Subunit Composition of the Purified Dihydropyridine Binding Protein from Skeletal Muscle†

Susan L. Hamilton,*,‡ Mary Jane Hawkes,‡ Kevin Brush,‡ and Richard Cook§
Department of Physiology and Molecular Biophysics and Howard Hughes Medical Institute and Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030
Ren-jie Chang and Henry M. Smilowitz
Department of Pharmacology, The University of Connecticut Health Center, Farmington, Connecticut 06032
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ABSTRACT: The dihydropyridine (DHP) receptor from rabbit skeletal muscle has been characterized by affinity labeling and purification. Two procedures were used for purification: one that was a procedure modified from that of Curtis and Catterall (1984) and one that employed an anti α_1 monoclonal antibody (Mab) affinity column. In addition, both digitonin and CHAPS solubilizations were utilized with each purification technique. The major findings are as follows: (1) In contrast to the behavior in digitonin, neither the 52K (β) nor the 140K (α_2) polypeptide quantitatively copurifies with the 170K (α_1) polypeptide when the purification is carried out in CHAPS. This has been shown by use of both wheat germ and monoclonal antibody columns. The digitonin-extracted receptor complex bound to the Mab affinity column loses α_2 and β when the digitonin is replaced by CHAPS, and when the complex is bound to a WGA column, a CHAPS wash causes dissociation of α_1, β, and γ from α_2. Loss of binding of dihydropyridines occurs with the CHAPS wash but can be partially restored by the addition of the CHAPS wash to the material eluted from the column with *N*-acetylglucosamine. (2) Although both detergents solubilized greater than 80% of the polypeptides associated with the DHP binding site, the ability of these proteins to bind dihydropyridines is reduced more by CHAPS treatment than by digitonin treatment, raising the possibility that subunit interactions contribute to high-affinity binding. Alternatively, CHAPS may remove tightly bound lipids necessary for binding or cause irreversible denaturation of the binding site. A particularly exciting consequence of this study is the utilization of the detergent CHAPS to dissociate the subunits. These polypeptides can then be purified, which may allow the assessment of their individual contributions to channel function.

Dihydropyridines modulate the activity of voltage-dependent calcium channels in cardiac and smooth muscle tissue. One of the richest sources of high-affinity dihydropyridine binding sites characterized to date is skeletal muscle t-tubule[1] membranes (Fosset et al., 1983; Glossmann & Ferry, 1985). It has been estimated, however, that the number of binding sites for dihydropyridines in this tissue is much greater than the number of functional voltage-dependent calcium channels (Schwartz et al., 1985). Rios and Brum (1987) have speculated that the DHP binding sites in skeletal muscle t-tubules play a role in voltage sensing and need not be conducting channels. Flockerzi et al. (1986), however, were able to demonstrate voltage-dependent calcium channel activity upon reconstitution of their purified DHP binding protein into bilayers. Muscle cells from dysgenic mice lack the α_1 subunit of the DHP receptor but have normal levels of α_2 (Knudson et al., 1989). A dihydropyridine-sensitive calcium channel with unusual properties has, however, been identified in these cells (Adams & Beam, 1989). Recently, Tanabe et al. (1988) have shown that microinjection of an expression plasmid containing the cDNA for the 170K dihydropyridine binding subunit of the skeletal muscle protein restores excitation–contraction coupling and voltage-dependent calcium channels in cultured muscle cells from dysgenic mice. These activities were sensitive to dihydropyridines. This observation supports the hypothesis that the DHP binding protein of skeletal muscle is both a calcium channel and a voltage sensor in the membrane. Whether the 170K polypeptide alone is sufficient to perform these functions or whether the other subunits are necessary for activity is not known.

Because the t-tubules are such an abundant source of DHP binding sites (Fosset et al., 1983), a number of laboratories (Glossmann & Ferry, 1983; Curtis & Catterall, 1984, 1986; Borsotto et al., 1985; Flockerzi et al., 1986; Vandaele et al., 1987; Vaghy et al., 1987; Sharp et al., 1987) have attempted to purify and characterize the DHP binding protein from skeletal muscle. There is considerable controversy over the subunit structure of the protein. A 170K polypeptide (α_1) has been shown to contain the binding site for dihydropyridines (Striessnig et al., 1987; Sharp et al., 1987; Takahashi et al., 1987) and for phenylalkylamines (Striessnig et al., 1987; Nakayama et al., 1987). The question arises, however, as to which of the other polypeptides found in partially purified

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* Author to whom correspondence should be addressed.
‡ Department of Physiology and Molecular Biophysics.
§ Howard Hughes Medical Institute and Department of Microbiology and Immunology.

[1] Abbreviations: BME, β-mercaptoethanol; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; cpm, counts per minute; DEAE, diethylaminoethyl; DHP, dihydropyridine; DHPR, dihydropyridine receptor; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NAG, *N*-acetylglucosamine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEG, polyethylene glycol 6000; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; t-tubule, transverse tubule; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WGA, wheat germ agglutinin.

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preparations are associated with the DHP binding polypeptide. Other candidates for subunits of the DHP binding protein are [from the nomenclature of Takahashi et al. (1987)] a 140K glycopolypeptide (α_2), which in the absence of reduction is disulfide bonded to a 33K polypeptide (δ), a 52K polypeptide (β), and another 30K polypeptide (γ). Both the α_1 and β subunits can be phosphorylated by cAMP-regulated protein kinase (Imagawa et al., 1987; Hosey et al., 1986) and, therefore, one or both of these subunits may be the site of the functionally important phosphorylation of the voltage-dependent channel (Reuter, 1983). Although there are some data to support each of these polypeptides as subunits of the DHP binding protein, Hofmann et al. (1987) recently questioned whether the α_2 and δ subunits are indeed subunits of the DHP binding protein or are contaminants since they did not stoichiometrically purify with the other subunits. A possible explanation of this observation was proposed by Takahashi et al. (1987), who demonstrated that dissociation of subunits occurs in some detergents. For this reason we choose to examine the purification and subunit composition of the [^{3}H]azidopine-labeled protein using the two detergents most commonly used for the purification of this protein, digitonin and CHAPS, and to analyze the ability of proteins solubilized in the different detergents to bind dihydropyridines.

Materials and Methods

Materials

[^{3}H]PN200-110 (82 Ci/mmol) and [^{3}H]azidopine (54 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Unlabeled nitrendipine was supplied by Miles Laboratories (Naperville, IL). Prestained electrophoresis standards were obtained from Diversified Biotech (Newton Centre, MA). Wheat germ agglutinin (WGA), digitonin, and CHAPS were purchased from Sigma (St. Louis, MO). Affi-Gel-10, Stains All, and nitrocellulose (0.2-μm mesh) were obtained from Bio-Rad (Richmond, CA). DEAE-Trisacryl M was purchased from IBF (Savage, MD). Coomassie Brilliant Blue R-250 was obtained from Serva (New York, NY).

