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Purification of the Calcium Antagonist Receptor of the Voltage-Sensitive Calcium Channel from Skeletal Muscle Transverse Tubules†

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ABSTRACT: [^{3}H]Nitrendipine binds with high affinity to a calcium antagonist receptor preferentially localized to the transverse tubule membrane of skeletal muscle. Digitonin was used to solubilize a [^{3}H]nitrendipine–receptor complex from transverse tubule membranes. The digitonin–[^{3}H]nitrendipine–receptor complex was purified 330-fold by a combination of wheat germ agglutinin chromatography, ion-exchange chromatography, and sedimentation through sucrose gradients to yield a preparation estimated to be 41% homogeneous on the basis of specific activity. Analysis by sodium dodecyl sulfate gel electrophoresis demonstrated that three polypeptides termed α [molecular weight (M_r) 130000], β (M_r 50000), and γ (M_r 33000) quantitatively comigrated with the [^{3}H]nitrendipine–receptor complex on sucrose gradients and represented 62% of the total protein staining. These three polypeptides are associated noncovalently. However, the apparent molecular weight of the α polypeptide is reduced from 160000 to 130000 upon reduction, consistent with the presence of an internal disulfide bond. Our results suggest that these three polypeptides are the subunits of the calcium antagonist receptor and are major components of the transverse tubule voltage-sensitive calcium channel.

In excitable cells, voltage-sensitive calcium channels mediate calcium-dependent depolarization and translate changes in membrane potential into an intracellular calcium signal that initiates a number of specific cellular functions including excitation–secretion and excitation–contraction coupling [reviewed in Hagiwara & Byerly (1981) and Tsien (1983)]. A group of compounds termed "calcium antagonists" block ion flux through calcium channels (Lee & Tsien, 1983) by binding to two distinct sites that comprise the calcium antagonist receptor (Triggle, 1982). Nitrendipine and other dihydropyridines bind with nanomolar affinity to a site that can either block (Triggle, 1982; Cauvin et al., 1983) or activate (Schramm et al., 1983) calcium channels. A second lower affinity site binds a diverse group of calcium antagonists that allosterically enhance (e.g., diltiazem) or inhibit (e.g., verapamil) [^{3}H]nitrendipine binding to the dihydropyridine recognition site (Murphy et al., 1983).

[^{3}H]Nitrendipine has been used as a chemical probe to begin the molecular characterization of the calcium channel. Radiation inactivation studies have indicated a probable size range of 180000–280000 for the membrane-bound calcium antagonist receptor in smooth muscle (Venter et al., 1983), skeletal muscle (Ferry et al., 1983; Norman et al., 1983), and brain (Norman et al., 1983). We have recently demonstrated that digitonin solubilizes both the dihydropyridine and verapamil/diltiazem recognition sites from rat brain membranes as an allosterically coupled complex (Curtis & Catterall, 1983). The solubilized calcium antagonist receptor appears to be a large glycoprotein on the basis of sedimentation through sucrose gradients and binding to immobilized wheat germ agglutinin (Curtis & Catterall, 1983; Ferry & Glossmann, 1983).

In skeletal muscle, voltage-dependent calcium currents flow almost exclusively across the transverse tubule system and are blocked by calcium antagonists (Almers et al., 1981; Chiarandini & Stefani, 1983). [^{3}H]Nitrendipine receptors are preferentially localized to the transverse tubule membrane, consistent with their association with calcium channels (Fosset et al., 1983; Glossmann et al., 1983). Since T-tubule membranes[1] are the most enriched source of calcium antagonist

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[1] Abbreviations: T-tubule membranes, transverse tubule membranes; WGA, wheat germ agglutinin; DEAE, diethylaminoethyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, poly(ethylene glycol); MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IgG, immunoglobulin G.

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receptors (Fosset et al., 1983) and the dihydropyridines block calcium currents in these membranes (Almers et al., 1981; Chiarandini & Stefani, 1983), they provide an excellent starting material for isolation of the calcium antagonist receptor and determination of its properties and relationship to the voltage-sensitive calcium channel. In this report, we describe the purification of a [^{3}H]nitrendipine–receptor complex from transverse tubules and present an initial analysis of its properties.

