Solubilization and Hydrodynamic Characterization of the Dihydropyridine Receptor from Rat Ventricular Muscle*

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The dihydropyridine receptor-calcium channel complex, prelabeled with (+)-[^3]H]PN200-110, was solubilized from rat heart membranes with a detergent mixture of digitonin and Triton X-100. The dissociation of (+)-[^3]H]PN200-110 was slow enough to permit the hydrodynamic characterization of the complex by means of sucrose gradient sedimentation and gel filtration. The hydrodynamic properties of the complex were determined in several detergents, including Tween 80, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS), and digitonin. Stokes radius values of 12.5, 15.4, and 21.0 S were obtained in sucrose gradients prepared in Tween 80, CHAPS, and digitonin, respectively. A Stokes radius of 86-87 Å was obtained in each of the three detergents. Determination of the partial specific volume of the protein-detergent complex in each case revealed that the differences in $\bar{\rho}$ values could be explained by the differences in the properties of the bound detergent species. Partial specific volumes of 0.796, 0.730, and 0.730 ml/g, corresponding to molecular weights of 595,000, 540,000, and 740,000 were obtained for the complex in Tween 80, CHAPS, and digitonin, respectively. This indicated that Tween 80 readily exchanged for the solubilizing mixture of digitonin and Triton X-100, whereas CHAPS did not. Detergent exchange with Tween 80 made it possible to determine the fractional contribution of the receptor protein to the molecular weight of the protein-detergent complex. The molecular weight of the dihydropyridine receptor-calcium channel complex was estimated to be 370,000. The protein-detergent complex was found to have a frictional coefficient of 1.39, consistent with a large transmembrane protein.

Calcium channels are involved in the regulation of a number of cellular functions, including neurotransmitter release, hormone secretion, and excitation-contraction coupling (1). In the heart, the voltage-sensitive calcium channel is responsible for the plateau phase of the action potential in Purkinje fibers and myocardiul cells and for the slow inward current found in cells of the sinoatrial node (2). Precise regulation of calcium influx is therefore important in regulating cardiac rate, rhythm, and contractility. Although the electrophysiological properties of the calcium channel in heart muscle are well characterized, little is known about its function and structure at the molecular level.

The study of the molecular properties of the calcium channel has become possible with the discovery that calcium influx in heart and other tissues can be regulated by a diverse group of compounds collectively referred to as calcium antagonists (3). These compounds bind to at least two distinct sites associated with the calcium channel (4). The binding of 1,4-dihydropyridines to one of these sites can either block (nitrendipine) or activate (BAY k 8644) the channel (5). The binding of other calcium antagonists to a second site allosterically either increases (diltiazem) or decreases (verapamil) the affinity of dihydropyridines for their binding site (6). Recently, a number of radiolabeled 1,4-dihydropyridines including [H]nitrendipine, [H]nimodipine, and (+)-[^3]H]PN200-110 have been introduced as specific, high affinity radioligands for binding sites associated with the calcium channel in heart and other tissues (7). The binding properties of these compounds have been studied extensively in a variety of tissues (8). The high affinities and slow dissociation rates of these radiolabeled compounds has made possible the study of the molecular properties of the dihydropyridine receptor by allowing it to be traced through a variety of experimental procedures.

Few studies of the molecular properties of the dihydropyridine receptor have been reported. Target size analysis of dihydropyridine receptors from brain (9), skeletal muscle (10), and smooth muscle (11) has indicated that the membrane-bound dihydropyridine receptor may have a molecular weight range of 180,000-280,000. A purified preparation of dihydropyridine receptor from skeletal muscle has revealed three protein components on sodium dodecyl sulfate gels: one large 145,000-dalton component, and two smaller, 35,000-50,000-dalton components (12, 13). [H]Azidopine, a 1,4-dihydropyridine arylazide photoaffinity ligand, has been shown to photoincorporate specifically into a 145,000-dalton protein from skeletal muscle membranes (14), suggesting that the large component previously identified contains the dihydropyridine receptor. In contrast, the dihydropyridine receptor purified from chick heart appears to be made up of three protein components of 60,000, 54,000, and 34,000 daltons (15), and [H]nitrendipine photoincorporates into a 33,000-dalton protein in canine cardiac membranes (16).

In this report we describe the solubilization and hydrodynamic characterization of the dihydropyridine receptor-calcium channel complex from rat heart membranes. Throughout this report we refer to the complex as the dihydropyridine receptor; however, pharmacological evidence suggests that the channel and the receptor are closely associated (17). Regulation of calcium channel function is important in the etiology and treatment of several cardiovascular disorders, including...
hypertension, cardiomyopathy, angina, and certain arrhythmias (18). A thorough knowledge of calcium channel structure and function is, therefore, prerequisite to understanding both normal cardiac function and the mechanisms of a number of cardiac diseases.