Methods

Membrane Preparation. Microsomal membranes were prepared by using a procedure modified from that of Rosemblatt et al. (1981) as follows: 100 g of frozen rabbit skeletal muscle was minced and mixed with 100 mL of 0.3 M sucrose, 20 mM Tris–maleate (pH 7.0) containing 100 μM PMSF, 1 μM aminobenzamidine, 1 μM pepstatin A, 1 μg/mL leupeptin, and 1 μg/mL aprotinin and was homogenized in a Waring blender for four 15-s bursts at low speed; 200 mL of 0.3 M sucrose and 20 mM Tris–maleate (pH 7.0) was added, and the suspension was homogenized for three 20-s bursts at high speed. This material was centrifuged at 8500g for 30 min. The pellets were rehomogenized in 300 mL of buffer for three 20-s bursts at high speed and spun at 8500g for 30 min. The supernatants were combined, and KCl was added to 0.5 M. This mixture was stirred for 1 h at 4 °C and centrifuged at 200000g for 45 min. The pellet was resuspended in 400 mL of 0.3 M sucrose and 20 mM MOPS (pH 7.4) and centrifuged again for 30 min at 200000g. This second pellet was resuspended in 400 mL of 20 mM MOPS (pH 7.4) and centrifuged again for 30 min at 200000g. The final pellet was resuspended in 30 mL of 20 mM MOPS (pH 7.4). We obtain 300–400 mg of protein using this procedure.

Preparation of WGA–Affi-Gel. Affi-Gel-10, an active *N*-hydroxysuccinimide ester on a cross-linked agarose gel, was added to a solution of 10 mg of WGA/mL of gel in 0.1 M $NaHCO_3$ (pH 8.4) and was shaken gently for 90 min at room temperature and then overnight at 4 °C. Glycine (0.1 M) was added to block any remaining active esters. The gel was shaken gently for 1 h and then transferred to a column and washed with 0.1 M $NaHCO_3$ (pH 8.4) until the gel was free of reactants as determined by A_{280nm}.

Radiolabeled DHP Binding. Membranes (5–25 μg) were incubated with [^{3}H]PN200-110 or [^{3}H]azidopine at concentrations from 0.05 to 2.0 nM in 2 mL of 50 mM MOPS (pH 7.4) for 2–3 h at room temperature in the dark. The binding was stopped by rapid filtration through Whatman GF/F filters. Each filter was washed five times with 5 mL of ice-cold distilled water and counted in 10 mL of Beckman HP/b.

Binding to detergent-solubilized protein was done in 250 μL of 50 mM MOPS, pH 7.4, and 0.25 mg/mL BSA at 4 °C for 1–2 h with [^{3}H]PN200-110 at concentrations ranging from 0.25 to 10 nM. Binding was stopped by PEG precipitation by addition of 3.5 mL of 10% polyethylene glycol 6000 (PEG) and 100 μL of a solution containing 5 mg/mL BSA and 5 mg/mL rabbit γ-globulin. After 15 min on ice, filtration was performed on Whatman GF/F filters followed by 3 × 4 mL washes of cold 10% PEG solution.

Purification of the DHP Binding Protein. Two hundred milligrams of membrane protein was diluted to 2 mg/mL in 1% detergent, 185 mM KCl, and 10 mM MOPS (pH 7.4) (buffer I) with the protease inhibitors at the concentrations mentioned previously. The membranes were solubilized in this buffer for 45 min at 4 °C. Insoluble material was removed by centrifuging for 30 min at 120000g. The solubilized membrane proteins were incubated with 30 mL of WGA–Affi-Gel and 95 mL of buffer I for 90 min with gentle shaking, loaded onto a column, and washed with 300 mL of buffer I containing detergent. The detergent concentration used throughout the remainder of the preparation was 1% for CHAPS and 0.1% for digitonin unless otherwise specified. Specifically bound material was eluted with 100 mL of 200 mM *N*-acetylglucosamine in detergent and buffer I. The pooled fractions from this elution were diluted 1:4 with 10 mM MOPS (pH 7.4) combined with 20 mL of DEAE-Trisacryl M and shaken gently at 4 °C for 30 min. The resin was washed batchwise with 200 mL of detergent, 20 mM NaCl, and 10 mM MOPS (pH 7.4) and eluted with 300 mM NaCl in detergent and 10 mM MOPS (pH 7.4). Protease inhibitors were included in all solutions.

A second procedure for purifying the DHP binding protein utilized an affinity column prepared by using an anti-170K antibody coupled to Bio-Rad Affi-Gel-10. The affinity column was prepared by coupling 20 mg of an antibody (78), purified from culture supernatant by protein A (Bio-Rad), to 1 mL of Affi-Gel-10 according to company instructions.

Membranes were solubilized with 1% digitonin or 2.5% CHAPS (final) in 25 mM MOPS, 185 mM KCl, 1 mM EDTA, and protease inhibitors. Detergent:protein ratio was 5:1 (digitonin) and 6–10:1 (CHAPS). Insoluble material was removed by ultracentrifugation (160000g for 40 min) and the supernatant incubated with 0.5 mL of the antibody-linked Affi-Gel-10 overnight with gentle rocking. The resin was packed into a column, washed with 20 mL of the above buffer containing 1% detergent followed by a 20 mL of buffer containing 0.3% detergent. Bound protein was eluted from the column with Bio-Rad buffer (pH 3.0) containing 0.3% detergent. Fractions (0.5 mL) were immediately neutralized with 65 μL of 1 M Tris (pH 9.0).

Preparation of Antibodies. Monoclonal antibody 78 (Smilowitz et al., 1987) was prepared by immunizing mice with a partially purified DHP binding protein (100 μg, 10%

DHPR) in complete Freund's adjuvant, followed by three boosts (100 μg) in incomplete Freund's adjuvant at 3-week intervals. The initial screening was performed by using an ELISA assay with the partially purified preparation and the final screening was by Western blotting.

Affinity Labeling. To label the DHP binding protein, membranes (2 mg/mL in 50 mM MOPS, pH 7.4) were incubated with 10 nM [^{3}H]azidopine for 2 h at 4 °C and then irradiated for 10 min at room temperature with a Blak-Ray lamp Model UVL-56 (366 nm) at a distance of 3 cm. To define nonspecific labeling, 10 μM nitrendipine was included in a parallel incubation solution.

SDS–PAGE. Samples in 2% SDS, 62.4 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue were (except where indicated in the figure legend) reduced in 20 mM DTT for 1 h at room temperature and alkylated with 50 mM *N*-ethylmaleimide for 15 min at room temperature. Aliquots (25–50 μL) of each sample were layered on SDS linear 5–20% polyacrylamide gels and electrophoresed as described by Laemmli (1970). Gels were stained either with Coomassie Brilliant Blue, Stains All, or silver stain (see below). Individual lanes from some Coomassie Brilliant Blue stained gels were removed, sliced into 1-mm slices, digested with NCS tissue solubilizer (Amersham), and counted in a liquid scintillation counter. Alternatively, radioactivity on a gel was visualized by fluorography using Autofluor (National Diagnostics) and Kodak X-Omat AR-5 film.

Electroelution and Preparation of Samples for Sequencing. Coomassie Brilliant Blue stained proteins in gel slices were electroeluted and prepared for sequencing by the technique of Hunkapillar et al. (1983).

Silver Staining. Gels that had been previously stained with Coomassie Brilliant Blue were washed extensively in 50% methanol and silver stained by using the technique of Wray et al. (1981).

Stains All Staining. Gels were stained with Strains All by using the technique of Ksiezak-Reding and Yen (1987).

N-Terminal Sequence Analysis. Electroeluted samples (50–500 pmol), which had been precipitated twice with ethanol to remove the Coomassie Brilliant Blue and excess SDS, were dried and resuspended in 0.1% (w/v) SDS and sequenced by using an Applied Biosystems 470A microsequencer (Foster City, CA) with a 120A PTH analyzer.

