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Dihydropyridine-Sensitive Calcium Channels in Cardiac and Skeletal Muscle Membranes: Studies with Antibodies against the α Subunits†

Masami Takahashi and William A. Catterall*

Department of Pharmacology, University of Washington, Seattle, Washington 98195

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ABSTRACT: Polyclonal antibodies (PAC-2) against the purified skeletal muscle calcium channel were prepared and shown to be directed against α subunits of this protein by immunoblotting and immunoprecipitation. These polypeptides have an apparent molecular weight of 162 000 without reduction of disulfide bonds. Under conditions where the functional properties of the purified skeletal muscle calcium channel are retained, β subunits (M_r 50 000) and γ subunits (M_r 33 000) are coprecipitated, demonstrating specific noncovalent association of these three polypeptides in the purified skeletal muscle channel. PAC-2 immunoprecipitated cardiac calcium channels labeled with [^{3}H]isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate ([^{3}H]PN200-110) at a 3-fold higher concentration than skeletal muscle channels. Preincubation with cardiac calcium channels blocked only 49% of the immunoreactivity of PAC-2 toward skeletal muscle channels, indicating that these two proteins have both homologous and distinct epitopes. The immunoreactive component of the cardiac calcium channel was identified by immunoprecipitation and polyacrylamide gel electrophoresis as a polypeptide with an apparent molecular weight of 170 000 before reduction of disulfide bonds and 141 000 after reduction, in close analogy with the properties of the α_2 subunits of the skeletal muscle channel. It is concluded that these two calcium channels have a homologous, but distinct, α subunit as a major polypeptide component.

Voltage-sensitive calcium channels play important roles in the regulation of the calcium-linked cellular functions including muscle contraction, neurotransmitter and hormone release, and calcium-dependent phosphorylation of intracellular proteins by controlling calcium influx from the extracellular environment (Hagiwara & Byerly, 1981; Tsien, 1983). Dihydropyridine calcium channel antagonists block calcium channel function in smooth, cardiac, and skeletal muscle cells at low concentrations (Triggle, 1981; Janis & Scriabine, 1983). High-affinity binding sites for [^{3}H]dihydropyridines have been identified in skeletal, smooth, and cardiac muscles and brain (Janis & Scriabine, 1983; Janis & Triggle, 1984) and have been successfully solubilized by digitonin and 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)[1] (Curtis & Catterall, 1983, 1984; Glossmann &

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Ferry, 1983; Borsotto et al., 1984b; Ruth et al., 1986; Ice et al., 1987). Dihydropyridine receptors have been purified to near-homogeneity from skeletal muscle T-tubular membranes (Curtis & Catterall, 1984; Borsotto et al., 1984a, 1985). After solubilization and purification in digitonin, the purified receptor consists of a noncovalently associated complex of α, β, and γ subunits having apparent molecular weights of 162000, 50000, and 33000, respectively (Curtis & Catterall, 1984). If disulfide bonds are reduced, α subunits are separated into two protein bands with apparent molecular weights of 165000 and 135000 (Curtis & Catterall, 1984; Curtis, 1986; Flockerzi et al., 1986). The purified dihydropyridine receptor has also been incorporated into phospholipid membranes and shown to mediate dihydropyridine-sensitive calcium conductance, providing evidence that these three classes of subunits are sufficient to mediate the physiological function of the calcium channel (Curtis & Catterall, 1986; Flockerzi et al., 1986).

While the skeletal muscle calcium channel has provided a valuable biochemical preparation for studies of the molecular properties of this membrane protein, its physiological properties, functional role in muscle contraction, and mechanism of regulation have not been studied as extensively as those of the cardiac calcium channel. Differences in single-channel conductance (Reuter et al., 1982; Affolter & Coronado, 1985), sensitivity to dihydropyridines (Triggle, 1981; Almers et al., 1981), and kinetics of voltage-dependent activation and inactivation (Affolter & Coronado, 1985; Hess et al., 1984) suggest important functional distinctions between these two channels. Moreover, a preliminary communication has concluded that cardiac calcium channels lack the α subunits that are the major components of the skeletal muscle channel (Rengasamy et al., 1985). In this report, we describe polyclonal antibodies that are directed against the α subunits of the skeletal muscle calcium channel and use them to examine the interactions among the polypeptide components of this purified protein and to identify a homologous α subunit of the calcium channel in cardiac sarcolemma.

Experimental Procedures

Materials. The catalytic subunit of type I cAMP-dependent protein kinase, prepared from bovine heart according to method 1 of Bechtel et al. (1977), was provided by Dr. S. Rossie of this laboratory. WGA–Sepharose was prepared as previously described (Hartshorne & Catterall, 1984). Drugs and chemicals were obtained from the following sources: digitonin, Sigma and ICN Biochemicals (Cleveland, OH); [^{3}H]-PN200-110, [γ-^{32}P]ATP, carrier-free Na^{125}I, and ^{125}I-labeled protein A, New England Nuclear (Boston, MA); ^{125}I-labeled Bolton–Hunter reagent, ICN Radiochemicals (Irvine, CA); Iodo-Beads, Pierce Chemical Co. (Rockford, IL); dithiothreitol, sodium dodecyl sulfate, and all gel electrophoresis reagents, Bio-Rad Laboratories (Richmond, CA); rabbit anti-mouse IgG (H + L) and rabbit anti-mouse Ig (G + A + M), Zymed Laboratories (San Francisco, CA); Freund's complete adjuvant, Calbiochem (San Diego, CA); Freund's incomplete adjuvant, Miles Scientific (Naperville, IL). All other chemicals were from Sigma.

Preparations of Membranes and Proteins. T-tubule membranes and the purified calcium channel were prepared from rabbit fast muscle as previously described (Curtis & Catterall, 1984; Glossmann et al., 1983). Cardiac sarcolemma was prepared from rabbit and bovine heart ventricles by the method of Brandt (1985) with the following modifications: the initial homogenization was done in 250 mM sucrose and 10 mM HEPES/Tris, pH 7.4, and the sucrose gradient sedimentation step was omitted. All of the solutions for preparation contained phenylmethanesulfonyl fluoride (1 mM) and pepstatin A (1 μM). In some experiments indicated in the text, 1,10-phenanthroline (1 mM), iodoacetamide (1 mM), antipain (1 μg/mL), and leupeptin (1 μg/mL) were added.

The γ subunit of the skeletal muscle calcium channel was separated from other subunits by SDS gel electrophoresis and was recovered by electroelution (Hunkapiller et al., 1983).

[^{3}H]PN200-110-labeled calcium channels for immunoprecipitation experiments were prepared as follows. Microsomal membranes of rabbit skeletal muscle and sarcolemmal membranes of rabbit and bovine heart were prelabeled with a saturating concentration of [^{3}H]PN200-110 and solubilized in 1% digitonin with a 5:1 (w/w) detergent to protein ratio in 75 mM NaCl and 10 mM HEPES/Tris, pH 7.4, at 4 °C for 30 min. Insoluble material was removed by centrifugation at 175000*g* for 45 min at 4 °C, and the solubilized material was diluted 1:1 with 75 mM NaCl and 10 mM HEPES/Tris, pH 7.4, to which had been added a 7-mL packed volume of WGA–Sepharose. After a 30-min equilibration, the Sepharose was packed in a 2 cm × 10 cm column and washed with 0.1% digitonin in buffer A (75 mM NaCl, 50 mM sodium phosphate, and 25 mM Tris, pH 7.4), and adsorbed glycoproteins were eluted with buffer A containing 100 mM *N*-acetyl-D-glucosamine and 0.1% digitonin.