Experimental Procedures

Preparations of T-Tubule Membranes. T-tubule membranes were prepared from rabbit skeletal fast (white) muscle by two different procedures: *procedure A* according to Rosemblatt et al. (1981) with the protease inhibitors phenylmethanesulfonyl fluoride (1 mM) and pepstatin A (1 μM) present in all solutions and *procedure B* according to Glossmann et al. (1983) with the protease inhibitors phenylmethanesulfonyl fluoride (1 mM), pepstatin A (1 μM), 1,10-phenanthroline (1 mM), iodoacetamide (1 mM), and antipain (1 mg/L) present in all solutions. Antibodies to T-tubule membranes prepared by procedure A stain only T-tubules and not sarcolemma or sarcoplasmic reticulum of intact muscle, indicating a high degree of purity of this preparation (Rosemblatt et al., 1981). T-tubule membranes prepared according to the more rapid procedure B seem less homogeneous by morphological criteria but have a similar high specific [^{3}H]nitrendipine binding activity (Glossmann et al., 1983). Measurement of [^{3}H]nitrendipine (New England Nuclear, Boston, MA) binding to T-tubule membranes was performed as previously described (Curtis & Catterall, 1983), and the calcium antagonist receptor content was determined as the B_{max} on Scatchard plots.

Preparation of the Soluble [^{3}H]Nitrendipine–Receptor Complex. T-tubule membranes were diluted to a protein concentration of 1 mg/mL (~6 nM receptor) and incubated with 2.8–8.4 nM [^{3}H]nitrendipine in solubilization buffer [185 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES/Tris (pH 7.4), and 100 μM diltiazem (Marion Laboratories, Kansas City, MO)] for 90 min at 4 °C. Membranes were sedimented, washed, and resuspended at a 5:1 (w/w) ratio of detergent to protein in 1% (w/v) digitonin in solubilization buffer containing the protease inhibitors of either procedure A or B as desired. After 40 min, insoluble material was removed by centrifugation at 175000*g* for 30 min. The soluble [^{3}H]nitrendipine–receptor complex was assayed by poly(ethylene glycol) (PEG) precipitation in the presence of IgG as carrier protein as previously described (Curtis & Catterall, 1983). All purification steps were carried out at 4 °C.

Chromatography on WGA–Sepharose. The solubilized [^{3}H]nitrendipine–receptor complex was diluted 1:1 (v/v) with solubilization buffer containing a 7-mL packed volume of WGA–Sepharose prepared as described by Hartshorne & Catterall (1981). After a 30-min equilibration, the WGA–Sepharose was packed in a 2 cm × 10 cm column and washed with solubilization buffer, and the receptor was eluted with 100 mM *N*-acetyl-D-glucosamine, 1 mM $CaCl_2$, 100 μM diltiazem, 0.1% digitonin, and protease inhibitors. The concentration of the [^{3}H]nitrendipine–receptor complex in each fraction was determined by liquid scintillation counting, and the fractions containing bound nitrendipine were pooled. The specific activity (picomoles of receptor per milligram of protein) of the pooled fractions was determined both from the total [^{3}H]nitrendipine and from PEG-precipitated [^{3}H]nitrendipine in an aliquot of the pool.

DEAE-Sephadex Chromatography. The pool of the [^{3}H]nitrendipine–receptor complex from the first WGA–Sepharose column was diluted with water to a conductivity equivalent to that of 20 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, 100 μM diltiazem, 0.1% digitonin, and protease inhibitors. The sample was loaded onto a 4 cm × 0.5 cm column of DEAE-Sephadex equilibrated and washed with the same buffer. The column was eluted with a 50-mL linear gradient from 20 mM to 300 mM NaCl in 50 mM Tris (pH 7.4), 1 mM $CaCl_2$, 100 μM diltiazem, 0.1% digitonin, and protease inhibitors. The fractions containing the [^{3}H]nitrendipine–receptor complex were pooled, and the concentration of the complex was determined.

Concentration by Chromatography on WGA–Sepharose. After chromatography on DEAE-Sephadex, a second WGA–Sepharose column (1-mL bed volume) was used to concentrate and further purify the [^{3}H]nitrendipine–receptor complex. Conditions were identical with those for the first WGA–Sepharose column except that 200 mM *N*-acetyl-D-glucosamine was used to elute the receptor.