Results

DHP Affinity Labeling of Microsomal Membranes. The affinity label [^{3}H]azidopine was used to modify covalently and identify the DHP binding protein in our membrane preparations. When the incubation is performed in the dark, this compound binds to membranes with an apparent dissociation constant of 0.6 ± 0.3 nM and a B_{max} of 14 ± 2 pmol/mg (n = 3). The maximum number of binding sites is similar to that found with [^{3}H]PN200-110. Upon irradiation of the membranes preincubated with [^{3}H]azidopine, the label is specifically incorporated into a polypeptide with an apparent molecular weight of 170K on SDS–polyacrylamide gels (Figure 1A). For Figure 1 the individual lanes from a gel were excised, sliced into 1-mm pieces, digested, and counted. The labeling of the 170K band is blocked by excess unlabeled nitrendipine (Figure 1B) and partially blocked by verapamil (Figure 1C). Some radioactivity is incorporated into a 100K band, but this is not blocked by either nitrendipine or verapamil. These findings are in agreement with the observations of other laboratories (Striessnig et al., 1987; Sharp et al., 1987).

Effect of Detergent Solubilization on DHP Binding. The two most commonly used detergents for the purification of the DHP receptor are digitonin (Glossmann & Ferry, 1983; Curtis & Catterall, 1984, 1986; Flockerzi et al., 1986; Vandaele et al., 1987; Vaghy et al., 1987; Sharp et al., 1987) and CHAPS (Glossmann & Ferry, 1983; Borsotto et al., 1985; Vandaele et al., 1987). Differences in the reported subunit structure and ability to bind dihydropyridines of protein purified in these two detergents led us to examine and compare the behavior of the DHP binding protein of skeletal muscle in these two detergents.

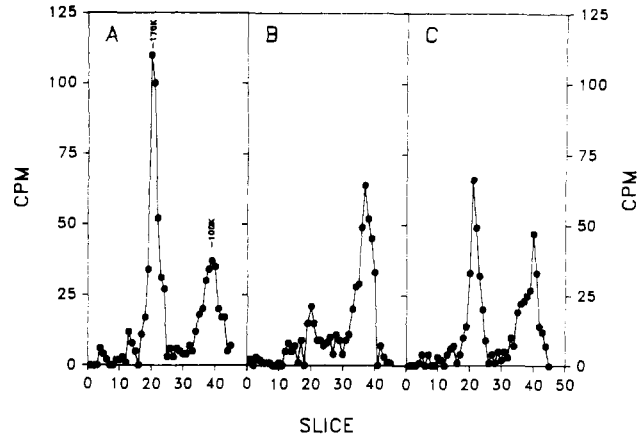

FIGURE 1: Affinity labeling of microsomal membranes with [^{3}H]-azidopine. Membranes were labeled with [^{3}H]azidopine as described in the text, and the individual lanes from the gel were excised, sliced into 1-mm pieces, digested with NCS (Amersham), and counted. (A) Control; (B) labeled in the presence of 1 μM nitrendipine; (C) labeled in the presence of 100 μM verapamil.

To determine the effect of detergent solubilization on the ability of the preparation to bind dihydropyridines, Scatchard analysis of the binding of [^{3}H]PN200-100 to membranes in the buffer used for solubilization, but without detergent, and to the preparations solubilized in either CHAPS or digitonin is shown in Figure 2A,B. Although the K_d was similar in CHAPS and digitonin, the B_{max} was greatly decreased in the former. As will be seen below, this is not due to a greater solubilization of binding sites in digitonin than in CHAPS, but rather to a loss in ability of CHAPS-solubilized proteins to bind dihydropyridines.

The largest fold purification of the DHP receptor, as will be seen below, is obtained by using a WGA–Affi-Gel column. Scatchard analysis of [^{3}H]PN200-110 binding to the preparations eluted from the WGA column is shown in Figure 2D. Binding to the material that does not stick to the column is shown in Figure 2C (note difference in scale). Less than 10% of the total binding sites are ever recovered in the WGA void. To examine further the nature of the detergent effects on binding, a digitonin-solubilized preparation was equilibrated with the WGA–Affi-Gel column, washed extensively with 0.1% digitonin, and then washed with either 1% digitonin or 1% CHAPS. After reequilibration of the column with 0.1% digitonin, [^{3}H]PN200-110 binding to the material eluted with *N*-acetylglucosamine was performed and is shown in Figure 3A. The CHAPS wash causes a dramatic decrease in binding. The material removed from the column with CHAPS did not bind [^{3}H]PN200-110. When this material is added back to the CHAPS- or digitonin-washed materials that have been eluted from the column with *N*-acetylglucosamine, it greatly enhances dihydropyridine binding (Figure 3B), giving a 2-fold increase with the material that was not washed with CHAPS and a 12.5-fold increases in that which was washed.

To analyze changes in the polypeptide composition of the DHP receptor upon treatment with different detergents, we

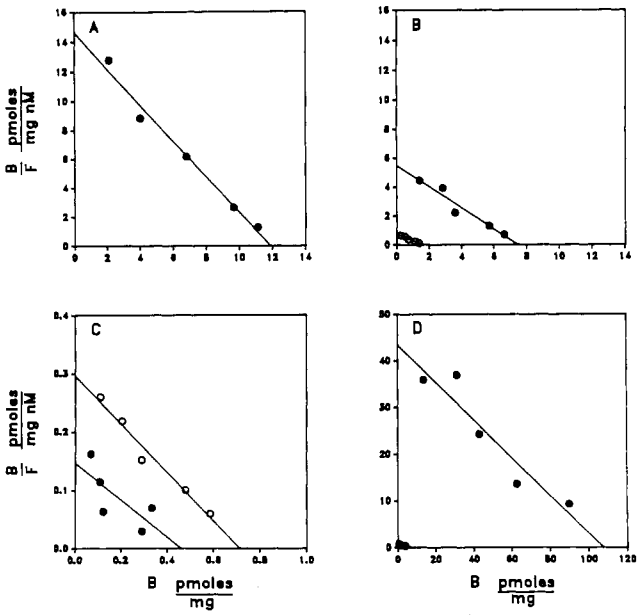

FIGURE 2: Scatchard analysis of the binding of [^{3}H]PN200-110 to membranes and detergent-solubilized preparations. Microsomal membranes or solubilized preparations were incubated with [^{3}H]-PN200-110 at concentrations from 0.2 to 10.0 nM in 250 μL of 50 mM MOPS (pH 7.4) and 0.20 mg/mL BSA for 2 h at 4 °C. A solution (100 μL) containing BSA/rabbit γ-globulin (5 mg/mL of each) was added, and proteins were precipitated with 3.5 mL of 10% PEG. After 15 min on ice, samples were filtered through Whatman GF/F filters and washed with 3 × 3.5 mL washes of 10% PEG. Filters were dried and counted after the addition of 100 μL of H_2O and 10 mL of Beckman ready protein scintillant. (A) Microsomal membranes (48 μg) in buffer I [185 mM KCl, 10 mM MOPS (pH 7.4)] were assayed under the same conditions as soluble protein. K_d = 0.81 nM; B_{max} = 11.9 pmol/mg. (B) Membranes (2 mg/mL) were solubilized with 1% digitonin or 1% CHAPS in buffer I for 30 min at 4 °C. Insoluble material was removed by centrifugation for 30 min at 120000g. Solubilized protein was assayed for binding by using procedures described under Methods. (○) Digitonin-solubilized protein (32 μg), K_d = 1.4 nM, B_{max} = 7.43 pmol/mg; (●) CHAPS-solubilized protein (43 μg), K_d = 0.99 nM, B_{max} = 1.65 pmol/mg. (C) 1% digitonin or 1% CHAPS soluble protein was shaken with 25 mL of WGA–Affi-Gel for 1 h at 4 °C and loaded onto a column. The column void was assayed for binding. (○) Digitonin WGA void (18 μg), K_d = 3.1 nM, B_{max} = 0.46 pmol/mg; (●) CHAPS WGA void (26 μg), K_d = 2.4 nM, B_{max} = 0.71 pmol/mg. (D) The WGA columns were washed with 125 mL of 0.1% digitonin (or 1% CHAPS) buffer I. Specifically bound protein was eluted with 50 mL of 0.1% digitonin (or 1% CHAPS), 200 mM N-acetylglucosamine, and 10 mM MOPS (pH 7.4). (○) Digitonin WGA elute (0.9 μg), K_d = 2.5 nM, B_{max} = 108 pmol/mg; (●) CHAPS WGA elute (0.9 μg), K_d = 8.4 nM, B_{max} = 6.78 pmol/mg.