The specific activity (picomoles of [^{3}H]PN200-110 bound per milligram of protein) of the pooled fraction was determined from the specifically bound [^{3}H]PN200-110. Negligible dissociation of [^{3}H]PN200-110 occurs during the purification process as assessed by poly(ethylene glycol) precipitation assays (Curtis & Catterall, 1983) or sucrose gradient analysis. Peak [^{3}H]PN200-110-labeled fractions were frozen in liquid N_2 for use in immunoprecipitation assays or for iodination with ^{125}I. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Radiolabeling of the Calcium Channel with ^{32}P and ^{125}I. Purified skeletal muscle calcium channel was phosphorylated as previously described by cAMP-dependent protein kinase (Curtis & Catterall, 1985) in the presence of 3 μM [γ-^{32}P]-ATP.

Proteins were iodinated with ^{125}I using either *N*-chlorobenzenesulfonamide immobilized to 2.8-mm nonporous polystyrene beads (Iodobeads) according to Markwell (1982) or ^{125}I-labeled Bolton–Hunter reagent (Bolton & Hunter, 1973). Fifteen Iodobeads were washed 2 times with 10 mL of 50 mM sodium phosphate, pH 7.4, and dried on Whatman 52 filter paper. Beads were then placed in a reaction vial, and 1 mCi of Na^{125}I in 500 μL of phosphate buffer was added. After 5 min at room temperature, 500 μL of protein solution (10 pmol of purified skeletal muscle calcium channel, 3 pmol of WGA–Sepharose-purified cardiac calcium channel, or 0.5 μg of purified γ subunit) in 50 mM sodium phosphate, pH 7.4, with 0.1% digitonin was added and incubated for 15 min at room temperature with gentle agitation. The iodination was terminated by removing the reaction mixture from the Iodo-

[1] Abbreviations: T-tubule, transverse tubule; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; WGA, wheat germ agglutinin; IgG, immunoglobulin G; BSA, bovine serum albumin; CMF-PBS, calcium/magnesium-free Dulbecco's phosphate-buffered saline; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHAPS, 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; TBS, 0.9% (w/v) NaCl/10 mM Tris-HCl, pH 7.4; TCA, trichloroacetic acid.

beads, and the free ^{125}I was removed by applying the 1-mL sample to a 1 cm × 25 cm column of a Bio-Gel P-6DG desalting gel equilibrated with 50 mM sodium phosphate, pH 7.4, 0.1% digitonin, and all six protease inhibitors described above for calcium channels or with 0.1% SDS for the γ subunit. The fractions containing iodinated protein were stored at −20 °C until use.

^{125}I-Labeled Bolton–Hunter reagent (250 μCi) was dried in a small reaction vial under a stream of nitrogen, and 200 μL of protein solution (8 pmol of purified skeletal muscle calcium channel or 1.2 pmol of WGA–Sepharose-purified cardiac calcium channel) in 0.1% digitonin, 75 mM NaCl, and 100 mM sodium borate, pH 8.4, was added and incubated at 4 °C with gentle agitation. After 60 min, 5 μL of 1 M Tris-HCl, pH 7.4, was added to stop the reaction, and the mixture was allowed to stand for an additional 5 min. The iodinated materials were applied to a Sephadex G-50 column equilibrated with 0.1% digitonin, 75 mM NaCl, and 25 mM HEPES/Tris, pH 7.4, with all six protease inhibitors. The excluded protein fraction was stored at −20 °C until use.

Production of Polyclonal Antisera. BALB/c mice were immunized by intraperitoneal injections of highly purified rabbit skeletal muscle calcium channel (10 μg of protein per animal) in Freund's complete adjuvant and boosted by three or four additional intraperitoneal or subcutaneous injections at 2–3-week intervals with the same amount of antigen in Freund's incomplete adjuvant. Blood was collected from the retroorbital venous plexus 5 days after the final boost. Preimmune serum was obtained from noninjected mice.

Immunoprecipitation of Radiolabeled Calcium Channels. The ^{32}P-labeled skeletal muscle calcium channel or the ^{125}I-labeled γ subunit of the skeletal muscle channel was incubated with various amounts of antiserum in buffer B consisting of 75 mM NaCl, 2.5 mM EDTA, 25 mM Tris, 50 mM sodium phosphate, 20 mM KF, and 1% (v/v) Triton X-100, pH 7.4, in a final volume of 100 μL for 4 h at 4 °C. Anti-mouse Ig (G + A + M) (10 μL/sample), protein A–Sepharose (2 mg/sample), and 1 mg/mL BSA in CMF-PBS (10 μL/sample) were mixed in a rotating shaker for 2 h at 4 °C, washed 3 times with buffer B, and added to the antigen–antibody mixture. The mixture was incubated in a rotating shaker for 1 h at 4 °C, washed 3 times with buffer B, and sedimented in a Beckman microfuge for 30 s. The amounts of ^{32}P and ^{125}I in the final pellet were determined by liquid scintillation and γ counting, respectively. Immunoprecipitation of ^{125}I-labeled cardiac calcium channel was performed with the same method described above except protein A–Sepharose (5 mg/sample), which was swelled with 1 mg/mL BSA in CMF-PBS and washed 3 times with buffer B, was used instead of protein A/antimouse Ig (G + A + M) complex and all six protease inhibitors were added throughout the immunoprecipitation.

For the radioimmune assay of [^3H]PN200-110-labeled calcium channel, WGA–Sepharose-purified channel was incubated with various amounts of antiserum in 0.5% digitonin/buffer A in a final volume of 100 μL for 4 h at 4 °C. Ten microliters of anti-mouse Ig (G + A + M), 2 mg of protein A–Sepharose, and 10 μL of 1 mg/mL BSA in CMF-PBS per sample were mixed for 2 h at 4 °C, washed 3 times with buffer A containing 0.5% digitonin, and added to the antigen–antibody mixture. The mixture was incubated in a rotating shaker for 1 h at 4 °C and washed 3 times with buffer A containing 0.5% digitonin as described above. The amount of [^3H]-PN200-110 in the final pellet was determined by liquid scintillation counting.

SDS–Polyacrylamide Gel Electrophoresis. Samples were mixed with concentrated sample buffer to give final concentrations of 2% (w/v) SDS, 8 mM EDTA, 4.8% (w/v) sucrose, 20 mM Tris-HCl, pH 7.6, and 20 mM NEM or DTT. Samples were placed in boiling water for 3 min and then analyzed in a discontinuous gel system according to Laemmli (1970) consisting of a 5% (w/v) stacking gel and a separation gel containing a linear 5–15% (w/v) acrylamide gradient. Gels were stained with Coomassie brilliant blue R250, destained, dried, and exposed to Kodak X-Omat film.