Sucrose Gradient Sedimentation. The peak of the [^{3}H]-nitrendipine–receptor complex pooled from the second WGA–Sepharose column (~1.5 mL) was layered onto a 38-mL linear gradient from 5 to 20% sucrose (density gradient centrifugation grade, Baker) in 5 mM MOPS (pH 7.4), 1 mM $CaCl_2$, 100 μM diltiazem, 0.1% digitonin, and protease inhibitors and sedimented for 1.5 h at 210000*g* in a VTi 50 vertical rotor. Fractions were collected from the bottom of the tube, and the concentration of the [^{3}H]nitrendipine–receptor complex in each fraction was determined by liquid scintillation counting. Gradient fractions including the peak were individually lyophilized and stored at −70 °C for up to 24 h prior to analysis by SDS gel electrophoresis.

SDS Gel Electrophoresis. Lyophilized samples were dissolved in sample buffer (3% SDS, 12 mM EDTA, 30 mM Tris-HCl, pH 7.6) at 100 °C in the presence of either 20 mM *N*-ethylmaleimide, to prevent spontaneous sulfhydryl oxidation, or 20 mM dithiothreitol, to reduce disulfide bonds, and placed in boiling water for 3 min. Samples were then analyzed on a discontinuous gel system according to Laemmli (1970) consisting of a stacking gel of 3% acrylamide and a running gel containing a 5–15% (w/v) linear acrylamide gradient. The gels were silver stained according to the method described by Oakley et al. (1980).

Protein Assays. Protein concentration was determined by using a modification of the Lowry assay (Peterson, 1977) and/or a dye binding assay (Bradford, 1976). These procedures gave similar results for digitonin-solubilized protein. A modification of the dye binding assay linear to 0.4 μg was used to quantitate protein in column profiles.

Results and Discussion

Solubilization of the Calcium Antagonist Receptor from Transverse Tubules. The results of Table I show that digitonin efficiently solubilizes the preformed [^{3}H]nitrendipine–receptor complex from skeletal muscle T-tubules. In the presence of a 2-fold excess of [^{3}H]nitrendipine to calcium antagonist receptors, 2.0 pmol of [^{3}H]nitrendipine was specifically bound per milligram of protein, and specific binding was 84% of total binding (Table I). Treatment with 1% digitonin solubilized 45% of the protein and 36% of the specific [^{3}H]nitrendipine–receptor complex as assessed by PEG precipitation and yielded a soluble extract containing 1.6 pmol of labeled complex per milligram of protein (Table I). Seventy-six percent of the [^{3}H]nitrendipine in the soluble extract represents the specific ligand–receptor complex.

Table I: Solubilization of the Calcium Antagonist Receptor from T-Tubule Membranes[a]

	protein (mg)	[^{3}H]nitrendipine bound: total pmol	total pmol/mg	nonspecific pmol	nonspecific pmol/mg	specific pmol	specific pmol/mg	% specific
T-tubule membranes	2.0	5.0	2.5	0.8	0.4	4.2	2.1	84
soluble extract (1% digitonin)	0.9	1.9	2.1	0.4	0.5	1.5	1.6	76

[a] T-tubule membranes prepared by procedure A were diluted to 1 mg of protein/mL and incubated with 8.4 nM [^{3}H]nitrendipine in solubilization buffer for 90 min at 4 °C. Membrane samples used to determine nonspecific binding contained 1 μM nitrendipine. Aliquots were removed to determine membrane-bound, total, and nonspecific binding by a rapid filtration assay. Membranes were then sedimented, washed, and solubilized in 1% digitonin. After another centrifugation to remove insoluble material, receptor solubilized from membranes labeled in the absence and presence of 1 μM nitrendipine was used to determine total and nonspecific [^{3}H]nitrendipine binding in the soluble extracts by the PEG precipitation assay (see Experimental Procedures).

Table II: Purification of T-Tubule Calcium Antagonist Receptor[a]

step (% dissociation)[b]	Ca antagonist receptor: pmol	%	protein: mg	%	specific act. (pmol/mg)	x-fold purification
T-tubule membranes	219	100	37	100	5.9	
digitonin extract	103	47	18	49	5.7	
WGA-Sepharose	81	37	0.48	1.3	170	30
DEAE-Sephadex (53%)	57	26	0.08	0.2	710	120
WGA-Sepharose (48%)	40	18	0.03	0.08	1250	210
sucrose gradient (27%)	28	13	0.02	0.04	1950[c]	330

[a] Values represent the mean of two purifications from procedure A membranes. [b] The picomoles of calcium antagonist receptor recovered was corrected for the percent dissociation of [^{3}H]nitrendipine from the receptor. % dissociation = [(receptor-bound cpm on column profile)/(total cpm on column profile)] × 100. [c] Specific activity of the peak fraction; all other values are from the pooled fraction.