have purified and characterized the receptor in each detergent.

Partial Purification of the DHP Binding Protein Using WGA–Affi-Gel. One procedure (method 1) to purify the DHP binding protein after solubilization in detergent utilized lectin affinity chromatography on WGA–Affi-Gel, a DEAE-Trisacryl M column, and a linear 5–20% sucrose gradient and is based on the procedure of Curtis and Catterall (1984). The yields of protein and picomoles of covalently bound [^{3}H]-azidopine at each stage of purification in the two detergents are summarized in Table I. Analysis by 1-mL G-50 columns of the binding after heat denaturation indicated that, on average, [^{3}H]azidopine was covalently coupled to 5–10% of the available sites. On the WGA column, the [^{3}H]azidopine label is purified 18- and 34-fold for CHAPS and digitonin, respectively. We obtain a 27-fold purification in CHAPS and a 138-fold purification in digitonin from the starting membranes to the material obtained on the sucrose gradient. As the starting membranes have 14 pmol of [^{3}H]azidopine binding sites/mg of protein, the number of binding sites in the final

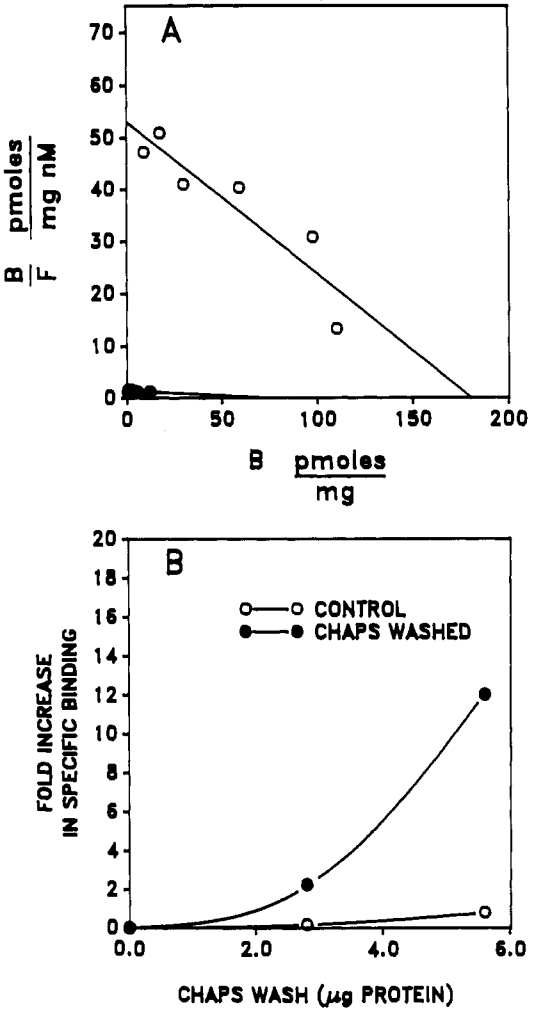

FIGURE 3: (A) Effect of CHAPS wash of digitonin preparation on [^{3}H]PN200-110 binding. A digitonin-solubilized preparation was divided into two parts and bound to a WGA–Affi-Gel column. One column was washed with 25 mL of 1% digitonin in buffer I while the second column was washed with 25 mL of 1% CHAPS with buffer I. Following reequilibration of the column with 0.1% digitonin, buffer I, the column was eluted with 200 mM N-acetylglucosamine, and binding to 25 μL of the eluate was performed as previously described. (●) CHAPS-washed WGA elute, K_d = 48 nM, B_{max} = 70 pmol/mg; (○) digitonin-washed WGA elute, K_d = 3.4 nM, B_{max} = 180 pmol/mg. (B) Effect of readdition of material removed in CHAPS wash. Proteins (0.15 μg) eluted from the WGA column without (a) and with (b) a CHAPS wash were incubated in 250 mL of 50 mM MOPS with [^{3}H]PN200-110 (12.5 nM) and the indicated amount of the CHAPS wash for 1 h at 4 °C and PEG precipitated as previously described. Assays were performed in duplicate. Binding was normalized to the amount obtained in the absence of the wash material to give x-fold increase.

preparation should be 370 and 1930 pmol/mg in CHAPS and digitonin, respectively. This is comparable to the number of DHP binding sites in CHAPS reported by Borsotto et al. (1985) for their preparation. Direct binding of [^{3}H]PN200-110 to a solubilized (unlabeled) preparation after elution from the WGA column (Figure 2D) gave an 8- and 19-fold purification for CHAPS and digitonin, respectively, suggesting some loss in the ability to bind dihydropyridines in both detergents. Addition of endogenous lipids obtained from a chloroform methanol extraction of skeletal muscle tissue increases this about 2–3-fold in digitonin, but no increase is seen with the CHAPS preparation (data not shown), suggesting that lipids play an important role in maintaining the high-affinity dihydropyridine binding site after detergent solubilization; however, the effect of lipids appears to be distinct from

Table I: Protein and Bound [^{3}H]Azidopine at Each Stage of the Purification[a]

fraction	CHAPS protein (mg)	CHAPS [^{3}H]azidopine (pmol)	CHAPS pmol/mg	digitonin protein (mg)	digitonin pmol	digitonin pmol/mg
microsomes	348	520	1.5	346	521	1.5
solubilized proteins	288	525	1.8	257	552	2.1
WGA–Affi-Gel column elute	3.0	82	27	2.8	144	51
DEAE-Trisacryl M column elute	1.9	52	27	2.1	84	40
sucrose gradient	0.6	24	40	0.4	83	207

[a] Protein was determined by the method of Lowry et al. (1951) and covalently bound [^{3}H]azidopine was determined by passing a heat-denatured aliquot of the sample through a 1-mL G-50 column.

that described in Figure 3B since the material in the CHAPS wash affects both the digitonin and CHAPS preparations.

The polypeptides associated with some of the different fractions in either digitonin or CHAPS are shown in Figure 4A, and the distribution of covalently bound [^{3}H]azidopine is shown in the fluorogram in Figure 4B. In this figure, as seen in the fractions from the DEAE-Trisacryl M column (lanes e and f), the CHAPS preparation has substantially less α_1 and β subunits than the digitonin preparation.

