the proportion of  $\alpha_2$  subunit protein appeared to vary in different preparations of purified

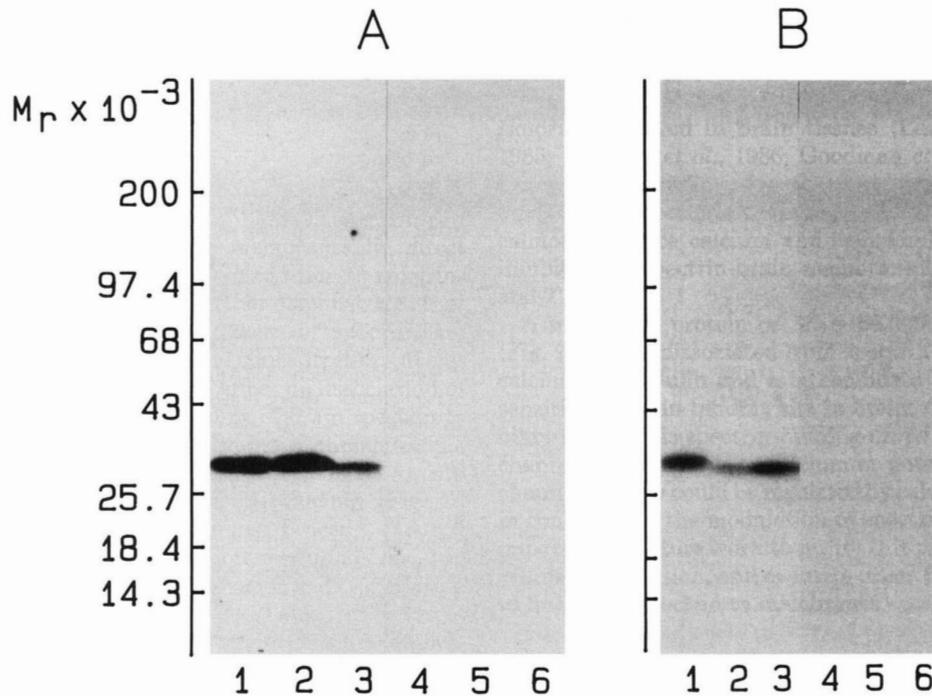


FIG. 6. Identification of  $\gamma$  subunit in partially purified dihydropyridine receptor from skeletal muscle membranes of various mammalian species. Membranes from various sources were solubilized with digitonin and partially purified dihydropyridine receptor was prepared as described under "Experimental Procedures." The partially purified dihydropyridine receptor was subjected to SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes. The transfer blots were then reacted with antibodies and the reactive bands visualized by autoradiography as described in the legend of Fig. 5. Lanes 1–3 were stained with GP16AP, affinity-purified antibody against the  $\gamma$  subunit of rabbit skeletal muscle dihydropyridine receptor. Lanes 4–6 were stained with an identical aliquot of antibody adsorbed with purified rabbit skeletal muscle dihydropyridine receptor. A, samples were: lanes 1 and 4, purified rabbit skeletal muscle dihydropyridine receptor (1  $\mu$ g); lanes 2 and 5, partially purified dihydropyridine receptor from guinea pig skeletal muscle triads (60  $\mu$ l); lanes 3 and 6, partially purified dihydropyridine receptor from dog skeletal muscle microsomes (60  $\mu$ l). Film was exposed for 18 h with a Du Pont Cronex Lightning Plus intensifying screen. B, samples were: lanes 1 and 4, purified rabbit skeletal muscle dihydropyridine receptor (1.5  $\mu$ g); lanes 2 and 5, partially purified dihydropyridine receptor from pig vastus intermedius muscle heavy microsomes (60  $\mu$ l); lanes 3 and 6, partially purified dihydropyridine receptor from pig longissimus dorsi muscle heavy microsomes (60  $\mu$ l). Film was exposed for 18 h with intensifying screen.