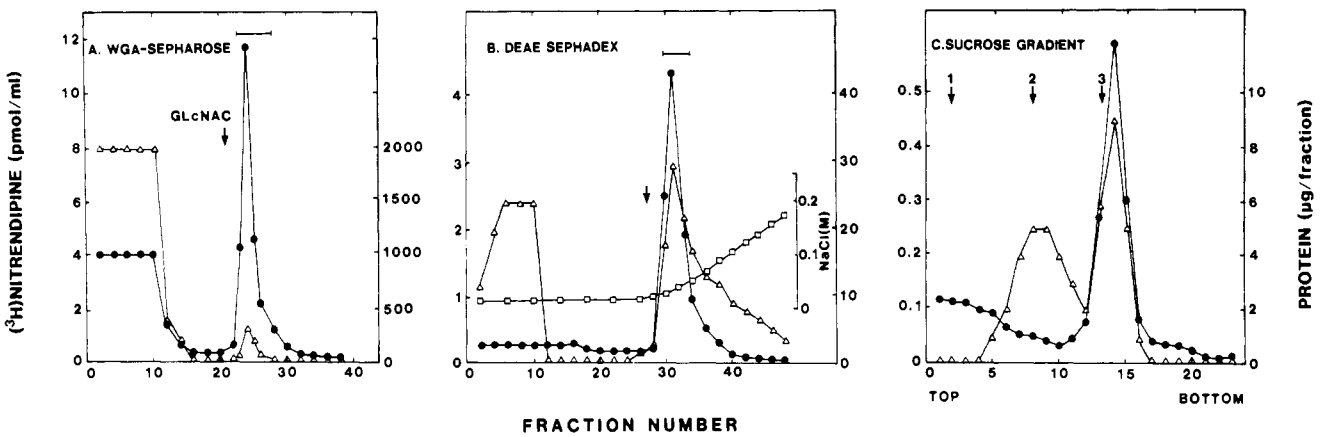

FIGURE 1: Profiles of WGA-Sepharose, DEAE-Sephadex, and sucrose gradient purification steps from procedure A membranes as described under Experimental Procedures: (▲) protein; (●) [^{3}H]nitrendipine; bars indicate the fractions pooled. (A) WGA-Sepharose: fractions 1–20, 5 mL; fractions 21–38, 1.8 mL. *N*-Acetyl-D-glucosamine (GlcNAc, 100 mM) was added at the arrow. (B) DEAE-Sephadex: fractions 1–10, 5 mL; fractions 11–26, 2 mL; fractions 27–48, 1.4 mL. The arrow indicates the beginning of the NaCl gradient (□). (C) Sucrose gradient: fractions, 1.8 mL; arrow 1, hemoglobin (4.5 S); arrow 2, catalase (11.2 S); arrow 3, thyroglobulin (19.2 S).

Although digitonin solubilizes the preformed [^{3}H]nitrendipine–receptor complex, digitonin extracts of unlabeled T-tubule membranes do not bind [^{3}H]nitrendipine specifically as previously observed for rat brain (Curtis & Catterall, 1983). This results, at least in part, from nonspecific binding of [^{3}H]nitrendipine to digitonin micelles. We have therefore purified the preformed [^{3}H]nitrendipine–calcium antagonist receptor complex.

Purification of the Calcium Antagonist Receptor. A sequence of four purification steps was used to purify the [^{3}H]nitrendipine–receptor complex (Figure 1, Table II). The solubilized [^{3}H]nitrendipine–receptor complex was first adsorbed quantitatively to WGA–Sepharose. Washing the resin released free [^{3}H]nitrendipine that was not precipitated by PEG and also removed 97% of the protein (Figure 1A). Elution with 100 mM *N*-acetyl-D-glucosamine resulted in recovery of the [^{3}H]nitrendipine–receptor complex with a 30-fold increase in specific activity (Table II). The large increase in specific activity indicates that T-tubule membranes, unlike sarcolemma, have relatively few glycoproteins that bind to WGA.