As can be seen in Table I, a substantially higher fraction of the [^{3}H]azidopine-labeled material is lost from the WGA column when the solubilization is performed in CHAPS as compared to digitonin, suggesting that the α_1 polypeptide may dissociate in CHAPS from the polypeptide which binds to the WGA column. The material that is eluted from the WGA column with *N*-acetylglucosamine (Figure 2B) and that which passes through the column (Figure 2C) bind [^{3}H]PN200-110 to a very limited extent when the solubilization is in CHAPS.

To characterize the CHAPS-solubilized material that does not bind to the WGA column, the material was concentrated and further fractionated on a DEAE-Trisacryl M column (Figure 5B). For comparison, the elution profile of the CHAPS-solubilized material that does bind to the WGA column is shown in Figure 5A. Although in most preparations the DEAE-Trisacryl M column was washed with 20 mM NaCl and eluted with 300 mM NaCl, we have found that the DHP binding material can be further fractionated by a three-step elution from this column as shown in Figure 5A,B, which will be further discussed in Figure 7. The material eluting from both columns with 200 and 300 mM NaCl was then sedimented on sucrose gradients. The profiles obtained for the material that did not bind to the WGA column is shown in Figure 5E,F, and the material from the WGA eluate is shown in Figure 5C,D for the 200 and 300 mM eluates of the DEAE-Trisacryl M columns. The [^{3}H]azidopine bound to protein found in the WGA void sediments, for the most part, more slowly than that which elutes from the column with *N*-acetylglucosamine. To establish that the radioactivity in the void is associated with the 170K polypeptide, the 170K region of an SDS gel of an aliquot of the fractions from the sucrose gradients shown in Figure 5E,F was excised from the gel, digested, and counted (Figure 5G,H). The affinity label covalently coupled to the 170K polypeptide coincided with the distribution of the radiolabel on the sucrose gradients.

Reequilibrium of the fraction that was not retained by the WGA–Affi-Gel column with fresh WGA–Affi-Gel resulted in only a small amount of additional binding to the column.

Characterization of the Polypeptide Components of the Purified DHP Binding Protein. The polypeptides found in the fractions from the sucrose gradient profiles of the purified CHAPS and digitonin preparations that bind to the WGA column are shown in Figure 6A,B. The major polypeptides of the most highly purified fractions from the sucrose gradients are 170K (α_1), 140K (α_2), and 52K (β) for the digitonin

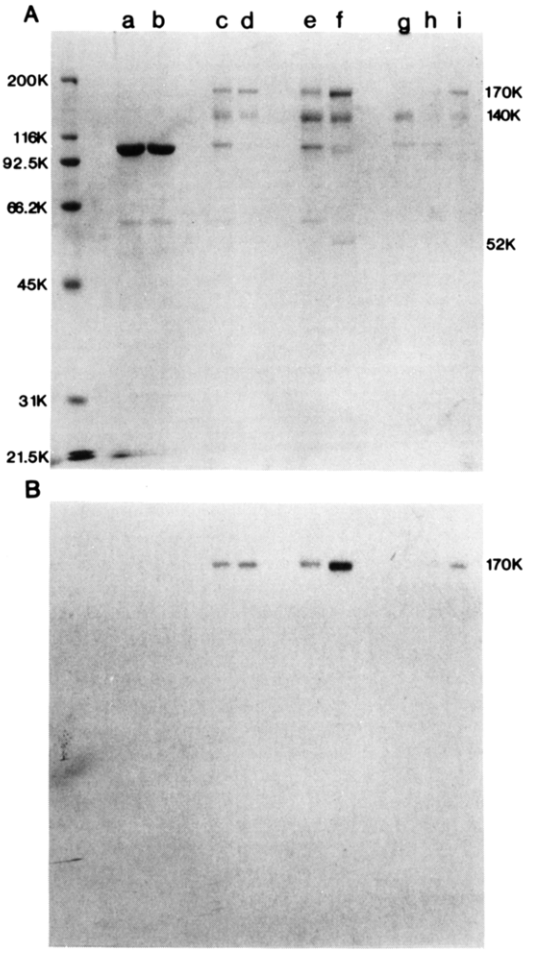

FIGURE 4: SDS gel electrophoresis and fluorography of fractions from digitonin- and CHAPS-solubilized preparations (method 1). (A) Microsomal membranes, affinity labeled with [^{3}H]azidopine, were solubilized in either CHAPS or digitonin. (Lane a) Proteins solubilized in CHAPS; (lane b) proteins solubilized in digitonin; (lane c) WGA column eluate of CHAPS preparation; (lane d) WGA column eluate of digitonin preparation; (lane e) DEAE-Trisacryl M column eluate (300 mM NaCl) of CHAPS preparation; (lane f) DEAE-Trisacryl M eluate (300 mM NaCl) of digitonin preparation; (lane g) fraction 4 from sucrose gradient of CHAPS preparation; (lane h) fraction 10 from sucrose gradient of CHAPS preparation; (lane i) fraction 11 from sucrose gradient of digitonin preparation. Sucrose gradient profiles are shown in Figure 6. (B) Autoradiogram of [^{3}H]azidopine photoaffinity labeled preparation gel shown above. The gel was soaked in autofluor, dried, and exposed for 6 days at −80 °C.

preparation. The 170K (α_1), 140K (α_2), 100K, and 58K polypeptides are the major polypeptides of the CHAPS preparation. The 58K polypeptide has been identified as calsequestrin by N-terminal sequencing of the first 40 amino acids (Table II). Also shown in Table II is the N-terminal sequence of α_2, and it is similar to that deduced by Nakayama et al. (1987). The N-terminal amino acid of the α_1 and 100K polypeptides was apparently blocked. The [^{3}H]azidopine label