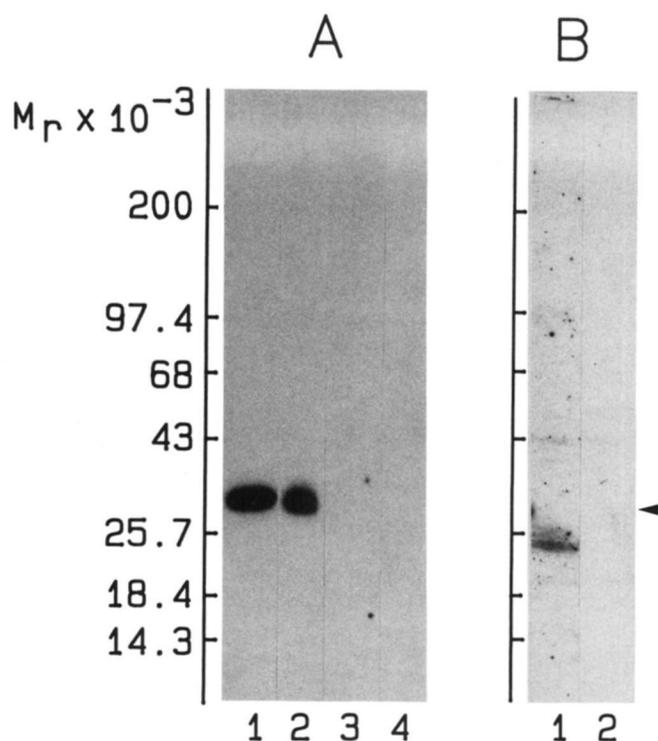
receptor and regarded the  $\alpha_2$  protein to be a contaminant of the dihydropyridine receptor. The  $\gamma$  subunit protein has not been reported in purified preparations of the dihydropyridine receptor from all laboratories (25, 45) and its relationship to the larger subunits has been previously unknown. Furthermore, the  $\alpha_2$  and  $\gamma$  subunits have not previously been rigorously shown to copurify with the 170-kDa dihydropyridine binding protein at each step of purification. For these reasons, there has still been confusion concerning the subunit composition of the dihydropyridine receptor and the role of the various proteins that have been reported in purified preparations of the receptor (46, 47).

In this report, we have produced polyclonal antibodies against the 32-kDa polypeptide ( $\gamma$  subunit) of the rabbit skeletal muscle dihydropyridine receptor and used them to characterize the association of the 32-kDa polypeptide with other subunits of the dihydropyridine receptor. We first examined the association of the  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$  subunits. In Fig. 2, the polyclonal antibodies were used to show that the 32-kDa polypeptide copurifies with the dihydropyridine binding subunit ( $\alpha_1$ ) and the  $\alpha_2$  subunit through WGA-Sepharose affinity chromatography and DEAE-cellulose ion exchange chromatography. In addition, the 32,000-Da polypeptide was immunoprecipitated by monoclonal antibodies against the  $\alpha_1$  subunit and by monoclonal antibodies against the  $\beta$  subunit (Fig. 3). These monoclonal antibodies have previously been

shown to immunoprecipitate the [ $^3$ H]PN200-110-labeled dihydropyridine receptor (18, 23). These results provide strong evidence that the digitonin-solubilized dihydropyridine receptor exists as a tight complex of  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$  subunits.