The [^{3}H]nitrendipine–receptor complex eluted from WGA–Sepharose adsorbed quantitatively to DEAE-Sephadex at low ionic strength (Figure 1B). Free [^{3}H]nitrendipine, which dissociated from the purified complex, was released during the wash along with a substantial fraction of the protein applied to the column. Elution of the column with a linear gradient of NaCl resulted in recovery of 70% of the [^{3}H]nitrendipine–receptor complex in a single peak between 30 and 50 mM NaCl (Figure 1B) with a 4-fold increase in specific activity (Table II).

The purified [^{3}H]nitrendipine–receptor complex was again adsorbed to a column of WGA–Sepharose. The column was washed and eluted with 200 mM *N*-acetyl-D-glucosamine. This procedure gave a substantial concentration of the receptor and a modest additional purification (Table II).

The final purification step was velocity sedimentation through a linear sucrose gradient to separate proteins by size.

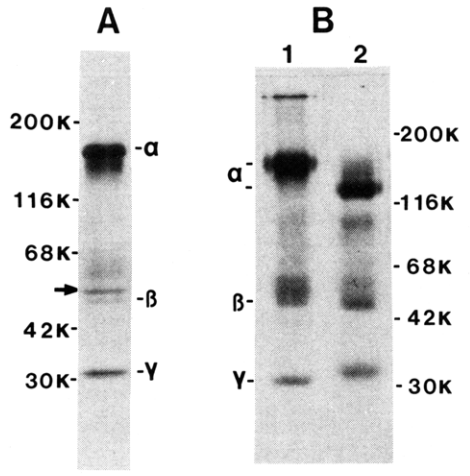

FIGURE 2: Subunit composition of the calcium antagonist receptor: effect of protease inhibitors and reduction. (A) Fraction 14 (500 ng, Figure 1C) from a four-step purification from procedure A was boiled in sample buffer in the presence of 20 mM *N*-ethylmaleimide and subjected to electrophoresis (see Experimental Procedures). A silver staining artifact is marked with the arrow. (B) SDS gel electrophoresis of the peak of the [^{3}H]nitrendipine–receptor complex from the sucrose gradients of a four-step purification with procedure B membrane and protease inhibitors (see Experimental Procedures). Samples (900 ng) were boiled in sample buffer with *N*-ethylmaleimide (lane 1) or dithiothreitol (lane 2) and subjected to electrophoresis.

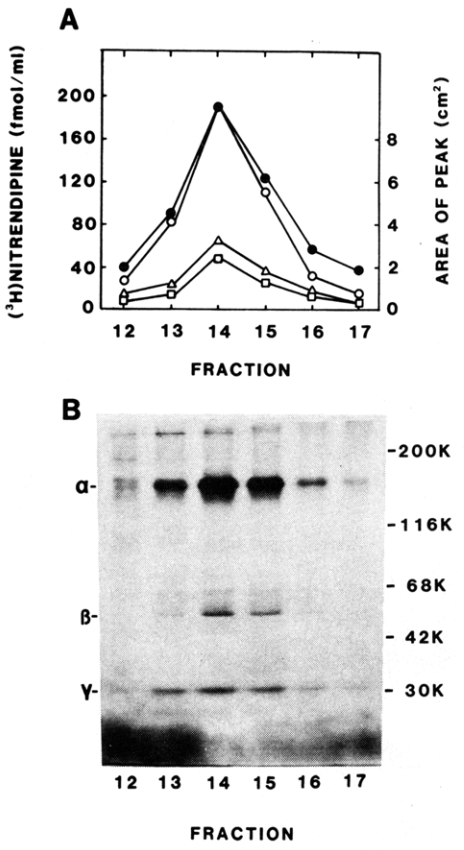

FIGURE 3: Comigration of α, β, and γ subunits with the [^{3}H]nitrendipine–receptor complexes on sucrose gradients. Procedure B membranes are purified through the four-step procedure with the complete protease inhibitor cocktail. Aliquots from the sucrose gradient were analyzed by scintillation counting to locate the [^{3}H]-nitrendipine–receptor complex (A, ●), and the indicated fractions containing receptor were lyophilized and then boiled in sample buffer containing 20 mM *N*-ethylmaleimide. Samples were subjected to electrophoresis on a 5–15% gradient gel (B). After being stained, the gels were scanned on a densitometer, and the area under α (○), β (□), and γ (△) bands was manually integrated and plotted vs. fraction number (A).