Immunoblot Analysis. Proteins were transferred from the polyacrylamide gel to nitrocellulose by using standard procedures (Burnette, 1981) in the presence of 0.1% (w/v) SDS. After protein transfer, the nitrocellulose paper was incubated in 5% (w/v) BSA and TBS [0.9% (w/v) NaCl/10 mM Tris-HCl, pH 7.4] for 3 h at room temperature and then with antiserum in 5% BSA/TBS overnight at 4 °C. Blots were washed 4 times with TBS containing 0.05% (v/v) Tween 20 and incubated with rabbit anti-mouse IgG at 1:50 dilution in TBS containing 1% BSA and 0.05% Tween 20 for 4 h at 4 °C. After being washed 4 times as described previously, the sheets were incubated with ^{125}I-labeled protein A [(4–5) × 10^6 cpm/mL] in TBS containing 5% BSA for 1 h at room temperature, washed 6 times with 0.05% Tween 20 in TBS, and autoradiographed on Kodak X-Omat film.

Sucrose Gradient Sedimentation and Determination of Sedimentation Coefficient. [^3H]PN200-110-labeled calcium channel was layered onto a 38-mL linear gradient from 5% to 20% sucrose in 0.1% digitonin, 1 mM $CaCl_2$, and 5 mM MOPS, pH 7.4, and sedimented for 1.5 h at 210000g in a VTi 50 vertical rotor. Fractions were collected from the bottom, and the amount of [^3H]PN200-110 in each fraction was determined by liquid scintillation counting. Thyroglobulin (19.2 S) and catalase (11.2 S) were used as standard proteins.

Results

Polyclonal Antibodies against the Purified Calcium Channel of Skeletal Muscle. An emulsion of a 1:1 mixture of 10 μg of highly purified skeletal muscle calcium channel and Freund's complete adjuvant was injected intraperitoneally into BALB/c mice. The mice were boosted 3 times by biweekly intraperitoneal or subcutaneous injection of the same amount of the channel mixed with Freund's incomplete adjuvant, and the titer of sera was tested 4–5 days after the final boost. For radioimmunoassay of the antisera, α and β subunits of the purified calcium channel of skeletal muscle were phosphorylated with ^{32}P by the catalytic subunit of cAMP-dependent protein kinase (Curtis & Catterall, 1985). As shown in Figure 1, all three antisera tested in this study immunoprecipitated the ^{32}P-labeled channel, but the titer of the individual sera varied over 100-fold. The antiserum having the highest titer, designated PAC-2, was used in these experiments. Immunoprecipitation of the ^{32}P-labeled calcium channel was detected at a concentration of PAC-2 above 0.001% (v/v) and reached a maximum at 0.1% (v/v). Normal serum precipitated only the background level of radioactivity even with the maximum amount tested.

Identification of the Antigenic Site of PAC-2 in T-tubule Membranes and Purified Calcium Channels from Rabbit Skeletal Muscle. T-tubule membrane proteins and purified skeletal muscle calcium channels were separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunostained with PAC-2 as described under Experimental Procedures. In both these preparations, PAC-2 stained a polypeptide with an apparent molecular weight of 162 000 before reduction of disulfide bonds (Figure 2, lanes 1 and 2),

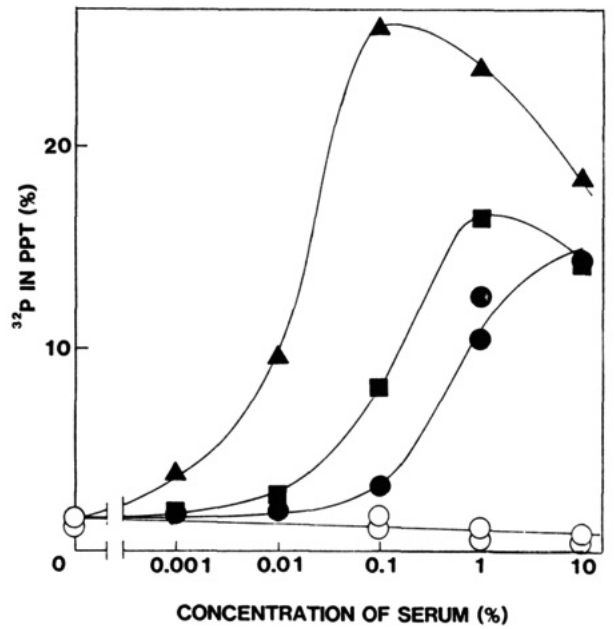

FIGURE 1: Immunoprecipitation of ^{32}P-labeled skeletal muscle calcium channel by three different polyclonal antisera against highly purified skeletal muscle calcium channel. Purified skeletal muscle calcium channel was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and incubated with the indicated concentrations of antisera from three different mice immunized with highly purified skeletal muscle calcium channel. These antisera were designated PAC-1 (●), PAC-2 (▲), and PAC-3 (■). Antigen/antibody complex was precipitated by adsorption to anti-mouse Ig (G + A + M)/protein A–Sepharose complex. The radioactivity recovered in the washed precipitate was expressed as a percent of the total radioactivity added. The background precipitation of radioactivity by preimmune serum is shown by open circles.

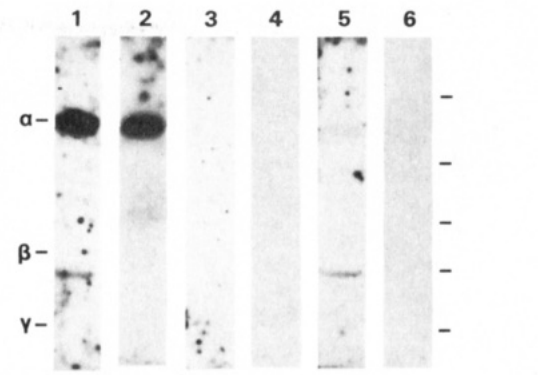

FIGURE 2: Immunoblotting of T-tubule membranes and purified calcium channel of rabbit skeletal muscle by PAC-2. T-tubule membrane proteins (190 μg per lane) (lanes 1, 3, and 5) and purified skeletal muscle calcium channel (2.2 μg per lane) (lanes 2, 4, and 6) were transblotted to a nitrocellulose sheet and immunostained by PAC-2 (lanes 1 and 2), preimmune serum (lanes 3 and 4), and PAC-2 preabsorbed with the purified calcium channel (lanes 5 and 6). The concentrations of all the sera were 0.3% (v/v). The migration positions of α, β, and γ subunits of the skeletal muscle calcium channel are indicated on the left. The migration positions of standard proteins indicated by horizontal bars correspond, from top to bottom, to the following molecular weights: 200 000, 116 000, 68 000, 42 000, and 30 000.