EXPERIMENTAL PROCEDURES

Materials—(+)-[3H]PN200-110 (specific activity 70–87 Ci/mmol) was obtained from New England Nuclear. [3H]Digitonin (specific activity 777 mCi/mmol) was a gift from Dr. R. Cerione (Duke University, Chapel Hill, NC). [3H]Triton X-100 (specific activity 75 mCi/mmol) was provided by Dr. E. Racker (Cornell University, Ithaca, NY). Nitrendipine (BAY E 5009) was supplied by Miles Institute for Preclinical Pharmacology (New Haven, CT). Diiatessin was obtained from Marion Laboratories (Kansas City, MO), digitonin from Fisher, and sucrose from Schwarz/Mann. All other materials were obtained from Sigma.

Preparation of Rat Heart Membranes—Male Wistar rats (200-250 g) were sacrificed by decapitation, and the hearts were quickly removed. Hearts were placed immediately in ice-cold 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4, or rapidly frozen in liquid Freon and stored at -80 °C for later use. The atria were dissected away and the ventricles were then weighed, minced with a knife, and homogenized in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4, using a Brinkmann Polytron (setting 7 for 3 × 10 s). This was followed by eight strokes in a tight fitting Dounce homogenizer. The homogenate was centrifuged at 20,000 × g for 15 min, and the supernatant was discarded. The pellet was washed twice by resuspension and centrifugation in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4 (30,000 × g for 15 min). The final pellet was resuspended in 20 ml/g original weight of heart, in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4.

Labeling of Membranes with (+)-[3H]PN200-110 and Detergent Solubilization—Membranes (4–5 mg of protein/ml) were incubated for 90 min at 20 °C in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4, with 3.0 nM (+)-[3H]PN200-110 and 10 μM diisothiocyanate in borosilicate glass test tubes. Non-specific binding was determined by labeling the membranes in the presence of 1 μM nitrendipine. Incubation was performed under a sodium vapor light to avoid breakdown of the dihydropyridines.

Following incubation, the membranes were washed twice with 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4 (20,000 × g for 15 min). In initial solubilization experiments, the pellets were resuspended in 0.1–0.5% CHAPS, cholate, digitonin, octylglycoside, Triton X-100, or Tween 80. For optimal solubilization, the final pellet was resuspended in 1.0% (v/v) digitonin (Fisher)/0.2% (v/v) Triton X-100 in 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl2, 1 mM PMSF, pH 7.4, with 3.0 μM (+)-[3H]PN200-110 and 10 μM diisothiocyanate in borosilicate glass test tubes. Non-specific binding was determined by labeling the membranes in the presence of 1 μM nitrendipine. Incubation was performed under a sodium vapor light to avoid breakdown of the dihydropyridines.

Detergent Precipitation—Solubilized dihydropyridine receptors were performed as previously described (19, 20) with only slight modification. Aliquots (0.5 ml) of solubilized material were incubated with 100 μl of γ-globulin and 0.5 ml of 20% (v/v) PEG 8000 in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4, for 15 min at 4 °C. Optimal precipitation of the dihydropyridine receptor was found to occur over the narrow range of 8–10% PEG. Samples were then filtered through Schleicher & Schuell glass fiber filters (No. 30). The filters were washed three times with 4 ml of 10% (v/v) PEG 8000 in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4. The filters were allowed to dry, and the amount of radioactivity remaining on the filters was determined using a xylene-based fluor (ACS, Amersham) in a Beckman liquid scintillation spectrometer at an efficiency of approximately 35%.

Gel Filtration—Gel filtration was performed using Sepharose 4B-CL at 4 °C using a column (1.5 × 50 cm) equilibrated in 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl2, 1 mM PMSF, pH 7.4, and either 1.0% (v/v) CHAPS, 1.0% (v/v) Tween 80, or 1.0% (v/v) digitonin. The column was calibrated with proteins of known Stokes radius. These included myoglobin (19 Å), ovalbumin (30 Å), and catalase (62.5 Å), β-galactosidase (69 Å), and thyroglobulin (86 Å). A 3-ml sample containing the solubilized (+)-[3H]PN200-110-labeled receptor was applied to the column and the column was developed using descending flow of 0.5 ml of H2O opaqued with 1% iodine.

The column was eluted with one of the above detergent mixtures, and 1.3-ml fractions were collected. In some experiments 10 μM diltiazem was included in the elution buffer. The void volume (V0) was determined with blue dextran and the total volume (Vt) with (+)-[3H]PN200-110. The distribution coefficient, Kd, was calculated from the relationship Kd = (Vt - V0)/(V0 - Vv), where Vv is the elution volume of the protein.

Protein Assay—Protein concentrations were determined using the Peterson modification (21) of the assay of Lowry et al. (22) with bovine serum albumin as the standard.