The major peak of protein in the gradient (Figure 1C, △) corresponds to the peak of the [^{3}H]nitrendipine–receptor complex. The maximum specific activity is 1950 pmol/mg (mean of two purifications, Table II). This represents an overall purification from the T-tubule membranes of 330-fold.

The molecular weight of the calcium antagonist receptor in skeletal muscle T-tubules has been estimated to be 210 000 by radiation inactivation (Norman et al., 1983). If the receptor binds 1 mol of nitrendipine, the specific activity of a homogeneous preparation would be 4760 pmol/mg. Because we have purified a reversible [^{3}H]nitrendipine–receptor complex, which even in the presence of diltiazem dissociates continually during our procedure, estimation of specific activity and extent of purification have required important assumptions. In the data of Table II, we have corrected for dissociation by measuring the free ligand released during each purification step and assuming that the receptor which releases the ligand remains in the purified fractions. This approach indicates a final specific activity of 1950 pmol/mg or a receptor purity of 41%. An alternative approach is to assume that dissociation during the purification proceeds at the same constant rate that is observed upon simple dilution of the [^{3}H]nitrendipine–receptor complex into excess nitrendipine ($t_{1/2}$ = 3.4 h, unpublished results). Over the 9 h of the purification, 84% dissociation is expected, and the final estimated specific activity would be 2030 pmol/mg for an estimated purity of 43%.

Subunit Composition of the Calcium Antagonist Receptor. In order to identify protein components of the calcium antagonist receptor, samples from each fraction of the sucrose gradient of Figure 1C were dissociated by boiling in SDS sample buffer containing *N*-ethylmaleimide and analyzed by SDS gel electrophoresis and sensitive silver staining (Oakley et al., 1980). Three protein bands were identified, which were present in fractions containing the calcium antagonist receptor and not in adjacent inactive fractions (Figure 2A). The α band (M_r 160 000) and the γ band (M_r 32 000) are stained well by the method used. The β band (M_r 53 000) stains weakly and is sometimes obscured by a silver stain artifact usually present in all gel lanes that has an R_f characteristic of a protein of M_r 57 000 (arrow in Figure 2A). The presence of these bands in individual sucrose gradient fractions is quantitatively correlated with the [^{3}H]nitrendipine–receptor complex (data not shown). They comprise approximately 60% of the silver stain intensity in the most highly purified fractions.

The quantity of the silver stain in the β band was much less than expected if 1 mol of β was present per mole of [^{3}H]nitrendipine receptor and, occasionally, polypeptides of low molecular weight were observed. These results suggested that the quantitiy of protein in the β band might have been reduced by proteolysis despite the presence of the two protease inhibitors used in procedure A. To examine this possibility, we prepared T-tubule membranes by a more rapid procedure (Glossmann et al., 1983) and included an additional three protease inhibitors throughout the purification as described for procedure B under Experimental Procedures. The protease inhibitors reduced [^{3}H]nitrendipine binding, and smaller amounts of labeled receptor were recovered. However, the amount of protein in the β band was doubled relative to the preparation carried out according to procedure A (compare Figures 2A and 3). All three of these bands appear to comigrate with the [^{3}H]nitrendipine–receptor complex in sucrose gradient sedimentation (Figure 3B). Densitometry scans revealed that the three polypeptides quantitatively comigrate with bound [^{3}H]nitrendipine (Figure 3A) and together comprise 65% of the stain intensity in these gel lanes. The presence of

these three bands in highly purified preparations of the calcium antagonist receptor and their quantitative comigration with the specifically labeled receptor in sucrose gradients indicate that they are subunits of the purified calcium antagonist receptor.