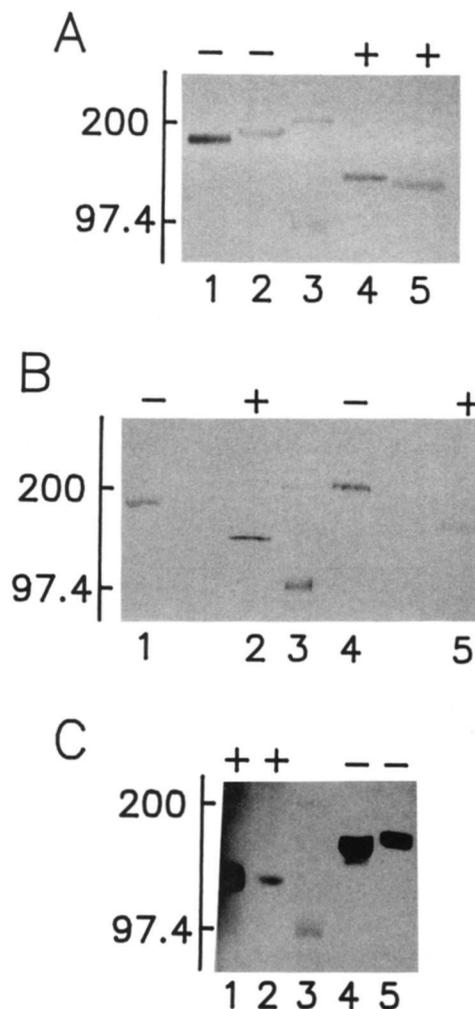
The antibodies against the  $\alpha_2$  and  $\gamma$  subunits were not used to directly immunoprecipitate the radioligand-labeled dihydropyridine receptor because these antibodies appear to preferentially bind denatured forms of the receptor, making immunoprecipitation inefficient. This preference is probably due to the fact that the antibodies were raised against proteins purified by SDS-PAGE. It also seems possible that the antibodies may inhibit radioligand binding or cause dissociation of the subunits by stabilizing nonnative conformations of the receptor. We are currently examining these possibilities.

We have also used the polyclonal antibodies to examine the relationship of the  $\gamma$  subunit to the larger subunits and to proteins reported in the purified dihydropyridine receptor preparations of other laboratories. In particular, Schmid *et al.* (25) demonstrated the presence of a 32-kDa protein in a preparation that was enriched in [ $^3$ H]dihydropyridine binding activity. This protein appeared only upon reduction and appeared to be disulfide linked to a protein with similar electrophoretic behavior to our  $\alpha_2$  subunit. To investigate the relationship of this protein to our 32-kDa  $\gamma$  subunit and  $\alpha_2$  subunit, we prepared polyclonal antibodies against both the nonreduced and reduced forms of the  $\alpha_2$  and  $\gamma$  subunits. We



**FIG. 7. Identification of  $\gamma$  subunit in partially purified dihydropyridine receptor from solubilized chicken and frog triads.** Chicken skeletal muscle triads and frog skeletal muscle triads were solubilized, and partially purified dihydropyridine receptor was prepared as described under "Experimental Procedures." Immunoblots were reacted with antibodies and visualized by autoradiography as described in the legend of Fig. 5. In *A*, lanes 1 and 3 contained 1.5  $\mu$ g of purified rabbit skeletal muscle dihydropyridine receptor; lanes 2 and 4 contained partially purified dihydropyridine receptor (60  $\mu$ l) from chicken skeletal muscle triads. Lanes 1 and 2 were reacted with GP16AP affinity-purified antibody against the  $\gamma$  subunit of the rabbit skeletal muscle dihydropyridine receptor and lanes 3 and 4 were reacted with GP16AP antibody previously adsorbed with purified rabbit skeletal muscle dihydropyridine receptor. Film was exposed for 20 h with intensifying screen. In *B*, lanes 1 and 2 contained partially purified dihydropyridine receptor from frog skeletal muscle triads (120  $\mu$ l). Lane 1 was stained with GP16AP antibody and lane 2 was stained with dihydropyridine receptor-adsorbed antibody. The arrowhead indicates the position of rabbit skeletal muscle  $\gamma$  subunit on these blots. Film was exposed for 3 days with intensifying screen.

have found that antibodies against the nonreduced and reduced forms of the  $\alpha_2$  subunit do not react with the 32-kDa  $\gamma$  subunit after separation by SDS-PAGE under either reducing or nonreducing conditions (Fig. 4, *C* and *D*). In addition, antibodies against the 32-kDa  $\gamma$  subunit do not react with any other components of the dihydropyridine receptor under either nonreducing or reducing conditions (Fig. 4, *A* and *B*). This data shows that the  $\alpha_2$  and  $\gamma$  subunits are distinct from the  $\alpha_1$  and  $\beta$  subunits of the dihydropyridine receptor and that the  $\gamma$  subunit is not a proteolytic fragment of a larger subunit or disulfide linked to another subunit. We have also shown that antiserum against the nonreduced form of the  $\alpha_2$  subunit recognizes proteins of 24 and 20 kDa after reduction of the dihydropyridine receptor (Fig. 4*D*). The 32-kDa  $\gamma$  subunit is also distinct from these proteins (Fig. 4). Affinity-purified antibodies against the isolated 24- and 20-kDa proteins have shown that these proteins are structurally related and disulfide linked to the  $\alpha_2$  subunit (Fig. 4, *E* and *F*). These proteins are probably identical to the  $\delta$  subunits (27 and 24 kDa) identified by Takahashi *et al.* (19) using hydrophobic photoaffinity probes. The proteins labeled by these photo-