Ion Exchange Chromatography—A column (2 × 20 cm) of DEAE-DE23 was preequilibrated at 4 °C with 50 mM Tris-HCl, 1 mM CaCl2, 1 mM PMSF, 1% Tween 80, pH 7.4. Five ml of digitonin/Triton X-100-solubilized material containing either [3H]digitonin (8 × 106 cpm) or the (+)-[3H]PN200-110-labeled dihydropyridine receptor was diluted 10-fold into 50 mM Tris-HCl, 1 mM CaCl2, 1 mM PMSF, 1% Tween 80, pH 7.4, and loaded on the column. The column was washed with 50 ml of 50 mM Tris-HCl, 1 mM CaCl2, 1 mM PMSF, 1% Tween 80, pH 7.4, and eluted with a 20 ml 0–1 M NaCl linear gradient in the same buffer. One-fifteenth fractions were collected and the [3H]PN200-110 peak or the (+)-[3H]PN200-110-dihydropyridine receptor peak was determined by assaying 100-μl aliquots of each fraction for radioactivity. Fractions containing the dihydropyridine receptor either prelabeled with (+)-[3H]PN200-110 or solubilized in the presence of [3H]digitonin (3 ml total) were loaded directly onto H2O sucrose gradients (5–20%) prepared in 1% Tween 80. Protein concentrations were determined prior to centrifugation and in each fraction following centrifugation.

Density Gradient Centrifugation—Linear 32-m sucrose gradients (5–20%) in either H2O or D2O containing 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl2, 1 mM PMSF, pH 7.4, were prepared with a Buchler Auto-Densi-Flow gradient maker in 2.5 × 75-cm polyallomer tubes. In addition, the gradients contained either 1.0% digitonin, 1.0% CHAPS, or 1.0% Tween 80. In some experiments the gradients also contained 10 μM diltiazem. Three ml of solubilized material containing 25 μl of catalase (10 mg/ml) and 50 μl of sucrose (1.0 mg/ml) were carefully layered on top of each gradient. An additional 1 ml of buffer was carefully layered on top of the solubilized material. The gradients were centrifuged at 200,000 × g in a Beckman VTI 80 vertical rotor for 1.5 or 2.5 h at 6–8 °C. The tubes were drained with a Buchler conductivity drainer and 0.65-ml fractions were collected. Each fraction was assayed for radioactivity, on cent sucrose, and catalase and β-galactosidase activities. The temperature of the gradients during centrifugation was corrected so that the observed smax values for catalase and β-galactosidase were 11.3 and 15.9 S, respectively. The corrected temperature was generally within 5 °C of the temperature to which the centrifuge was set.

Computation of the Sedimentation Coefficient and Partial Specific Volume—The sedimentation coefficient (smax) and the partial specific volume (ρ*) of the protein-detergent complex were determined using the procedure described by Smigel and Fleischer (23), modified for use with a reorienting gradient in a vertical rotor. Measurements of refractive index were used to calculate density and viscosity gradients in the tube following centrifugation, which were present in an orientation perpendicular to that existing during the actual centrifugation. The centrifuge tube can be modeled accurately by a cylinder and half sphere. The distance from the center of rotation was determined by adding the distance from the center of the rotor to the inside edge of the tube. The vertical distance down the tube was converted to a distance from the inside edge of the tube. This was done by measuring the distance 75 cm from the top of the operating pressure to the inside edge of the tube. The temperature of the tube was assumed to be equal to the average of the temperatures of the fractions. The density of the fractions was calculated by adding the density of the fraction from the center of the rotor to the inside edge of the tube. The density of each fraction was calculated using the relationship n = (Vf/V0) - (V0/Vv), where Vf is the volume of the fraction.

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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part of the tube, \( d_1 \) and \( d_2 \) are the distances from the center of the tube, and \( \delta \) is \( d_2 - d_1 \). \( \alpha \) is the constant (\( d_2/r \)) and \( \alpha_2 \) is the constant (\( d_2/r \)). The horizontal density and viscosity gradients were then used to integrate a numerically the sedimentation equation using Simpson's rule. Because of the short centrifugation times used, acceleration and deceleration were a significant portion of the total sedimentation time. The force-time integral corrected for acceleration and deceleration was adapted from Young (24):

\[
\int f_r dt = \left[ (T + A) + D/3 \right]/15
\]

where \( T \) is the time at full speed, \( A \) is the acceleration time, and \( D \) is the deceleration time. This yields a series of \( s_{20,w} \) and \( \nu^* \) values for both H2O and D2O. The actual values of \( s_{20,w} \) and \( \nu^* \) for the protein detergent complex are those values at the intersection of the H2O and D2O curves.