Effect of Reduction of Disulfide Bonds on Subunits of the Calcium Antagonist Receptor. After alkylation of sulfhydryl groups with *N*-ethylmaleimide, the calcium antagonist receptor consists of three subunits (Figure 2B, lane 1). In order to determine whether the α, β or γ protein bands represent covalently linked polypeptides, the subunit composition was also determined in the presence of a reducing agent to separate subunits linked by interchain disulfide bonds. Treatment of a peak fraction from the sucrose gradient with dithiothreitol, a strong reducing agent, did not produce additional protein bands that comigrated with the [^{3}H]nitrendipine–receptor complex (Figure 2B, lane 2). The M_r 100 000 band visible in reduced samples does not comigrate with the [^{3}H]nitrendipine–receptor complex and has an electrophoretic mobility identical with that of Ca^{2+}-ATPase, the major protein in the starting material (Rosemblatt et al., 1981). There was a small shift in the apparent molecular weight of β to 50 000 and of γ to 33 000 upon reduction. In contrast, the apparent molecular weight of the α subunit decreased substantially from 160 000 to 130 000 upon reduction (Figure 2B, lane 2). If milder reducing conditions were used (20 mM β-mercaptoethanol), the α subunit migrated as a 160 000/130 000 doublet. If samples fully reduced with dithiothreitol to the form having M_r 130 000 were then treated with iodoacetamide to inactivate the excess reducing agent, the samples also displayed the 160 000/130 000 doublet (data not shown). These results are more consistent with the reversible reduction of an intrachain disulfide bond within the α subunit itself than with the dissociation and association in SDS of an additional undetected subunit. Although the majority of single polypeptide chains exhibit either no change or an increase in apparent molecular weight upon reduction, some instead show a decrease in molecular weight (Dunker & Rueckert, 1969) consistent with an increased binding of SDS upon reduction (Fish et al., 1970). To obtain accurate determination of the molecular weight of polypeptides on SDS gels, it is necessary to completely reduce samples to allow a linear conformation (Fish et al., 1970). Therefore, the appropriate assignments of molecular weight to the subunits of the calcium antagonist receptor should be 130 000 for α, 50 000 for β, and 33 000 for γ.

Molecular Structure of the Calcium Antagonist Receptor of the Voltage-Sensitive Calcium Channel. The results presented in this report confirm that, as in brain (Curtis & Catterall, 1983), the calcium antagonist receptor of the T-tubule membrane of skeletal muscle is a large membrane glycoprotein. The major components of the calcium antagonist receptor of the voltage-sensitive calcium channel are α with M_r 130 000, β with M_r 50 000, and γ with M_r 33 000. These subunits associate through noncovalent interactions. The molecular weight of the T-tubule calcium antagonist receptor has been estimated to be 210 000 by radiation inactivation (Norman et al., 1983). A complex of the α, β, and γ subunits that we have identified as components of the calcium antagonist receptor would be consistent with this size estimate.

The α subunit appears to have an intrachain disulfide linkage whose reduction allows increased binding of SDS and increased mobility on SDS gels. The β subunit in particular was sensitive to proteolysis during preparation (Figure 2). Preliminary data from cross-linking experiments confirmed that α and β are part of the same receptor complex (B. M. Curtis and W. A. Catterall, unpublished results). In addition, labeling of the nitrendipine binding site in situ provides evidence for polypeptides of the size of β and γ. Covalent incorporation of an isothiocyanate derivative of [^{3}H]nitrendipine specifically labels a protease-sensitive polypeptide of M_r 45 000 in cardiac and smooth muscle membranes (Venter et al., 1983). High-intensity irradiation of cardiac membranes with bound [^{3}H]nitrendipine covalently labels a polypeptide of M_r 30 000 (Campbell et al., 1983). The similarity of molecular weights and protease sensitivity suggests a relationship between the β and γ subunits of our purified calcium antagonist receptor and these polypeptides labeled by dihydropyridines in situ.

It is of interest to briefly compare the structure of the calcium antagonist receptor of the voltage-sensitive calcium channel with the structure of the voltage-sensitive sodium channel. Both are large membrane glycoproteins of 200 000–300 000 daltons and consist of one large subunit and two smaller subunits (Catterall, 1982). This overall structural similarity may reflect similar structural requirements for rapid voltage-gated movements of ions across a phospholipid bilayer. As for the sodium channel (Tamkun et al., 1984), reconstitution studies of highly purified preparations of the calcium antagonist receptor will be required to determine if these three subunits are responsible for the physiological functions of the calcium channel.

Registry No. Calcium, 7440-70-2; nitrendipine, 39562-70-4.