which is the same value as that of the α subunits of purified calcium channels. This staining was not observed with preimmune serum (lanes 3 and 4) or with PAC-2 preabsorbed with the purified skeletal muscle calcium channel (lanes 5 and 6), indicating that this band contains the α subunits of the skeletal muscle channel. PAC-2 did not bind to proteins corresponding to β and γ subunits of the calcium channel. A group of faint protein bands having apparent molecular weights between 61 000 and 78 000 was occasionally observed in the purified calcium channel stained by PAC-2 (lane 2), but not by preimmune serum (lane 4) or by preabsorbed PAC-2 (lane 6). Since these polypeptides were not observed in T-tubule membranes and their content varied greatly among the different preparations, they are likely to be proteolytic fragments of the α subunits. In T-tubule membrane samples, a small peptide of 40 000 daltons was immunostained by PAC-2 (lane 1). However, this staining was not blocked after the absorption of PAC-2 with the purified skeletal muscle calcium channel (lane 5), indicating that peptide is not a component of the calcium channel. Therefore, we conclude that the major recognition sites of PAC-2 in T-tubule membranes are on the α subunits of skeletal muscle calcium channels and that recognition is specifically blocked by the pretreatment of PAC-2 with the purified channel.

Coimmunoprecipitation of the α, β, and γ Subunits of the Purified Skeletal Muscle Calcium Channel by PAC-2 under Nondenaturing Conditions. The purified calcium channel from skeletal muscle is a complex of α, β, and γ subunits (Curtis & Catterall, 1984). All of these polypeptides quantitatively comigrate during velocity sedimentation in sucrose gradients (Curtis & Catterall, 1984). In order to provide further evidence that these three polypeptides are specifically associated, the subunits were radiolabeled with either ^{32}P or ^{125}I, and immunoprecipitation by PAC-2 was examined under both native and denaturing conditions. In the presence of 3 μM [γ-^{32}P]ATP, both α and β subunits were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Curtis & Catterall, 1985; Figure 3B, lane 1). After phosphorylation, the preparation was divided into three samples. The first was boiled in the presence of 0.1% SDS, mixed with excess Triton X-100, and passed through a Sephadex G-50 column to remove SDS. The second was treated with 0.1% SDS without boiling in the presence of an excess of Triton X-100, and the SDS was immediately removed by gel filtration on Sephadex G-50. The third sample was applied to a Sephadex G-50 column without pretreatment with SDS. The flow-through of each column was used for immunoprecipitation. PAC-2 immunoprecipitated ^{32}P-labeled calcium channels from all three preparations, and there was no difference in the concentration of PAC-2 which gave half-maximum [0.01% (v/v)] and maximum precipitation [0.1% (v/v)], indicating that the apparent affinity of PAC-2 for the antigen was not changed before and after treatment with SDS (Figure 3A). In contrast, the maximum level of immunoprecipitation was reduced 40% by boiling the channel in the presence of SDS (Figure 3A, ■). Addition of SDS in the presence of an excess amount of Triton X-100 without boiling did not cause a decrease in immunoprecipitation (Figure 3A, ▲). In all three samples, normal serum precipitated only the background level of radioactivity.

When the phosphorylated calcium channel was analyzed by SDS gel electrophoresis, the α and β subunits of the calcium channel, the catalytic subunit of protein kinase (41 000 daltons), and two unidentified proteins of 75 000 and 32 000 daltons were found to be phosphorylated (Figure 3B lane 1). When the subunits of the calcium channel were associated, PAC-2 precipitated both α and β subunits (lanes 2 and 3). However, if the calcium channel was dissociated into subunits by boiling in SDS, PAC-2 precipitated only α subunits (lane 4). These results show that PAC-2 recognizes α subunits but does not recognize the SDS-denatured β subunit.

Purified calcium channels that have been phosphorylated at 37 °C contain a substantial fraction of β subunit that has dissociated from α subunits (Curtis & Catterall, 1985). After

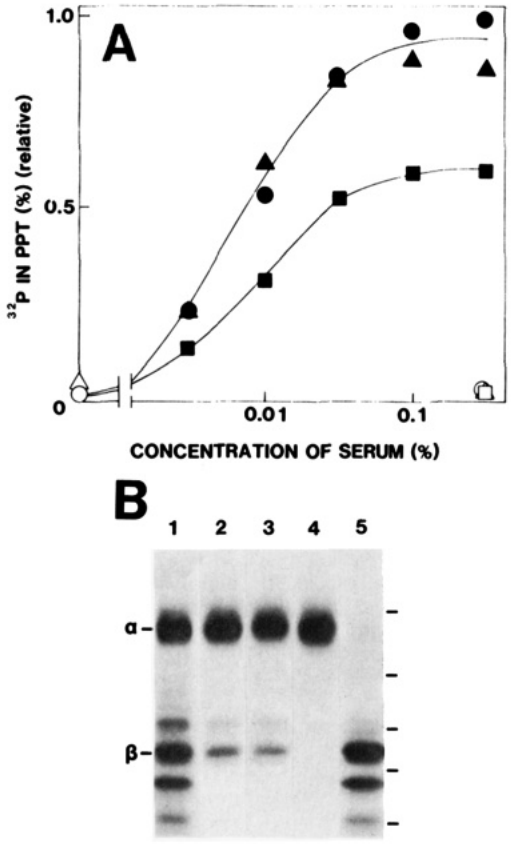

FIGURE 3: Immunoprecipitation of phosphorylated α and β subunits of skeletal muscle calcium channel by PAC-2 before and after dissociation into its subunits. (A) α and β subunits of skeletal muscle calcium channel were phosphorylated with ^{32}P by the catalytic subunit of cAMP-dependent protein kinase and immunoprecipitated by PAC-2 after the following treatments: (1) 45 μL of ^{32}P-labeled calcium channel was mixed with 5 μL of 1% SDS, boiled for 2 min, mixed with 150 μL of buffer B to exchange SDS with Triton X-100, applied to a 2-mL Sephadex G-50 column prespun with buffer B, and centrifuged at 1000g for 1 min (■). (2) 45 μL of ^{32}P-labeled calcium channel was mixed with 150 μL of buffer B and then mixed with 5 μL of 1% SDS just before application of the Sephadex G-50 column as in (1) (▲). (3) 45 μL of ^{32}P-labeled calcium channel was mixed with 150 μL of buffer B and 5 μL of water and applied to a Sephadex G-50 column as in (1) (●). The radioactivity recovered in the precipitate is expressed as a percent of the maximum amount of ^{32}P precipitated by excess PAC-2. The background precipitation of radioactivity by preimmune serum is shown by open symbols. (B) ^{32}P-Labeled calcium channels and the supernatants and pellets of the immunoprecipitations at 0.1% PAC-2 from the experiment of panel A were analyzed by SDS gel electrophoresis and autoradiography: lane 1, ^{32}P-labeled skeletal muscle calcium channel; lane 2, immunoprecipitate from treatment 3 (see above); lane 3, immunoprecipitate from treatment 2; lane 4, immunoprecipitate from treatment 1; lane 5, supernatant from treatment 3. The migration positions of α and β subunits of the skeletal muscle calcium channel are indicated on the left of the figure. The migration positions of standard proteins indicated by horizontal bars correspond, from top to bottom, to the following molecular weights: 200 000, 116 000, 68 000, 42 000, and 30 000.

the immunoprecipitation of the native phosphorylated calcium channel with excess PAC-2, dissociated β subunit remained in the supernatant while α subunits were completely precipitated (Figure 3, lane 5). This result indicates that PAC-2 does not recognize either the native or the SDS-denatured β subunit. The coimmunoprecipitation of α and β subunits must therefore result from specific interactions between these two polypeptides in the purified calcium channel.