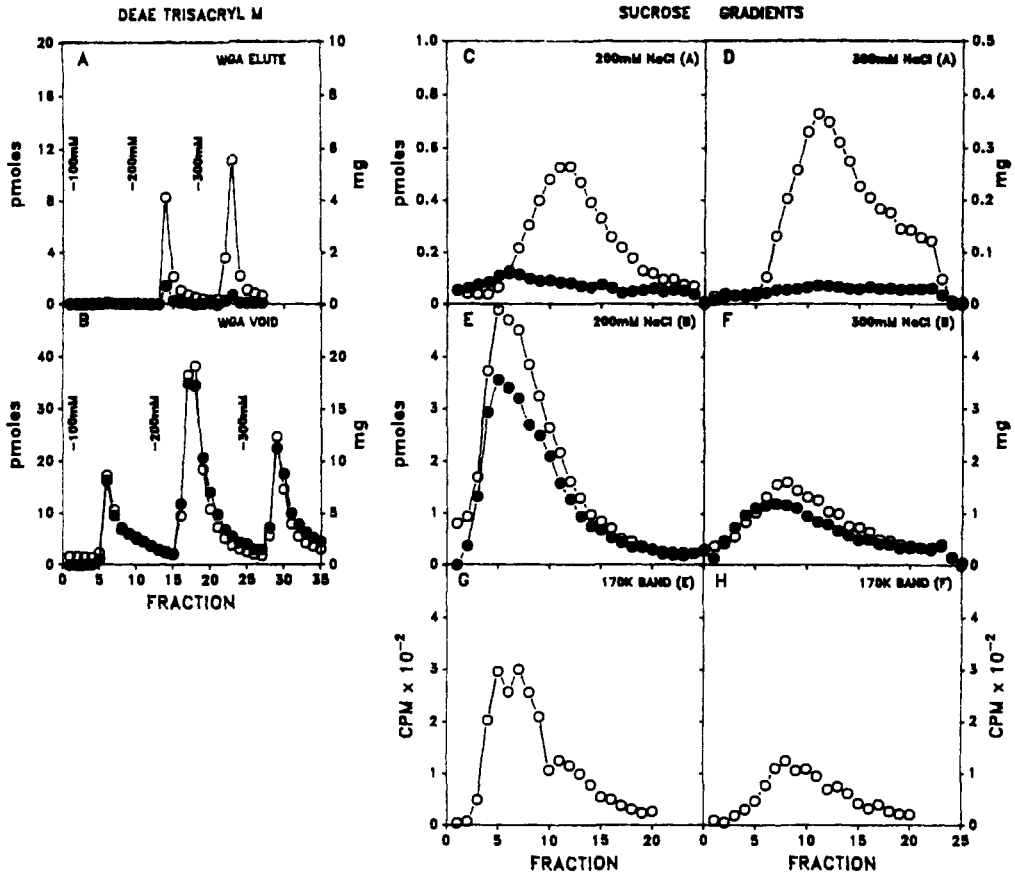

FIGURE 5: DEAE-Trisacryl M and sucrose gradient profiles of CHAPS-solubilized protein after fractionation on a WGA column. Following separation of the CHAPS-solubilized protein (460 mg) into a fraction that bound to the WGA column (3 mg) and one that did not (320 mg), these samples were further fractionated by a salt step elution of a DEAE-Trisacryl M column. (○) pmol; (●) protein. (A) DEAE-Trisacryl M column elution of CHAPS-solubilized proteins that were eluted from the WGA column with *N*-acetylglucosamine; (B) DEAE-Trisacryl M column elution of CHAPS-solubilized proteins that do not bind to WGA column; (C) 5–20% sucrose gradient profile of 200 mM NaCl elute from column shown in (A); (D) 5–20% sucrose gradient profile of 300 mM NaCl elute from column shown in (A); (E) 5–20% sucrose gradient profile of 200 mM NaCl elute from column shown in (B); (F) 5–20% sucrose gradient profile of 300 mM NaCl elute from column shown in (B); (G) [^{3}H]azidopine cpm from 170K band of SDS gel of 50-μL aliquots of fractions across the sucrose gradient shown in (E) (the 170K region of the gel was excised, digested, and counted as previously described); (H) [^{3}H]azidopine cpm from 170K band of SDS gel of 50-μL aliquots of fractions across the sucrose gradient shown in (F).

Table II: N-Terminal Sequence Analysis of Major Polypeptides in Partially Purified DHP Binding Preparations

170K	Apparently blocked.
140K	EPFPSAVTIKSWVDKMQESLV
100K	Apparently blocked.
58K	EEGLDFPEYDGVDRVINVNAKNYKNVFKKYEVLALLYHEPPKDDKAS

is found in the 170K band (Figure 6E,F). Other bands that can sometimes be detected in both CHAPS and digitonin by silver staining include a 30K polypeptide and a 25K polypeptide. In CHAPS, but not in digitonin, most of the α_2 polypeptide sediments slower than the α_1 polypeptide that is eluted from the WGA column. As shown in Figure 5, the α_1 polypeptide that does not bind to the WGA column in CHAPS (i.e., that found in the column void) also sediments more slowly than that which binds to the column. There is, however, always in this preparation some of the α_2 polypeptide cosedimenting with the α_1 polypeptide after purification on a WGA column, suggesting that for α_1 to purify on the WGA column it must be in association with α_2. In addition, this polypeptide appears to always cosediment with the α_1 polypeptide in digitonin. Very little of the 52K (β) polypeptide can be detected in any fraction of the CHAPS preparation, presumably due to loss from the WGA column.

If, prior to the sucrose gradient, a three-step elution (100, 200, 300 mM NaCl) of the DEAE-Trisacryl M column is used, the α_2 subunit (and possibly β) can be also seen to separate from the α_1 subunit in CHAPS (Figure 7) but not in digitonin (data not shown).

As shown in Figure 3, a CHAPS wash of the digitonin-solubilized preparation bound to the WGA column causes a loss in the ability of the *N*-acetylglucosamine-eluted material to bind [^{3}H]PN200-110. To examine changes in the polypeptide composition of the digitonin preparation after treatment with CHAPS, we pooled the peak functions of these sucrose gradients of the digitonin preparation and reequilibrated this material with the WGA column in 1% CHAPS. The polypeptides applied to the column are shown in Figure 8, lane b. The polypeptides eluted from the column with *N*-acetylglucosamine after reequilibrium with 0.1% digitonin are shown in Figure 8, lane c. The CHAPS treatment removes most of the α_1 subunit and essentially all of both the β and γ subunits from the WGA-eluted proteins. These results suggest either that one or more of these polypeptides are necessary for high-affinity [^{3}H]PN200-110 binding or that CHAPS treatment irreversibly modifies the α_1 subunit conformation. We cannot yet discriminate between these two possibilities.

Purification of the DHP Receptor Using an Anti-170K Antibody Affinity Column. The observation that the α_1, α_2, and β polypeptides dissociate from one another in CHAPS but not in digitonin has been confirmed by using an anti-170K antibody column for the purification instead of the WGA column.

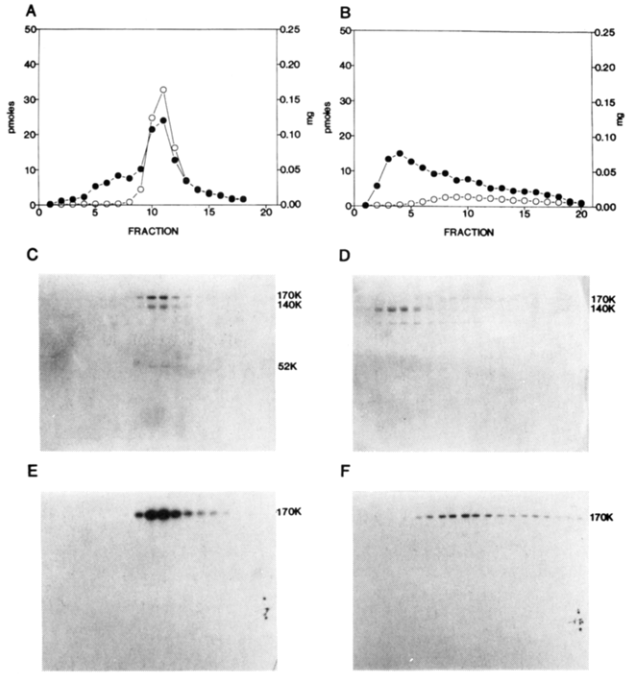

FIGURE 6: Sucrose gradient profiles of partially purified preparations in CHAPS or digitonin. The 300 mM NaCl (1.5 mL) elutions from the DEAE-Trisacryl M column of the preparation described in Figure 5 were layered separately on 38 mL of linear 5–20% sucrose gradients containing 1% CHAPS or 0.1% digitonin, 185 mM KCl, and 10 mM MOPS (pH 7.4) and sedimented for 2.5 h at 210000*g* in a VTI 50 rotor (Beckman); 1.8-mL fractions were collected from the top (left). Aliquots (50 μL) were placed in SDS sample buffer, reduced and alkylated as previously described, and electrophoresed on 5–20% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue. (A) Digitonin preparation [(○) pmol of [^{3}H]azidopine bound; (●) protein]; (B) CHAPS preparation [(○) pmol of [^{3}H]azidopine bound; (●) protein]; (C) SDS–PAGE of proteins in (A); (D) SDS–PAGE of proteins in (B); (E) Fluorogram of (C); (F) Fluorogram of (D).