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Reactions with the Oxidized Iron Protein of *Azotobacter vinelandii* Nitrogenase: Formation of a 2Fe Center†

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ABSTRACT: The Fe-S center of oxidized Fe protein from *Azotobacter vinelandii* nitrogenase is decomposed by α,α'-dipyridyl in a biphasic process. In the presence of MgATP, 2 Fe are immediately removed by chelation while the additional irons are removed only after several hours. A slower biphasic Fe release also was observed in the presence of chelator alone. MgADP prevented the Fe release by chelator. An intermediate in the reaction was isolated containing 2 Fe. The visible spectrum of the intermediate was similar to that of 2Fe-2S ferredoxins (ϵ_{max} at 325, 416, and 460 nm of 16.1, 11.3, and 9.0 mM^{-1} cm^{-1}). The 2Fe form was electron paramagnetic resonance (EPR) silent until partially reduced with sodium dithionite. The EPR spectral properties were similar to 2Fe-2S ferredoxins; namely, the Fe center had resonances at $g = 2.00$, 1.94, and 1.92 which were detectable, essentially unbroadened at 70 K. The results suggest that in the oxidized (2+) state Fe protein can undergo a 4Fe to 2Fe conversion.

Dinitrogen reduction requires two enzyme components: the MoFe protein (dinitrogenase), which is the site of substrate reduction, and the Fe protein (dinitrogen reductase), which is the ATP-dependent reductant for the MoFe protein. The Fe protein is a dimer of identical subunits and generally is accepted to contain 4 Fe and 4 inorganic S that can be extruded as a single 4Fe-4S cluster (Gillum et al., 1977; Averill et al., 1978). Recently, we found cysteinyl residues 97 and 132 in reduced Av2[1] subunits to be the four terminal Fe ligands (Hausinger & Howard, 1983). This is consistent with a symmetrical arrangement of the Fe cluster between subunits. In contrast, Braaksma et al. (1983) report 8 Fe and 8 S for the fully active Av2 dimer. At present, it is difficult to reconcile their values with the activity, Fe quantitation, and proposed ligands given by others [see Burgess (1984) for a comparison of the literature].

The Fe protein, upon binding MgATP, appears to undergo a conformational change as measured by shifts in the electron paramagnetic resonance (EPR) spectra, magnetic circular dichroic spectra, and redox potential (Zumft et al., 1973, 1974; Stevens et al., 1979). The most striking effect of nucleotide binding is the altered Fe center reactivity with chelators (Walker & Mortenson, 1974; Ljones & Burris, 1978; Hausinger & Howard, 1983). For example, α,α'-dipyridyl with MgATP removed 4 mol of Fe/mol of reduced Av2 ($t_{1/2} \sim 3$ min), whereas α,α'-dipyridyl with MgADP or alone complexed less than 0.2 Fe/mol after 5 h (Hausinger & Howard, 1983). For the reversibly or irreversibly oxidized protein, Walker & Mortenson (1974) and Ljones & Burris (1978) found the Fe center was chelated independent of MgATP. As part of our studies attempting to identify the specific thiol ligands in different Av2 oxidation states, we repeated these experiments. In this paper, we report both qualitative and quantitative differences with the previous work on oxidized Fe protein. Our results indicate a selective abstraction of half the Fe from reversibly oxidized Av2. The chelation is prevented by MgADP and is enhanced by MgATP. The modified protein containing half the original Fe has visible and EPR spectra characteristic of a 2Fe-2S ferredoxin. Our results suggest the 4Fe-Av2 Fe-S center has different properties from those of the 4Fe-4S centers of bacterial ferredoxins or aconitase, which can undergo 4Fe → 3Fe interconversions (Kent et al., 1982; Moura et al., 1982). Furthermore, the Av2 protein structure probably has different constraints on the Fe-S center in the reversibly oxidized and reduced states.

Materials and Methods

Nitrogenase enzymes were isolated from *Azobacter vinelandii* (ATCC 13705) by a modification of a procedure described by Burgess et al. (1980). Av2, assayed at 30 °C and saturating MoFe protein, typically had a specific activity of ~2200 $nmol \cdot min^{-1} \cdot mg^{-1}$ for acetylene reduction and ~3200 $nmol \cdot min^{-1} \cdot mg^{-1}$ for hydrogenase activity (Shah et al., 1972). Protein concentrations for all experiments were determined by amino acid analysis using the known composition for Av2 (Hausinger & Howard, 1982). [Protein determination by the Hartree method gave variable results including ~60% overestimation of protein in some cases (Hartree, 1972).] All procedures were performed under a purified Ar atmosphere with a Schlenk gas manifold for transfer of solutions and

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[1] Abbreviations: Av2, Fe protein from *Azotobacter vinelandii* nitrogenase; 4Fe-Av2 and 2Fe-Av2, 4Fe and 2Fe forms of Av2; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance.

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