To examine coimmunoprecipitation of all three of the subunits of the purified channel, purified preparations of skeletal muscle calcium channels were labeled with ^{125}I, im-

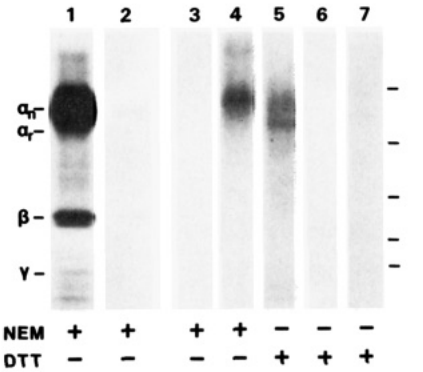

FIGURE 4: Immunoprecipitation of ^{125}I-labeled skeletal muscle calcium channel by PAC-2. Purified calcium channel of rabbit skeletal muscle was iodinated by the Bolton–Hunter (lanes 1 and 2) and Iodobead methods (lanes 3–7) and incubated with PAC-2 (lanes 1, 4, and 5), preimmune serum (lanes 2, 3, and 6) and PAC-2 preabsorbed with purified skeletal muscle calcium channel (lane 7). The final concentration of antiserum was 0.1% (v/v). The resulting antigen/antibody complexes were precipitated by adsorption to protein A–Sepharose, boiled in SDS in the presence of NEM or DTT as indicated, and analyzed by SDS gel electrophoresis. The migration positions of the α subunit under nonreducing conditions (α_n), the lower molecular weight form observed under reducing conditions (α_r), and the β and γ subunits are indicated on the left of the figure. The migration positions of standard proteins indicated by bars correspond, from top to bottom, to the following molecular weights: 200 000, 116 000, 68 000, 42 000, and 30 000.

munoprecipitated by PAC-2, and analyzed by SDS–polyacrylamide gel electrophoresis. When the purified skeletal muscle calcium channel was iodinated by the Bolton–Hunter method, the α and β subunits were clearly identified after immunoprecipitation (Figure 4, lane 1). The apparent molecular weights of the iodinated α and β subunits were 163 000 and 54 000, respectively, without reduction, in close agreement with previous estimates of 162 000 and 50 000. The γ subunit was poorly labeled but could also be identified in these immunoprecipitates as a polypeptide with an apparent molecular weight of 33 000 by this method (Figure 4, lane 1). The α-, β-, and γ-polypeptides were not immunoprecipitated by preimmune serum, confirming the identification of these polypeptides as subunits of the calcium channel (Figure 4, lane 2).

When the purified skeletal muscle calcium channel was labeled with ^{125}I by the Iodobead method, immunoprecipitated, and analyzed by SDS gel electrophoresis, only the α subunit appeared labeled (Figure 4, lane 4). After reduction with dithiothreitol, the α subunits migrated as two diffuse bands of 162 000 and 135 000 daltons (Figure 4, lane 5) as observed previously (Curtis, 1986; Flockerzi, et al., 1986). The α subunits were not immunoprecipitated by preimmune serum (lanes 3 and 6) or by PAC-2 that had been preabsorbed with the purified calcium channel (lane 7), confirming identification of these bands as calcium channel components. Labeled protein bands corresponding to the β and γ subunits of the purified calcium channel were not observed in Iodobead-labeled samples due to a low level of iodination by this method. The results of immunoprecipitation of samples labeled by these two different methods support our previous conclusion that the α-, β-, and γ-polypeptides are noncovalently associated subunits of a purified calcium channel complex.

In order to verify that the γ subunit is not directly immunoprecipitated by PAC-2, we isolated it by electroelution from SDS–polyacrylamide gels and labeled it with ^{125}I by the Iodobead method (Markwell, 1982). After the unreacted Na^{125}I was removed by gel filtration, 67% of the total radioactivity was TCA precipitable. Preimmune serum and PAC-2, at

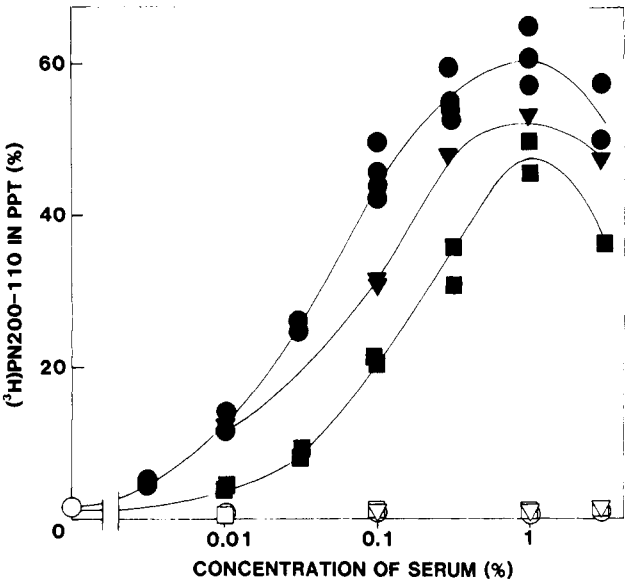

FIGURE 5: Immunoprecipitation of [^{3}H]PN200-110-labeled calcium channels solubilized from skeletal muscle and heart. Equal amounts of [^{3}H]PN200-110-labeled calcium channels from rabbit skeletal muscle (●, ○), rabbit heart (▼, ▽), or bovine heart (■, □) were incubated with the indicated concentrations of PAC-2 (closed symbols) or preimmune serum (open symbols), and antigen/antibody complex was precipitated by adsorption to anti-mouse Ig (G + A + M)/protein A–Sepharose complex. The radioactivity recovered in the precipitate was expressed as the percentage of total [^{3}H]PN200-110-labeled calcium channel.

serum concentrations of 0.1%, precipitated only 0.2% and 0.4% of the radioactivity, respectively, and these values were not increased further by increasing the amounts of serum added. These results confirm the conclusion from immunoblots (Figure 2) that PAC-2 does not recognize γ.