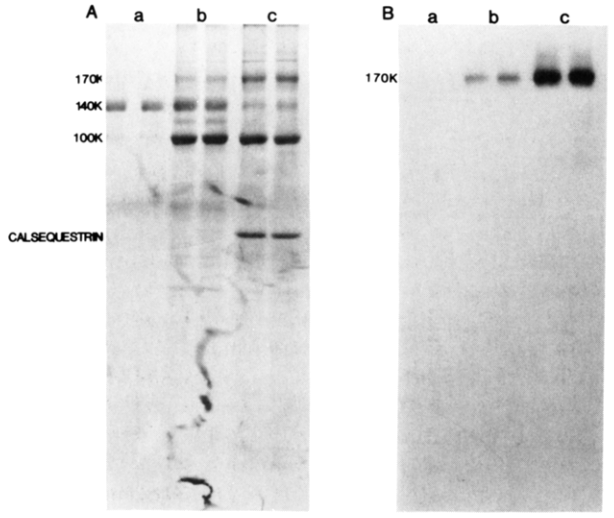

FIGURE 7: SDS–PAGE and fluorography of CHAPS-solubilized proteins step-eluted with NaCl from a DEAE-Trisacryl M column. CHAPS-solubilized proteins eluted from the WGA column with *N*-acetylglucosamine were applied to a DEAE-Trisacryl M column and step-eluted with (a) 100 mM NaCl, (b) 200 mM NaCl, and (c) 300 mM NaCl. Figure includes two preparations. (A) Coomassie Brilliant Blue stained gel; (B) Fluorogram.

A monoclonal antibody (Smilowitz et al., 1987) prepared by immunization of mice with a partially purified DHP binding protein and selected on the basis of ability to immunoprecipitate bound [^{3}H]PN200-110 (Figure 9B) and to bind to the 170K polypeptide (Figure 9A) was used to prepare an antibody affinity column. To purify the DHP binding protein (method 2) with this antibody affinity column, 30 mg of membranes (28 pmol of DHP binding sites/mg of protein) was solubilized with either digitonin or CHAPS, and the binding proteins were purified as described under Methods. In digitonin approximately 300 μg of protein was recovered in the pH 3 eluate,

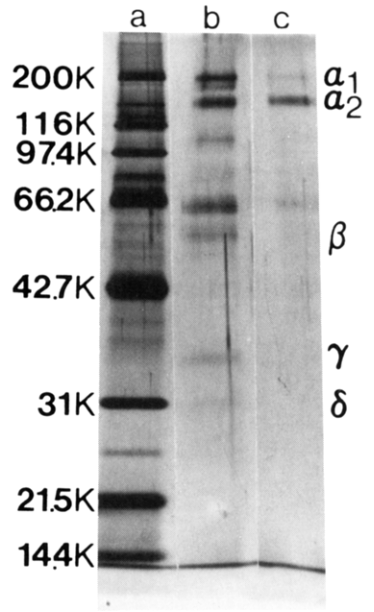

FIGURE 8: Effect of 1% CHAPS on polypeptides of a digitonin-solubilized preparation bound to WGA–Affi-Gel column. Pooled peak fractions of the sucrose gradient of the digitonin preparation were incubated 1 h at 4 °C in 1% CHAPS with 1 mL of WGA–Affi-Gel. The column was washed with 0.1% digitonin and then eluted with NAG in 0.1% digitonin. Samples were electrophoresed on a SDS–10% polyacrylamide gel and silver stained. (Lane a) Molecular weight markers; (lane b) polypeptides in peak fractions from sucrose gradient of DEAE-Trisacryl–200 mM NaCl elution of digitonin-solubilized preparations; (lane c) polypeptides in NAG eluate.

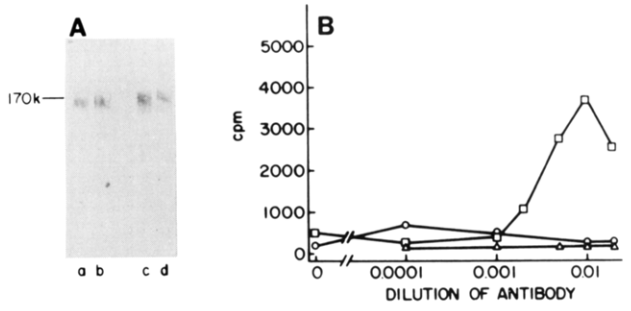

FIGURE 9: Characterization of Mab 78. (A) Immunoblot with Mab 78. Lanes a and c and lanes b and d contain the partially purified DHP receptor with 500–1000 and 1000–2000 pmol of [^{3}H]PN200-110 binding sites/mg of protein, respectively. (Lanes a, b) Unreduced; (lanes c, d) reduced. The Western blots were incubated with a 1/1000 dilution of Mab 78 for 1 h at 37 °C and, after washing, with a 1/1000 dilution of HRP-labeled goat anti-mouse antibody (Hyclone). (B) Immunoprecipitation with antibody 78. T-tubule membranes (0.2 mg/mL, 32 pmol of DHP binding sites/mg) in 50 mM Tris were labeled with 10 mM [^{3}H]PN200-110 for 30 min at 4 °C in the dark in the absence (□) and presence of 4 μM unlabeled PN200-110 (△). The membranes were solubilized for 30 min at 4 °C with 1% digitonin (detergent:protein = 5:1) and centrifuged at 160000*g* for 40 min; 10-μL aliquots of the extract containing 0.3 pmol of DHP receptor were incubated for 4 h at 4 °C with various dilutions of antibody 78 or normal mouse IgG (Sigma) (○). Protein A–Sepharose reagent [prepared according to the method of Takahashi and Catterall (1987)] was incubated with each of the reaction mixtures overnight at 4 °C. Complexes were collected by sedimentation (8000*g* for 1.5 min) and washed twice in 0.5% digitonin in PBS. Pellets were dissolved in 100 μL of 0.5% SDS overnight and assayed by scintillation counting.

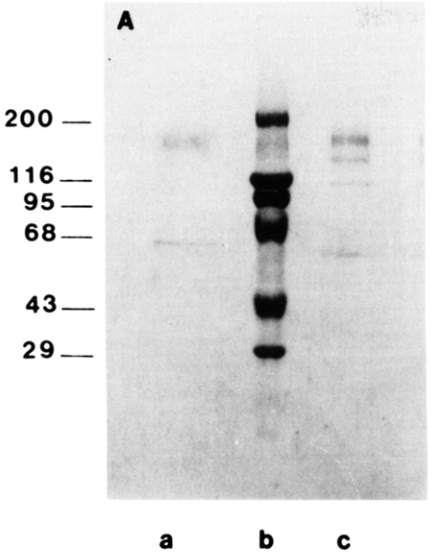

FIGURE 10: Comparison of CHAPS- and digitonin-solubilized proteins purified on a antibody affinity column. The preparations eluted from the antibody column after solubilization in CHAPS (lane a) and in digitonin (lane c) were electrophoresed on SDS gels in the presence of reducing agents. Each of these lanes contains 5 μg of protein. Lane b contains molecular weight standards.