Calculation of the Molecular Weight and Frictional Ratio—Although digitonin is effective in solubilizing membrane-bound proteins without altering their conformation, a problem is encountered if it is present during hydrodynamic studies. The partial specific volume of digitonin is 0.738 ml/g, which is similar to that of many proteins. In order to determine a shift in the sedimentation rate of a solubilized protein in D2O versus H2O relative to the proteins used as standards, the detergent which is bound to the solubilized protein must have a partial specific volume significantly different from that of the proteins used as standards, which presumably do not bind detergent. A shift in the sedimentation rate in D2O versus H2O allows the determination of the partial specific volume of the protein-detergent complex, and thus permits the calculation of the fractional contribution of both detergent and protein to the molecular weight of the complex. The partial specific volume of the detergent must also be significantly different from that of the solubilized protein in order to make this calculation. Using the Stokes radius \( R_s \) computed from Sepharose 6B-CL chromatography in which 1.0% digitonin was exchanged for 1.0% Tween 80 and \( s_{20,w} \) and \( \nu^* \) values obtained from sedimentation in which 1.0% digitonin was exchanged for 1.0% Tween 80, the molecular weight of the protein-detergent complex (M*) is given by the equation:

\[
M^* = \left[ s_{20,w} - 6\pi n_{20,w} N \cdot R_s \right] / \left[ 1 - \nu^* / p_{20,w} \right]
\]

where \( p_{20,w} \) and \( n_{20,w} \) are the density and viscosity of water, respectively, at 20 °C (25). Using a partial specific volume for Tween 80 of 0.896 ml/g and assuming a partial specific volume of 0.735 ml/g for the protein, the weight fraction of the protein (Xp) and detergent (Xd) can be determined using the relationship:

\[
\nu^* = 0.735 \text{ ml/g} \cdot X_p + 0.896 \text{ ml/g} \cdot X_d
\]

where \( \nu^* \) is the partial specific volume of the protein-detergent complex. The molecular weight of the dihydropyridine receptor is \( X_p \cdot M^* \). The frictional ratio can be calculated according to the formula:

\[
\tilde{f}/f = R_s \left[ 4\pi N / 3 M^* \cdot \left( \nu^* + 6 / p_{20,w} \right) \right]^{1/2}
\]

where \( \delta \) is 0.2 g of solvent as suggested by Tanford (26).

RESULTS

Solubilization of the Dihydropyridine Receptor—Table I summarizes the results of solubilization experiments with CHAPS, cholate, digitonin (Sigma and Fisher), octylglucoside, Triton X-100, and Tween 80. All detergents were tested at concentrations ranging from 0.1-5.0% and at various ionic strengths (adjusted with KCl) in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4. Maximum solubilization generally occurred at concentrations of 1.0% and the number of specific counts remaining in the pellet following solubilization. Aliquots of supernatant were assayed for solubilized receptor-bound (+)-[3H]PN200-110 by PEG precipitation, as described under "Experimental Procedures" (27). Protein assays of membranes (the presence of detergent) and the solubilized fraction were used to determine the percentage of membrane protein that was solubilized (A). A, solubilization with increasing concentrations of digitonin, B, solubilization with 1.0% digitonin and increasing concentrations of Triton X-100.

<table>
<thead>
<tr>
<th>Detergent*</th>
<th>Solubilized*</th>
<th>Recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS (±10% glycerol)</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>Cholate</td>
<td>36</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Digitonin (Sigma)</td>
<td>20</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Digitonin (Fisher)</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td>Octylglucoside</td>
<td>80</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>75</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tween 80</td>
<td>24</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Digitonin (Fisher)/ Triton X-100</td>
<td>75</td>
<td>50</td>
</tr>
</tbody>
</table>

* Concentrations ranged from 0.5-5.0%, in 25 mM HEPES-NaOH, 1 mM CaCl2, pH 7.4, containing 0-500 mM KCl. Detergent/protein ratios (w/w) ranged from 1:1 to 10:1. Maximum solubilization determined as the difference between total specific counts in membranes prior to solubilization and the number of specific counts remaining in the pellet following solubilization. Percentage of total labeled recoverable by PEG precipitation.

The total number of binding sites, with 1.0% digitonin. The efficacy of digitonin solubilization increases to 75% of the total number of binding sites with the addition of increasing concentrations of Triton X-100 up to 0.5%. Recovery of the dihydropyridine receptor (as defined by PEG precipitation of specific radioactivity described under "Experimental Procedures") varied with the concentration of Triton X-100. Fig. 1B demonstrates that concentrations of Triton X-100 exceeding 0.5% resulted in the loss of PEG-precipitable radioactivity. Selective solubilization of the dihydropyridine receptor is demonstrated in Fig. 1B when the percentage of solubilized receptors exceeds the percentage of solubilized protein. As a result of these studies, we routinely used 1.0% digitonin (Fisher) combined with 0.2% Triton X-100 to solubilize the dihydropyridine receptor from rat heart membranes.