Immunoreactivity of PAC-2 with Rabbit and Bovine Cardiac Dihydropyridine-Sensitive Calcium Channels. The dihydropyridine-sensitive calcium channels of rabbit skeletal muscle T-tubule membranes and rabbit and bovine cardiac sarcolemma were prelabeled with [^{3}H]PN200-110, solubilized with 1% digitonin, purified by chromatography on WGA–Sepharose, and used for radioimmune assay. Figure 5 shows the concentration dependence of immunoprecipitation of the [^{3}H]PN200-110-labeled calcium channels by PAC-2. Substantial immunological reactivity with calcium channels from all three tissues was observed. The [^{3}H]PN200-110-labeled channel recovered in the precipitate increased with increasing PAC-2 concentration and attained maximum values of 60%, 52%, and 48% of total [^{3}H]PN200-110-labeled channels added from rabbit skeletal muscle, rabbit heart, and bovine heart, respectively. The immunoreactivity of PAC-2 with the skeletal muscle calcium channel was greater than with the cardiac channel. Half-maximal immunoprecipitation of skeletal muscle channel was obtained at an antiserum concentration of 0.04% (v/v). In contrast, a concentration of 0.06% (v/v) was required to obtain equivalent immunoprecipitation of the [^{3}H]PN200-110/channel complex from rabbit heart. These results indicate that the dihydropyridine-sensitive calcium channels in heart and skeletal muscle are antigenically related but not identical.

The immunoreactivity of PAC-2 also differed between species. A serum concentration of 0.15% (v/v) was necessary to obtain half-maximal immunoprecipitation of bovine heart calcium channels, indicating that there are species-specific regions in the structure of cardiac calcium channels.

Absorption of the Immunoreactivity of PAC-2 with ^{32}P-Labeled Skeletal Muscle Calcium Channel by Pretreatment with Skeletal and Cardiac Muscle Calcium Channels. The difference in immunoreactivity of cardiac and skeletal muscle calcium channels observed in Figure 5 might result from a reduced average affinity of PAC-2 antibodies for the cardiac channel or from the presence of antibody populations that recognize epitopes on the skeletal muscle calcium channel exclusively. To determine whether heart calcium channels could react with all the PAC-2 antibodies against skeletal muscle calcium channels, the purified skeletal calcium channel was phosphorylated with ^{32}P and then immunoprecipitated with PAC-2 that had been previously incubated with increasing amounts of partially purified unlabeled calcium channels from either skeletal muscle or heart. As shown in Figure 6, immunoprecipiation of the ^{32}P-labeled channel was progressively decreased by increasing the amount of skeletal muscle calcium channels used for preabsorption and was completely blocked by 1 pmol of the channel per microliter of PAC-2. Complete block of the immunoreactivity of PAC-2 was also achieved by pretreating with highly purified skeletal muscle calcium channel (data not shown). In contrast, immunoprecipitation was reduced to 51% of the maximum level by 0.3 pmol of heart calcium channel per microliter of PAC-2, but the extent of inhibition was not further increased with increasing amounts of the cardiac channel. These results show that only a fraction of the immunoreactivity of PAC-2 can be blocked by cardiac calcium channel, suggesting that only part of the structure of skeletal muscle calcium channel is immunologically homologous to the cardiac channel.

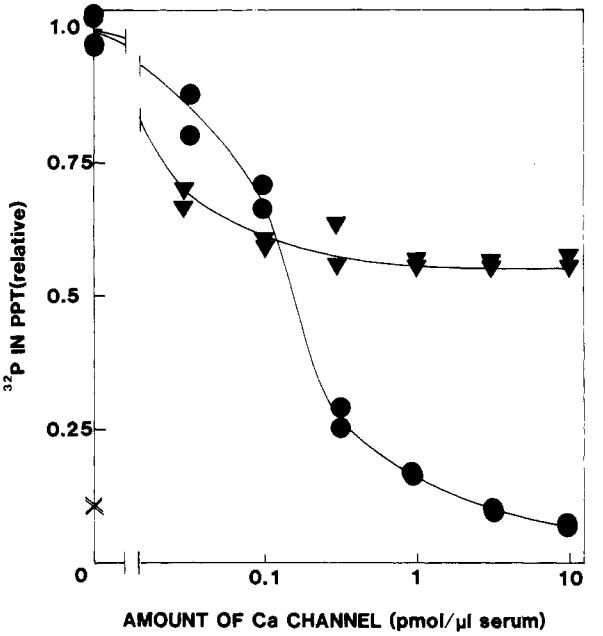

FIGURE 6: Absorption of the immunoreactivity of PAC-2 by pretreatment with rabbit skeletal and cardiac muscle calcium channels. PAC-2 at a final concentration of 0.01% was mixed with various amounts of the partially purified skeletal (●) and cardiac (▼) muscle calcium channels. After overnight incubation, ^{32}P-labeled skeletal muscle calcium channel (8900 cpm per sample) was added, and the resulting antigen/antibody complex was precipitated by absorbing to protein A–Sepharose/anti-mouse Ig (G + A + M) complex. The radioactivity in the precipitates with preabsorbed PAC-2 is presented as the fraction of that with unabsorbed PAC-2. The background immunoprecipitation without antiserum was determined in parallel experiments (×).

Identification of an α Subunit of the Calcium Channel of Rabbit Heart by Immunoprecipitation. In order to identify the immunoreactive polypeptide in the rabbit heart, sarcolemmal membrane proteins were solubilized in digitonin, pu-

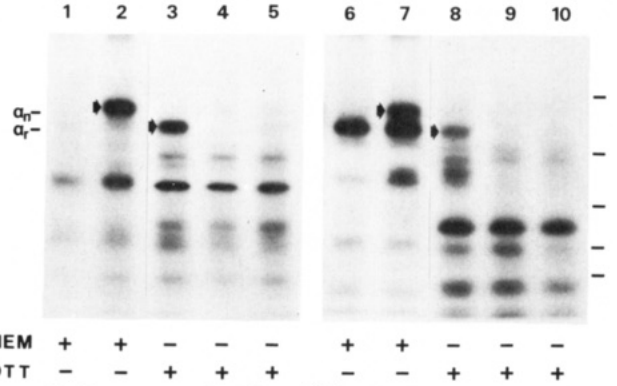

FIGURE 7: Immunoprecipitation of ^{125}I-labeled α subunit of rabbit cardiac calcium channel by PAC-2. Partially purified calcium channel from rabbit heart was iodinated by the Bolton–Hunter (lanes 1–5) or Iodobead methods (lanes 6–10) and immunoprecipitated by PAC-2 (lanes 2, 3, 7, and 8), preimmune serum (lanes 1, 4, 6, and 9), and PAC-2 preabsorbed with purified skeletal muscle calcium channel (lanes 5 and 10). The final concentration of antiserum was 0.3% (v/v). The resulting antigen/antibody complex was precipitated by adsorption to protein A–Sepharose, boiled in SDS in the presence of NEM or DTT as indicated, and analyzed by SDS gel electrophoresis. The migration positions of α subunits of skeletal muscle calcium channel before reduction of disulfide bonds and the lower molecular weight form observed after reduction are indicated as α_n or α_r, respectively. *Arrows* indicate the migration positions of α_n and α_r from cardiac muscle. The migration positions of standard proteins indicated by horizontal bars correspond, from top to bottom, to the following molecular weights: 200 000, 116 000, 68 000, 42 000, and 30 000.

rified by WGA–Sepharose chromatography, and iodinated with ^{125}I by the Bolton–Hunter and Iodobead methods. In preparations labeled by the Bolton–Hunter method, a sharp protein band having a molecular weight of 141 000 under reducing conditions was specifically immunoprecipitated by PAC-2 (Figure 7, lane 3). Immunoprecipitation of this protein was not observed with preimmune serum (lane 4) and was markedly reduced in intensity with PAC-2 that had been previously absorbed with purified skeletal muscle calcium channel (lane 5). This specifically immunoprecipitated protein migrated with an apparent molecular weight of 170 000 without reduction of disulfide bonds (lane 2) and was not observed with immunoprecipitation by preimmune serum (lane 1). Several other protein bands were precipitated nonspecifically by PAC-2, preimmune serum, and blocked PAC-2 (lanes 1–5), but no other protein bands were specifically precipitated by PAC-2.