while approximately 200 μg was recovered in CHAPS. Figure 10A shows the Coomassie Brilliant Blue stain of the purified protein from the CHAPS (lane a) and digitonin preparations (lane c). In digitonin the major polypeptides have apparent molecular weights of 170 000–175 000 (α_1), 140 000 (α_2), and 52 000–58 000 (β). This latter band is not calsequestrin as evidenced by an immunoblot with anti-calsequestrin antibody (kindly provided by Dr. Larry Jones). Minor Coomassie Brilliant Blue staining bands are also seen at 100K and 70K. The ratio of 170K:140K under reducing conditions is approximately 1:1. In CHAPS the major bands are the 170K and a 55–60K band. The lower molecular weight band in the CHAPS preparation (52–60K) is mostly calsequestrin, as determined by immunoblotting with anti-calsequestrin antibody. In the CHAPS preparation both the 52K (β) and the 140K (α_2) bands are apparently absent from the material eluted from the column. The α_2 polypeptide can be recovered from the proteins that do not bind to the antibody column by passing this material through a WGA–Affi-Gel column (data not shown). Hence, again, the α_2 and β polypeptides appear to dissociate from α_1 in CHAPS. Further evidence that α_1 dissociates from α_2 and β in CHAPS was obtained by washing the antibody affinity column containing the bound digitonin-extracted protein with CHAPS. The α_2 polypeptide (and possibly β) is eluted from the column with CHAPS, and the α_1 polypeptide remains bound but does elute from the column at low pH. This latter fraction is depleted in β. Heat denaturation of the digitonin preparation also results in the dissociation of the α_1 and α_2 polypeptides. Use of the anti-α_1 affinity column and a CHAPS wash allows the purification of α_1 free of the other subunits.

Discussion

The studies described in this paper were undertaken to resolve some of the discrepancies regarding the polypeptide composition of the DHP binding preparations from rabbit skeletal muscle by performing a detailed analysis in the two detergents most commonly used to purify the DHP binding protein. We have identified and partially purified the DHP binding protein from this tissue using either covalently attached [^{3}H]azidopine or the binding of [^{3}H]PN200-110 to follow the purification. The affinity label is located in a 170K (α_1) polypeptide. Other polypeptides found in some preparations include a 140K glycopolypeptide (α_2), a 58K polypeptide, a 52K (β) polypeptide, and a 30K/25K polypeptide. In CHAPS the DHP binding protein, after purification on a WGA–Affi-Gel column, a DEAE-Trisacryl M column, and a sucrose gradient, has 370 pmol of DHP binding sites/mg of protein. In digitonin the preparation has about 1930 pmol of binding sites/mg of protein. If the DHP binding protein is composed of only the α_1 polypeptide, the purified protein would be expected to bind 5900 pmol/mg. The CHAPS preparation is, therefore, 6.3% α_1, while the digitonin preparation is 33% α_1.

The α_2 polypeptide appears always to be associated with the α_1 polypeptide in digitonin but does not quantitatively copurify with this subunit in CHAPS. The β polypeptide also appears to dissociate from the other subunits in CHAPS. Our evidence that the α_1, α_2, and β subunits dissociate in CHAPS but less so in digitonin is that as follows: (1) CHAPS solubilization is accompanied by a loss in the ability of α_2 to bind to an anti-α_1 affinity column. (2) Conversely, a substantial fraction of α_1 in the CHAPS preparation cannot bind to a WGA–Affi-Gel column, which appears to quantitatively retain α_2. (3) A CHAPS wash of a digitonin preparation bound to the anti-α_1 column releases α_2. (4) A CHAPS wash of a digitonin preparation bound to WGA–Affi-Gel releases α_1 and β. There are several possible explanations for these observations: (1) The α_2 and β polypeptides are not subunits of the DHP binding protein but can copurify with it in digitonin but less so in CHAPS. (2) The α_2 and β polypeptides are subunits of the binding protein, but part of the complex falls apart during the purification in CHAPS. Our data do not completely resolve these questions. However, evidence to suggest that the polypeptides are components of a larger oligomeric complex include (1) the [^{3}H]azidopine-labeled α_1 that does not bind to the WGA–Affi-Gel in CHAPS sediments more slowly than that which eluted from the column and (2) a substantial fraction of the α_2 polypeptide obtained from the WGA–Affi-Gel column after solubilization in CHAPS sediments more slowly than the remainder, which cosediments with the small amount of α_1 recovered from the WGA column. The finding that a monoclonal antibody specific for α_2 can immunoprecipitate bound [^{3}H]PN200-110 from a detergent-solubilized preparation (Vandaele et al., 1987) supports our conclusion that α_2 and α_1 are part of an oligomeric complex.

Finally, we have some data to suggest that components other than α_1 are necessary for high-affinity binding of dihydropyridines. We show that CHAPS solubilization under the conditions described in this paper is accompanied by a much greater loss in the number of high-affinity binding sites for [^{3}H]PN200-110 than digitonin solubilization. Loss of binding in CHAPS was not, however, observed by Borsotto et al. (1985). Although different assay techniques were used by these workers (G-50 columns), we have recently demonstrated that using G-50 columns to separate bound from free [^{3}H]-PN200-110 gives results qualitatively similar to those reported here. Binding using G-50 columns is somewhat higher than that detected by PEG precipitation, presumably due to loss of some protein with the latter technique. It should also be noted that Curtis and Catterall (1984) did not obtain dihydropyridine binding to preparations solubilized in digitonin. Again, we have no explanation for the difference between their data and ours. Further support for a model in which α_1 alone after solubilization on CHAPS cannot bind dihydropyridines is given by the finding that a CHAPS wash of a digitonin preparation bound to a WGA column causes loss of ability

of the material retained by the column to bind [^{3}H]PN200-110, and this binding is not recovered in the CHAPS wash. However, readdition of the material removed by the CHAPS wash to the material specifically eluted from the WGA column partially restores binding, strongly suggesting that irreversible denaturation of the binding site does not explain loss of DHP binding. Whether all of the subunits are required for high-affinity dihydropyridine binding is not yet known. The ability of the material removed from digitonin-purified preparation with a CHAPS wash to partially restore binding may reflect either a reassociation of α_1 in the wash with other subunits that were retained by the WGA column or the stimulation of binding by some factor in the CHAPS wash (e.g., subunit, lipid) of the residual α_1 polypeptide that is still associated with α_2 and hence retained on the WGA column. The CHAPS wash alone does not bind dihydropyridines. The active components in the reconstitution are currently being investigated. The ability of lipids to stimulate binding of the digitonin preparation but not the CHAPS preparation suggests that lipids alone cannot fully explain the enhancement of DHP binding shown in Figure 3B.

Perhaps the most exciting consequence of this study is our ability to purify the α_1 and α_2 polypeptides free of one another. Purified α_1 and α_2 polypeptides should allow us to begin to assess their relative contributions to the function of the voltage-dependent calcium channel upon reconstitution, a goal that we are currently pursuing. Reconstitution of calcium channel activity from partially purified dihydropyridine binding proteins has been accomplished after solubilization with both digitonin (Curtis & Catterall, 1986; Flockerzi et al., 1986; Smith et al., 1987) and CHAPS (Coronado et al., 1986). In agreement with our observation that CHAPS solubilization causes loss of binding sites, channels reconstituted from the CHAPS-solubilized and -purified preparation do not appear to be sensitive to dihydropyridines (Coronado et al., 1986), whereas the digitonin preparations do appear to show this sensitivity (Curtis & Catterall, 1986; Flockerzi et al., 1986; Smith et al., 1987).

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Registry No. CHAPS, 75621-03-3; digitonin, 11024-24-1; Affi-Gel-10, 60454-66-2.

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