**FIG. 8. Identification of  $\alpha_2$  subunits in chicken and frog triads and rat brain synaptosomes.** Membranes from various sources were subjected to SDS-PAGE under nonreducing (20 mM NEM) or reducing (20 mM DTT) conditions as indicated by the - and + signs, respectively. Proteins were then transferred to Immobilon-P membranes and stained with GP5SA, anti- $\alpha_2$  subunit epidermal protein-column adsorbed ascites (*A* and *B*) or GP13VSA anti- $\alpha_2$  subunit ascites (adsorbed on WGA void column and epidermal protein column) (*C*) by the indirect immunoperoxidase method as described under "Experimental Procedures." The samples on the blots are: *A*, lanes 1 and 4, rabbit skeletal muscle triads (50  $\mu$ g); lanes 2 and 5, chicken breast muscle triads (200  $\mu$ g); *B*, lanes 1 and 2, rabbit skeletal muscle triads (250  $\mu$ g); lanes 4 and 5, frog skeletal muscle triads; *C*, lanes 1 and 4, rabbit skeletal muscle triads (50  $\mu$ g); lanes 2 and 5, rat brain synaptosomes (250  $\mu$ g). Lane 3 of each blot is molecular weight standards. Numbers at the left of each blot refer to the size of the molecular weight standards and are expressed as  $M_r \times 10^{-3}$ . The molecular weight standards are: myosin,  $M_r \times 10^{-3} = 200$  and phosphorylase *b*,  $M_r \times 10^{-3} = 97.4$ .

affinity probes also appeared only upon reduction of the dihydropyridine receptor. The 24- and 20-kDa proteins and the weakly stained 16-kDa protein recognized by our antibodies are also probably identical to the proteins of 32, 29, and 26 kDa identified by Lazdunski and co-workers (25), which are disulfide linked to a 140-kDa protein and are present in a purified preparation that is highly enriched in [ $^3$ H]dihydropyridine binding activity (45). This group also showed, using immunoblot techniques and one-dimensional proteolytic mapping, that the 32-, 29-, and 26-kDa proteins are structurally related (45). The data presented in this report also confirm that the 140-kDa protein, identified by the same group as a component of the dihydropyridine receptor (45), is

identical to the  $\alpha_2$  subunit in the purified receptor preparations of a number of laboratories.

The 32-kDa  $\gamma$  subunit of the dihydropyridine receptor shares certain characteristics with the 38-kDa  $\beta$  subunit of the skeletal muscle  $\text{Na}^+$  channel (53) and the 36-kDa  $\beta_1$  subunit of the brain  $\text{Na}^+$  channel (54, 55). The dihydropyridine receptor  $\gamma$  subunit is heavily glycosylated (19) and is noncovalently associated with a multisubunit complex including the  $\alpha_1$  subunit, which is highly homologous to the  $\text{Na}^+$  channel  $\alpha$  subunit (43). The skeletal muscle  $\text{Na}^+$  channel  $\beta$  subunit and the brain  $\beta_1$  subunit are also highly glycosylated and are noncovalently associated with the  $\text{Na}^+$  channel  $\alpha$  subunit (53–55). Similar to the brain  $\beta_1$  subunit (54), the dihydropyridine receptor  $\gamma$  subunit is strongly labeled by a hydrophobic photoaffinity probe (19) indicating possible hydrophobic regions. These similarities suggest that the  $\gamma$  subunit of the dihydropyridine receptor may be analogous in structure and/or function to the  $\text{Na}^+$  channel  $\beta$  subunit. Further work will be required to determine whether significant homology between the primary structures of the two proteins actually exists. Although no clear function has yet been identified for the  $\text{Na}^+$  channel  $\beta$  subunits, it has been suggested that the brain  $\beta_1$  subunit may affect the channel gating function (56). In preliminary experiments (57), affinity-purified antibodies against the dihydropyridine receptor  $\gamma$  subunit inhibited the activity of  $\text{Ca}^{2+}$  channels reconstituted in lipid bilayers. This suggests that the  $\gamma$  subunit is closely associated with the  $\text{Ca}^{2+}$  channel and could be involved in modulation of channel function.