In preparations labeled by the Iodobead method, this same protein band with a molecular weight of 141 000 under reducing conditions was immunoprecipitated by PAC-2 (Figure 7, lane 8) but not by preimmune serum or PAC-2 blocked by incubation with purified skeletal muscle calcium channels (lanes 9 and 10). This specifically immunoprecipitated protein migrated with a molecular weight of 170 000 without reduction of disulfide bonds as observed with samples labeled with Bolton–Hunter reagent (lane 7) and was not precipitated by preimmune serum (lane 6). With samples labeled by the Iodobead method, one of the major nonspecifically precipitated proteins had an apparent molecular weight of 140 000 under nonreducing conditions (lanes 6 and 7) but was clearly resolved from the specifically precipitated protein migrating with an apparent molecular weight of 170 000 under these conditions. A protein with an apparent molecular weight of 80 000 was specifically immunoprecipitated in some experiments (lanes 8–10), but its variable appearance and its similarity in size to the major proteolytic fragment of the α subunits of the skeletal muscle calcium channel led us to conclude that it is produced by proteolysis.

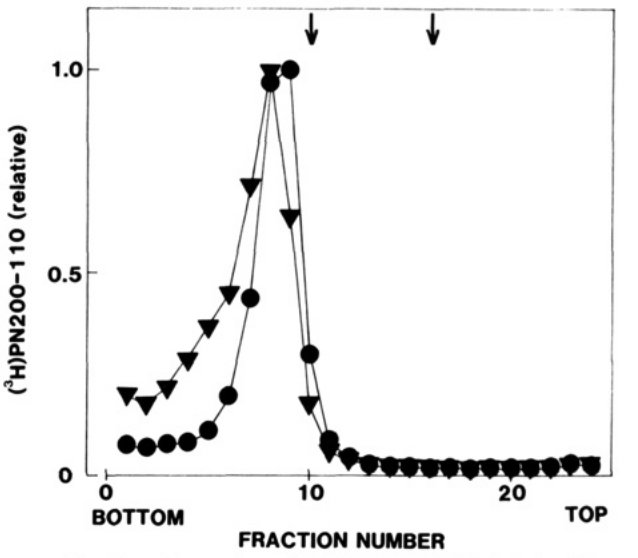

FIGURE 8: Cosedimentation of [^{3}H]PN200-110-labeled calcium channel from rabbit skeletal (●) and cardiac muscle (▼) in centrifugation through sucrose density gradients. 7.5 pmol of skeletal calcium channel and 2.8 pmol of cardiac calcium channel were loaded onto two 5–20% (w/v) linear sucrose gradients containing 0.1% digitonin, 1 mM $CaCl_2$, and 5 mM MOPS, pH 7.4. Samples were sedimented for 1.5 h at 210000*g* in a VTi50 vertical rotor. After fractionation, [^{3}H]PN200-110 in each tube was measured by scintillation counting and expressed relative to the value in the peak fractions. The migration of the protein standards catalase (11.2 S) and thyroglobulin (19.2 S) was determined on a third gradient (arrows).

The protein of 170 000 daltons specifically immunoprecipitated by PAC-2 has the same apparent molecular weight as an α subunit of the skeletal muscle calcium channel before and after reduction of disulfide bonds and is antigenically related. It is the only protein specifically precipitated by PAC-2 under conditions which precipitate dihydropyridine-sensitive calcium channels. These results provide direct identification of an α subunit of the cardiac dihydropyridine-sensitive calcium channel.

Cosedimentation of Skeletal and Cardiac Muscle Calcium Channels by Centrifugation through a Sucrose Density Gradient. The results obtained in this study indicate that the cardiac calcium channel contains a large subunit which is homologous to α subunits of the skeletal muscle channel. In additon to the α subunits, the calcium channel purified from skeletal muscle contains β and γ subunits (Curtis & Catterall, 1984), suggesting that the calcium channel has an oligomeric structure. To compare the oligomeric size of the skeletal muscle and the cardiac calcium channel, sedimentation velocity through 5–20% sucrose density gradients was measured for digitonin-solubilized and partially purified skeletal muscle and cardiac channels. As shown in Figure 8, both channels sedimented as a single peak at nearly the same velocity. The *s* values were estimated to be 21 and 21.6 S for the skeletal and cardiac muscle channels, respectively, by using catalase and thyroglobulin as the standards. This result suggests that the subunit composition of the cardiac calcium channel may resemble that of the skeletal muscle channel.

Discussion

Antibodies against the Purified Skeletal Muscle Calcium Channel Recognize the α Subunits. Purified calcium channel preparations contain multiple polypeptide components (Curtis & Catterall, 1984, 1985). We have designated the largest and most prominent of these the α subunits of the channel. These polypeptides migrate as a single protein band with an apparent

molecular weight of 162000 before reduction of disulfide bonds but are separated into two protein bands with molecular weights of 162000 and 135000 after reduction (Curtis & Catterall, 1984; Curtis, 1986; Flockerzi et al., 1986). Purified preparations also contain β subunits of M_r 50000 and γ subunits of M_r 33000. The presence of β- and γ-polypeptides is less consistent in preparations from different laboratories and different animal species (Borsotto et al., 1984a, 1985). Here we show that polyclonal antisera raised in multiple mice by injection of preparations containing α, β, and γ subunits have their primary immunoreactivity against the α subunits. In immunoblots of the purified calcium channel or of T-tubule membranes, only α subunits are specifically stained. In immunoprecipitation experiments, only α subunits are precipitated under conditions which cause dissociation of β and γ. Evidently, the primary antigenic sites of the calcium channel are located on the α subunits.

Antibodies against the α Subunits of the Skeletal Muscle Calcium Channel Coprecipitate β and γ. The copurification of the α-, β-, and γ-polypeptides (Curtis & Catterall, 1984) and their quantitative comigration in sedimentation experiments (Curtis & Catterall, 1984, 1985, 1986) suggest that they are associated in a complex that retains calcium channel activity in purified form. However, the stoichiometry of these peptides as revealed by staining and radiolabeling procedures can be quite variable. Therefore, additional evidence on this point is desirable. The results presented here show that, under conditions where the native conformation of the purified calcium channel is retained, both β- and γ-polypeptides are immunoprecipitated by antibodies against α subunits. These results support the conclusion that these three classes of polypeptides are specifically associated components of the purified calcium channel from skeletal muscle.