Dissociation of (+)-[3H]PN200-110 from rat heart membranes occurs slowly, as demonstrated in Fig. 2A. Four hours after a 10-fold dilution into binding media containing 1 μM

Fig. 1. Solubilization of the dihydropyridine receptor from rat heart membranes. Membranes were labeled with 3.0 nM (+)-[3H]PN200-110 in the presence or absence of 1 μM nitrrendipine, washed, and resuspended with the indicated concentration of detergent as described under "Experimental Procedures." After 5 min at 24 °C and 25 min at 4 °C, samples were spun at 100,000 g for 1 h. Soluble (O) dihydropyridine receptor was determined as the difference between total specific counts in membranes prior to solubilization and the number of specific counts remaining in the pellet following solubilization. Aliquots of supernatant were assayed for solubilized receptor-bound (+)-[3H]PN200-110 by PEG precipitation, as described under "Experimental Procedures" (27). Protein assays of membranes (the presence of detergent) and the solubilized fraction were used to determine the percentage of membrane protein that was solubilized (A). A, solubilization with increasing concentrations of digitonin, B, solubilization with 1.0% digitonin and increasing concentrations of Triton X-100.
ble receptors were diluted 10-fold into 25 mM HEPES-NaOH, 1 mM CaCl₂, pH 7.4, at 20 °C containing 1 μM nitrendipine (O), 100 μM verapamil, and 1 μM nitrendipine (A), or 10 μM diltiazem and 1 μM nitrendipine (Δ), and assayed by filtration or PEG precipitation, respectively, at the indicated time points. Specifically bound (+)-[3H]PN200-110 was determined as the difference between samples labeled in the presence and absence of 1 μM nitrendipine.

**Table II**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Tween 80</th>
<th>CHAPS</th>
<th>Digitonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>s_{20,w} (× 10^{-10} s)</td>
<td>12.5</td>
<td>15.4</td>
<td>21.0</td>
</tr>
<tr>
<td>ϕ* (ml/g)</td>
<td>0.796</td>
<td>0.730</td>
<td>0.730</td>
</tr>
<tr>
<td>Rₜ (× 10⁻⁸ cm)</td>
<td>86</td>
<td>86</td>
<td>87</td>
</tr>
<tr>
<td>Mₚ</td>
<td>595,000</td>
<td>540,000</td>
<td>730,000</td>
</tr>
<tr>
<td>Mₚ</td>
<td>370,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f/f₀</td>
<td>1.39</td>
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</tr>
</tbody>
</table>

nitrendipine, 50% of the radioligand remains bound. As shown in the figure, 10 μM diltiazem reduces the dissociation rate, whereas 100 μM verapamil enhances the dissociation rate significantly. Fig. 2B demonstrates that the dihydropyridine binding site prelabeled with (+)-[3H]PN200-110 retains these relative binding characteristics in the solubilized state; however, the apparent rate of dissociation increases 2-fold.

**Gel Filtration and Sucrose Gradient Sedimentation**—We have used both gel filtration and sucrose density gradient centrifugation as methods of detergent exchange to circumvent the problems associated with use of digitonin in determining the protein molecular weight (see “Experimental Procedures”). The hydrodynamic properties of the dihydropyridine receptor (Table II) have been determined in digitonin (ϕ = 0.738 ml/g), CHAPS (ϕ = 0.810 ml/g) at Tween 80 (ϕ = 0.896 ml/g). Fig. 3 demonstrates that Sepharose 6B-CL gel chromatography of 1.0% digitonin/0.2% Triton X-100-solubilized material containing the (+)-[3H]PN200-110-labeled dihydropyridine binding site yields a single peak of radioactivity in columns equilibrated and eluted with 1.0% CHAPS. The specificity of (+)-[3H]PN200-110 binding is demonstrated by the absence of a peak when binding was performed in the presence of 1 μM nitrendipine. Similar results were obtained with Sepharose columns equilibrated and eluted with either 1.0% Tween 80 or 1.0% digitonin (results not shown). A Stokes radius of 86–87 Å was determined for the dihydropyridine receptor-detergent complex in CHAPS, Tween 80, and digitonin (Table II).