The disulfide-linked 16–24-kDa  $\delta$  subunit(s) of the dihydropyridine receptor superficially resembles the disulfide-linked 33-kDa  $\beta_2$  subunit of the brain  $\text{Na}^+$  channel (19, 56). However, if truly homologous to the brain  $\beta_2$  subunit, the  $\delta$  polypeptides would be expected to be disulfide linked to the  $\alpha_1$  subunit, which has been shown to be highly homologous to the  $\text{Na}^+$  channel  $\alpha$  subunit (43). Instead, they are linked to the  $\alpha_2$  subunit, which does not resemble any known protein (58). Thus, a possible parallel relationship of the dihydropyridine receptor  $\delta$  subunit(s) to the brain  $\text{Na}^+$  channel  $\beta_2$  subunit is not yet clear.

Dihydropyridine receptors are known to be present in skeletal muscle from many different species. Therefore,  $\gamma$  subunits should also be detectable in species other than rabbit. We have used polyclonal antibodies against the rabbit skeletal muscle 32-kDa  $\gamma$  subunit to examine whether similar proteins are present in skeletal muscle of other species. Affinity-purified antibodies against the rabbit skeletal muscle 32-kDa  $\gamma$  subunit specifically labeled proteins similar in size to the rabbit  $\gamma$  subunit in skeletal muscle of a number of species including dog, guinea pig, pig, chicken, and frog (Figs. 5–7). In pig,  $\gamma$  subunits from heavy microsomes prepared from a muscle with predominantly slow (type II) fibers appeared to be identical in molecular weight to those from heavy microsomes prepared from muscle with predominantly fast (type I) fibers. Some difference in the intensity of staining of the two fiber types was noted, suggesting a possible difference in distribution of the 32-kDa  $\gamma$  subunit detected by the antibody between the two fiber types. These results are consistent with results of immunofluorescence studies in rabbit type I and type II myofibers using antibodies against the  $\alpha_1$  and  $\beta$  subunits (48). However, we cannot exclude the possibility that microsomes from the two muscle types may not fractionate identically during the microsome prep. Therefore, more comparable means of sample preparation (such as direct SDS extraction of crude homogenates from equivalent masses of tissue) will be necessary to confirm these results by the

immunoblot technique using anti- $\gamma$  subunit antibodies.

Proteins similar to the rabbit skeletal muscle  $\alpha_2$  subunit have previously been identified in a variety of species and organs (21, 25, 49–52, 59). However, the methods of data presentation in many of these reports make direct comparisons between different samples difficult. We have used anti- $\alpha_2$  subunit antibodies to demonstrate the presence of  $\alpha_2$  subunits in membranes from a variety of species and organs including chicken and frog triads and rat brain synaptosomes. In each case, direct comparison can be made to the rabbit skeletal muscle dihydropyridine receptor (Fig. 8). The anti- $\alpha_2$  subunit antibodies described have also recognized the mouse dihydropyridine receptor and have proven useful in the characterization of the biochemical defect in *mdg* mice, which are affected by an inherited disease causing muscular dysgenesis (60).

In conclusion, our data demonstrates that the 32-kDa  $\gamma$  subunit is an integral and distinct component of the dihydropyridine receptor from rabbit skeletal muscle. Our data also confirms the existence of small ( $\delta$ ) subunits disulfide linked to the  $\alpha_2$  subunit. Therefore, the dihydropyridine receptor consists of five immunologically distinct polypeptides including  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits.

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