Structural Homology of the Cardiac and Skeletal Muscle Calcium Channels. Antibodies that react specifically with the α subunits of skeletal muscle calcium channels also immunoprecipitate solubilized, [^{3}H]PN200-110-labeled calcium channels from cardiac sarcolemma, indicating that homologous antigenic determinants are present on the cardiac channel. However, cardiac calcium channels can only block a fraction of the immunoreactivity of our antibodies. Therefore, the skeletal muscle calcium channel must also contain antigenic sites that are not present on the cardiac channel. These tissue-specific regions of the channel structure may play a role in the different functional properties of cardiac and skeletal muscle calcium channels (Triggle, 1981; Reuter et al., 1982; Affolter & Coronado, 1985; Almers et al., 1981; Hess et al., 1984).

Comparison of the sedimentation behavior of [^{3}H]PN200-110-labeled, digitonin-solubilized calcium channels from cardiac and skeletal muscle suggests additional molecular similarities. The sedimentation coefficients of 21 and 21.6 S, respectively, indicate that each of these proteins is a large detergent–protein complex containing multiple polypeptide components including one or more α subunits. The molecular weight of the cardiac calcium channel has been estimated to be 370000 by analysis of its hydrodynamic properties after exchange into a low-density detergent and correction for the contribution of bound detergent (Horne et al., 1986). This size is sufficient to include multiple α subunits or α subunits in combination with smaller polypeptides like the β and γ subunits of the purified skeletal muscle channel.

The immunoreactive component of the cardiac calcium channel identified in the present study resembles one of the α subunits of the skeletal muscle channel in molecular properties. It has an apparent molecular weight of 170000 when disulfide bonds are not reduced, compared to 162000–167000 for the skeletal muscle channel. Its apparent molecular weight is reduced to 141000 upon reduction of disulfide bonds as previously observed for a portion of the protein in the α-subunit band of the purified skeletal muscle calcium channel (Curtis & Catterall, 1984; Curtis, 1986; Flockerzi et al., 1986). These similarities in immunoreactivity, subunit and oligomeric molecular weights, and electrophoretic behavior upon reduction of disulfide bonds indicate that the α subunits of the cardiac and skeletal muscle calcium channels are homologous, but not identical, proteins which are present in a similar oligomeric complex.

Previous studies of the cardiac calcium channel suggested a substantially different polypeptide composition from that of the skeletal muscle channel. Campbell et al. (1984) reported photoaffinity labeling of a protein with an apparent molecular weight of 32000 with nitrendipine in contrast to the consistent photolabeling of the α subunits of the skeletal muscle calcium channel by dihydropyridines (Ferry et al., 1984; Galizzi et al., 1986). Moreover, Rengasamy et al. did not identify a protein with the properties of the α subunits in partially purified preparations of the cardiac calcium channel (Rengasamy et al., 1985). In contrast to these earlier studies, Schmid et al. (1986) used antibodies against a component of the purified skeletal muscle calcium channel with an apparent molecular weight of 32000–34000 to identify cross-reacting proteins in cardiac muscle membranes. In reduced preparations, polypeptides with apparent molecular weights of 32000–34000 were labeled in immunoblots. In preparations with intact disulfide bonds, polypeptides with apparent molecular weights of 170000 were observed. Those results were interpreted to indicate the presence of a disulfide-linked complex with a molecular weight of 170000 containing the immunoreactive polypeptide, but the other components of such a complex were not identified. Our results directly identify an α subunit with an apparent molecular weight of 141000 as a major component of the cardiac dihydropyridine-sensitive calcium channel. Since this polypeptide migrates with an apparent molecular weight of 170000 when disulfide bonds are not reduced, it may be linked through disulfide bonds to the polypeptide with an apparent molecular weight of 32000–34000 detected previously (Campbell et al., 1984; Schmid et al., 1986). Our results clearly establish the presence of an α subunit with an apparent molecular weight of 141000 as a component of the cardiac calcium channel as well as the skeletal muscle calcium channel.

Recently, we have found that the two polypeptides resolved from the α subunit band of purified skeletal muscle calcium channels (Curtis & Catterall, 1984; Curtis, 1986; Flockerzi et al., 1986) represent two different, noncovalently associated, α subunits which we designate α_1 and α_2. α_1 (M_r 175000 contains the dihydropyridine binding site, cAMP-dependent protein kinase phosphorylation site(s), and substantial hydrophobic domain(s). α_2 (M_r 140000) is heavily N-glycosylated and is disulfide linked to a 24000–27000-dalton glycopeptide (Takahashi et al., 1987). Thus, the α subunit of the cardiac calcium channel which has been identified in the present studies resembles the α_2 subunit of the skeletal muscle calcium channel.

Subsequent to preparation of this paper for publication, Cooper et al. (1987) reported a 900-fold purification of the cardiac dihydropyridine-sensitive calcium channel. The predominant protein component of their preparation has apparent molecular weights of 170000 before reduction and 140000 after reduction of disulfide bonds. It is therefore similar to

the α_2 subunit we have identified by immunoprecipitation in this report and to the α_2 subunit of the skeletal muscle calcium channel. We assume that the cardiac calcium channel also contains an α_1 subunit like the skeletal muscle channel but that this subunit has not been recovered intact in the purified preparations of Cooper et al. (1987). A preliminary report of photoaffinity labeling of cardiac sarcolemmal polypeptides of 185 000 and 60 000 daltons with azidopine (Sharp et al., 1987) supports this assumption. Further studies are necessary to establish the complete subunit structure of the cardiac dihydropyridine-sensitive calcium channel.

Molecular Properties of Dihydropyridine-Sensitive Calcium Channels from Different Excitable Tissues. Several lines of evidence suggest that dihydropyridine-sensitive calcium channels may have similar molecular properties in a wide range of tissues. [^{3}H]PN200-110-labeled calcium channels from rabbit ileum longitudinal smooth muscle are immunoprecipitated by PAC-2, indicating the presence of a homologous protein component (Ice et al., 1987). Antibodies against a component of the purified skeletal muscle calcium channel with apparent molecular weights of 32 000–34 000 recognize polypeptides in smooth muscle with molecular weights of 170 000 before reduction and 32 000 after reduction as in cardiac and skeletal muscle (Schmid et al., 1986a). Antibodies against the α subunit of the skeletal muscle calcium channel recognize a similar polypeptide in immunoblots of brain proteins (Schmid et al., 1986b). [^{3}H]PN200-110-labeled calcium channels from rabbit brain are also immunoprecipitated by PAC-2, and the immunoreactive polypeptide has apparent molecular weights of 170 000 before reduction and 142 000 after reduction (Takahashi & Catterall, 1987). These results suggest that dihydropyridine-sensitive calcium channels in all excitable cells have a homologous, but not identical, α_2 subunit as one component, which may be linked by disulfide bonds to a polypeptide with an apparent molecular weight of 27 000–32 000. The different functional properties of dihydropyridine-sensitive calcium channels may result from variations on a common structural theme. It is now important to determine the complete subunit structures of calcium channels in these different excitable tissues.

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