Sedimentation of 1.0% digitonin/0.2% Triton X-100-solubilized material containing the (+)-[3H]PN200-110-labeled receptor produced peaks of radioactivity corresponding to s_{20,w} values of 12.5, 15.4, and 21.0 S, when placed on gradients prepared in 1.0% Tween 80, 1.0% CHAPS, and 1.0% digitonin, respectively (Fig. 4). The differential sedimentation rate exhibited by the solubilized dihydropyridine receptor in sucrose/H₂O versus sucrose/D₂O gradients prepared in 1.0% Tween 80 is shown in Fig. 5. The protein sedimented ahead of catalase in sucrose/H₂O gradients but behind catalase in sucrose/D₂O gradients. The sedimentation analysis described under “Experimental Procedures” is shown in Fig. 6. The sucrose/H₂O and sucrose/D₂O curves intersect at an s_{20,w} value of 12.5 and partial specific volume of 0.796 ml/g. The results of sedimentation analyses with gradients prepared in 1.0% Tween 80, 1.0% CHAPS, and 1.0% digitonin (Table II) demonstrate that the molecular weight and partial specific volume of the protein-detergent complex vary with the detergent composition of the sucrose gradient. The complex has an apparent molecular weight of 730,000 in gradients prepared in 1.0% digitonin. Molecular weights of 595,000 and 540,000 are calculated for gradients prepared in 1.0% Tween 80 and 1.0% CHAPS, respectively. A partial specific volume of 0.73 ml/g, similar to that of digitonin, was found in gradients.
Dihydropyridine Receptor from Rat Ventricle

FIG. 4. Detergent effects on sucrose gradient sedimentation of the dihydropyridine receptor. Thirty-two-ml linear (6-20%) sucrose/H$_2$O density gradients containing 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl$_2$, and either 1.0% Tween 80, 1.0% CHAPS, or 1% digitonin were prepared as described under "Experimental Procedures." Three ml of 1% digitonin/0.2% Triton X-100-solubilized material containing the (+)-[${}^{3}$H]PN200-110-labeled dihydropyridine receptor and the standards catalase and $\beta$-galactosidase were layered on each gradient and sedimented for 2.5 h at 200,000 $\times$ g in a Beckman VTi-50 vertical rotor. Fractions (0.65 ml) were collected from the top of the tube and assayed for radioactivity and catalase and $\beta$-galactosidase activity. Micellar-associated, (+)-[${}^{3}$H]PN200-110 runs at the top of the gradient. (+)-[${}^{3}$H]PN200-110-labeled dihydropyridine receptor peaks in 1% Tween 80 (O), 1% CHAPS (C), and 1% digitonin (A), and are indicated by arrows. The positions of catalase and $\beta$-galactosidase, as shown by arrows, are independent of the detergent used in preparing the gradient.

FIG. 5. Sedimentation of the (+)-[${}^{3}$H]PN200-110-labeled dihydropyridine (DHP) receptor into sucrose/H$_2$O and sucrose/D$_2$O gradients containing 1.0% Tween 80. Thirty-two-ml linear (6-20%) sucrose/H$_2$O and sucrose/D$_2$O density gradients containing 185 mM KCl, 25 mM HEPES-NaOH, 1 mM CaCl$_2$, and 1.0% Tween 80 were prepared as described under "Experimental Procedures." Three ml of 1% digitonin/0.2% Triton X-100-solubilized material containing the (+)-[${}^{3}$H]PN200-110-labeled dihydropyridine receptor and catalase as a standard were layered on each gradient and sedimented for 1.5 h at 200,000 $\times$ g in a Beckman VTi-50 vertical rotor. Fractions (0.65 ml) were collected from the top of the tube and assayed for radioactivity and catalase activity. Micellar-associated, (+)-[${}^{3}$H]PN200-110 runs at the top of the gradient. Peaks of (+)-[${}^{3}$H]PN200-110-labeled dihydropyridine receptor and catalase are indicated by arrows in H$_2$O (O) and D$_2$O (C).

prepared in either 1.0% CHAPS or 1.0% digitonin. A partial specific volume of 0.796 ml/g was determined in gradients prepared in 1.0% Tween 80.

Characterization of Detergent Exchange—Fig. 3 demonstrates that [${}^{3}$H]Triton X-100 is not associated with the detergent-protein complex following Sepharose 6B-CL gel chromatography of digitonin/Triton X-100-solubilized material in columns equilibrated and eluted with 1.0% CHAPS. The molar ratio of total Triton X-100 to total protein in peak fractions containing the dihydropyridine receptor after gel filtration was estimated to be 2:1 (assuming a protein $M_r$ of 370,000). Assuming the dihydropyridine receptor exchanges detergent in a manner similar to that of the total protein, the contribution of Triton X-100 to the total molecular weight of the protein-detergent complex would be less than 0.3% following gel filtration. Similar results were obtained in 1.0% Tween 80.

To determine whether complete detergent exchange with Tween 80 occurs during sucrose gradient sedimentation, the digitonin/Triton X-100-solubilized dihydropyridine receptor either prelabeled with (+)-[${}^{3}$H]PN200-110 or solubilized in the presence of [${}^{3}$H]digitonin (8 $\times$ 10$^6$ cpm) was adsorbed to a DEAE-column and washed with 1.0% Tween 80 prior to sedimentation as described under "Experimental Procedures." The wash procedure resulted in removal of 99.8% of the [${}^{3}$H]digitonin. The molar ratio of total digitonin to protein following the wash procedure was estimated to be 6:1 (assuming an average protein $M_r$ of 100,000). Fig. 7 demonstrates that further detergent exchange occurs during sucrose gradient sedimentation, as [${}^{3}$H]digitonin does not appear to be associated with the bulk protein. The molar ratio of residual digitonin to protein at the location of the dihydropyridine receptor in the gradient was 4:1 (assuming a protein $M_r$ of 370,000).

Assuming the dihydropyridine receptor exchanges detergent in a manner similar to that of the total protein, the contribution of digitonin to the total molecular weight of the protein-detergent complex would be less than 0.7%. The (+)-[${}^{3}$H]PN200-110-labeled receptor, after washing on the DEAE-column, sediments the same distance as the digitonin/Triton X-100-solubilized receptor loaded directly on a sucrose gradient. This indicates that essentially complete detergent exchange occurs during sucrose gradient sedimentation alone.

Determination of the Protein Molecular Weight—An esti-
The results of gel chromatography and sucrose gradient sedimentation experiments in which the solubilizing detergent, 1.0% digitonin/0.2% Triton X-100, has been exchanged for 1.0% Tween 80. Calculations are based on the observed $s_{20,w}$ for the complex of 0.796 ml/g, a known value of $\bar{v}$ for the detergent component of 0.806 ml/g, and an estimate of $\bar{v}$ for the protein of 0.735 ml/g. Combined with the Stokes radius determined from gel exclusion chromatography in 1.0% Tween 80, calculations yield a protein-detergent complex molecular weight of 595,000. An estimate of the protein molecular weight can be determined by calculating the fractional contribution of the detergent to the molecular weight of the complex (see “Experimental Procedures”). The molecular weight of the dihydropyridine receptor was estimated to be 370,000. A frictional ratio ($f/f_o$) of 1.39 was determined for the protein detergent complex. These data are summarized in Table II.

**DISCUSSION**

Specific detergents are often required to solubilize integral membrane proteins from a particular cell type. The specificity of the detergent is most likely determined by the lipid composition of the immediate protein environment (27). In order that the protein maintain its membrane-associated conformation in soluble form, the detergent must substitute effectively for the endogenous membrane lipid. Bile acids and nonionic detergents satisfy this requirement by apparently binding to the hydrophobic regions of a protein. Detergents such as digitonin, CHAPS, and Triton X-100 have been used successfully in numerous solubilization studies involving a variety of proteins (28).

The dihydropyridine receptor has been solubilized from brain (19) and skeletal muscle (12, 20) with either CHAPS or digitonin. CHAPS has also been used in combination with various concentrations of glycerol and/or phospholipids in order to stabilize the receptor in soluble form (29). In the present study, the combination of digitonin (Fisher) and Triton X-100 proved most effective in solubilization and recovery of the dihydropyridine receptor from heart muscle. This detergent combination has also been used in solubilization of the a-adrenergic receptor from canine heart tissue (30). We found that concentrations of Triton X-100 greater than 0.5% in the solubilization mixture decreased the recovery of the solubilized receptor assayed by PEG precipitation. A loss of (+)-[^3H]PN200-110 binding was also demonstrated by gel filtration and density gradient centrifugation in 1.0% Triton X-100. The mechanism for this is not clear.

Evidence that the dihydropyridine receptor-complex has been solubilized in the present study is provided both by specific (+)-[^3H]PN200-110 binding and by the allosteric regulation of (+)-[^3H]PN200-110 dissociation by verapamil and diltiazem. Allosteric regulation of dihydropyridine binding has been demonstrated in a number of membrane preparations, including brain, smooth muscle, and skeletal muscle (4, 6), as well as in soluble preparations of brain and skeletal muscle (19, 20). This suggests that the allosteric binding sites are an integral part of the dihydropyridine-receptor complex.

Competitive binding studies have shown that verapamil and diltiazem modulate dihydropyridine binding at a site separate from the dihydropyridine binding site and that verapamil and diltiazem may interact with separate allosteric sites (31, 32). In the present study, the rate of dissociation of (+)-[^3H]PN200-110 appears to increase 2-fold with solubilization, which may reflect some conformational change; however, the relative allosteric effects of verapamil and diltiazem are still present, suggesting that all three binding sites are still intact. Similar results have been demonstrated in brain and skeletal muscle (19, 20).

The $s_{20,w}$ value of the solubilized dihydropyridine receptor-detergent complex varies with the detergent composition of the sucrose density gradient in which it is sedimentsed. This is apparent in previous studies where $s_{20,w}$ values of 12.9 and 19.2 S are reported for CHAPS- and digitonin-solubilized receptor sites from skeletal muscle (20) and brain (19), respectively. Values of 11.4, 14.4, and 21.0 S have been reported for the dihydropyridine receptor in a study in which a CHAPS-glycerol mixture was used both in solubilization and sedimentation procedures (29). In the present study, we have demonstrated $s_{20,w}$ values of 12.5, 15.4, and 21.0 S for the digitonin/Triton X-100-solubilized dihydropyridine receptor in sucrose gradients prepared in Tween 80, CHAPS, and digitonin, respectively. This suggests that the variability in sedimentation lies with the detergent component of the complex and not with the protein. To test this hypothesis, we determined the partial specific volume of the protein-detergent complex in each gradient by comparing sedimentation velocities in sucrose/H$_2$O versus sucrose/D$_2$O. Gradients were prepared in Tween 80, CHAPS, and digitonin, which have partial specific volumes of 0.896 (33), 0.810, and 0.738 ml/g (34), respectively. Assuming that the protein has a partial specific volume of 0.735 ml/g, the partial specific volume of the protein-detergent complex would be expected to vary from its value according to the partial specific volume of the bound detergent species. One would predict that the protein-detergent complex would have a partial specific volume of 0.735 ml/g in digitonin, with increased values in CHAPS and Tween 80, assuming that effective detergent exchange had occurred during sedimentation.

As shown in Table II, the protein-detergent complex has the same partial specific volume, 0.73 ml/g, in gradients prepared in both CHAPS and digitonin. In contrast, the
partial specific volume in Tween 80, 0.796 ml/g, is significantly higher. This suggests that digitonin readily exchanges with Tween 80 but not with CHAPS. The difference in $\bar{s}_{20,w}$ values in CHAPS versus digitonin is explained, then, by a difference in molecular weight of the protein-detergent complex. This probably represents a difference in the amount of digitonin bound to the protein in each case. CHAPS, which has a very high critical micellar concentration, may be less likely to associate with the protein-detergent complex at concentrations present in the gradient (28). The difference in $\bar{s}_{20,w}$ values in Tween 80 versus CHAPS is explained by differences in the partial specific volume of the protein-detergent complex in each detergent. The molecular weight of the complex is similar in the two detergents. Thus, the differences in $\bar{s}_{20,w}$ values in the present study, as well as those reported (12.9 and 19.2 S) in previous studies (19, 20), can be explained on the basis of differences in the properties of the bound detergent species. It is more difficult to interpret the results of the study in which three $\bar{s}_{20,w}$ values were obtained in a single gradient (29). It has been suggested that these represent oligomeric forms of the dihydropyridine receptor; however, it would be necessary to determine the partial specific volume and Stokes radius of each form to verify this.

We have found that the dihydropyridine receptor is a nonglobular protein, as suggested by a frictional ratio $(f/f_0)$ of 1.39 for the protein-detergent complex. The protein has an approximate molecular weight of 370,000. The frictional coefficient suggests that the complex behaves as a hydrodynamic particle with the shape of either a prolate ellipsoid with an axial ratio of 3.6 or an oblate ellipsoid with an axial ratio of 3.3. The axial ratios are determined assuming the degree of hydration to be 0.2 g of solvent/g of protein as suggested by Tanford (26). The accuracy of the axial ratio estimates therefore depends on the accuracy of this assumption; however, the results are consistent with the idea that the dihydropyridine receptor is a large, transmembrane protein.

Theoretical limitations on the accuracy of sedimentation values determined in H$_2$O versus D$_2$O have been described in the literature (35). The method involves a number of assumptions. The calculation of the Stokes radius requires that the standards used do not bind appreciable detergent. This assumption appears reasonable from the work of Helenius and Simons (36), showing that hydrophobic proteins bind little or no Triton X-100. The binding of detergent is assumed to be the same in H$_2$O and D$_2$O and to be independent of sucrose concentration. A practical problem is that of maintaining constant temperature at high speeds in the ultracentrifuge. The distance traveled by a sedimenting particle is dependent on viscosity and density, which are dependent on the temperature. The rotor temperature is assumed to be that which gives the standards used (catalase and $\beta$-galactosidase) their correct $\bar{s}_{20,w}$ and $\bar{v}$ values. The value of 0.735 ml/g is often assumed to be the partial specific volume of a typical nonglycosylated protein. This probably represents the largest source of error in this method, considering the wide range (340,000–410,000) calculated by the possible limits of $\bar{v}$, 0.72–0.75 ml/g.

The method we have used is extremely useful for preliminary characterization of proteins not readily obtainable in pure form. In contrast to skeletal muscle, there are relatively few dihydropyridine binding sites located in heart muscle. The dihydropyridine receptor-calcium channel complex, however, plays a major role in the regulation of intracellular calcium concentration, and in excitation-contraction coupling. Altered regulation of intracellular calcium concentration is a component of a number of cardiovascular diseases including hypertension, cardiomyopathy, and certain arrhythmias (18). These disease conditions emphasize the importance of understanding the structural and functional properties of the dihydropyridine receptor.

REFERENCES