HETEROGENEITY OF CALCIUM CHANNELS FROM A PURIFIED DIHYDROPYRIDINE RECEPTOR PREPARATION

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ABSTRACT Dihydropyridine receptors were purified from rabbit skeletal muscle transverse tubule membranes and incorporated into planar lipid bilayers. Calcium channels from both the purified dihydropyridine receptor preparation and the intact transverse tubule membranes exhibited two sizes of unitary currents, corresponding to conductances of 7 ± 1 pS and 16 ± 3 pS in 80 mM $BaCl_2$. Both conductance levels were selective for divalent cations over monovalent cations and anions. Cadmium, an inorganic calcium channel blocker, reduced the single channel conductance of calcium channels from the purified preparation. The organic calcium channel antagonist nifedipine reduced the probability of a single channel being open with little effect on the single channel conductance. The presence of two conductance levels in both the intact transverse tubule membranes and the purified dihydropyridine receptor preparation suggests that the calcium channel may have multiple conductance levels or that multiple types of calcium channels with closely related structures are present in transverse tubule membranes.

INTRODUCTION

The dihydropyridines (DHPs) as exemplified by nitrendipine and nifedipine have been shown to inhibit calcium influx through many types of voltage-dependent calcium channels (for review see reference 30). High affinity binding sites for these compounds have been identified in a variety of tissues (34). Although there is substantial evidence that DHPs modulate calcium channel function by interacting directly with the calcium channel molecule or a closely associated protein, it is not yet clear whether the high affinity DHP binding sites actually represent DHP-sensitive calcium channels. The picture has been complicated by the observations that a large fraction of the DHP-binding sites in skeletal muscle do not appear to be functional calcium channels (26, 31).

We sought to clarify the relationship between the DHP receptor and calcium channels still further by purifying the receptor from skeletal muscle transverse tubule (T-tubule) membranes, then incorporating the purified receptor into planar lipid bilayers. In this study we show that purified DHP receptor preparations reconstituted into planar lipid bilayers exhibit two divalent cation–selective conductance levels of 7 and 16 pS, similar in single channel conductance to calcium channels reported in a variety of intact preparations (21, 23, 35). The larger conductance level is sensitive to DHPs and appears to be very similar to the calcium channel described by Flockerzi et al. (15), and to the calcium channel identified in intact rat T-tubule membranes by Affolter and Coronado (1). The smaller conductance level may be similar to the 10.6 pS T-tubule calcium channel described by Rosenberg et al. (27). It is not known whether these two conductance levels represent two different calcium channel types or whether they represent two conductance levels of a single calcium channel molecule. However, the presence of at least two calcium channel conductance levels in both intact T-tubule membranes and highly purified DHP receptor preparations suggests that the molecular structures underlying these conductance levels may be very closely related.

A preliminary account of this work was presented to the Society for General Physiology (32).

METHODS AND MATERIALS

Transverse Tubule Membrane Preparation

T-tubule membranes were prepared from rabbit back muscle according to the method of Glossmann et al. (16) in the presence of protease inhibitors (1 mM iodoacetamide, 1 μM Pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride). Membranes were suspended in 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, at a protein concentration of 5–10 mg/ml, and stored at −70°C. These preparations contained 15–90 pmol of [^{3}H]PN200-110 binding sites per mg of membrane protein.

Purification of the DHP Receptor

The DHP receptor was purified from T-tubule membranes as described by Curtis and Catterall (12). The following modifications were intro-

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duced into this procedure: (*a*) To improve the yield of labeled DHP receptor, the receptor was labeled with [^{3}H]PN200-110 after rather than before solubilization; (*b*) The DHP receptor was eluted from the first wheat germ agglutinin (WGA)-Sepharose column in a low ionic strength buffer and applied directly to DEAE-Sephadex without further dilution; and (*c*) Diltiazem, a calcium channel antagonist that would interfere with the detection of functional reconstituted calcium channels, was not included in any of the solutions used for purification. Membranes were solubilized in 1% digitonin, 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, at a final protein concentration of 2.5 mg/ml. After centrifugation at 175,000 *g* for 30 min to remove insoluble material, the soluble receptor was labeled with [^{3}H]PN200-110 at a total concentration of 2.5 nM, which was sufficient to label 5–10% of the total receptor. The labeled receptor was absorbed to WGA-Sepharose washed with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, then washed with 20 mM NaCl, 25 mM Tris-HCl, pH 7.4, 0.1 M sucrose, 1 mM $CaCl_2$, and finally eluted with 100 mM *N*-acetylglucosamine, 20 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$. The eluted receptor, detected by bound [^{3}H]PN200-110, was applied directly to DEAE-Sephadex equilibrated in 20 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, and eluted with a linear 20–200 mM NaCl gradient. The peak receptor fractions from this step were concentrated by a second WGA-Sepharose step and further purified by sedimentation on linear 5–20% sucrose gradients (in 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$), as described by Curtis and Catterall (12). All solutions used for the purification contained 0.1% digitonin, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μM Pepstatin A.

[^{3}H]PN200-110 Binding Assays

[^{3}H]PN200-110 binding to intact T-tubule membranes was measured by rapid filtration through GF/C filters (Whatman Inc., Clifton, NJ), as previously described by Curtis and Catterall (11). Membrane samples containing a total of 2.5 μg protein in a volume of 0.5 ml, binding solution (150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$) were incubated for 90 min at 4°C with the desired final concentration of [^{3}H]PN200-110. Nonspecific binding was measured in the presence of 1 μM unlabeled nifedipine.

Reversible [^{3}H]PN200-110 binding to the soluble DHP receptor was measured using a Sephadex G-50 centrifugation assay. Samples of the soluble receptor (0.1–1.25 μg of protein) were incubated in 0.25 ml of binding solution containing 0.05% digitonin and the desired final concentration of [^{3}H]PN200-110 for 90 min at 4°C. Bound ligand was measured by centrifuging 200-μl aliquots of the samples through 2-ml Sephadex G-50 columns that had been equilibrated with binding solution (no digitonin), exactly as described previously (9). Protein recovery from the columns averaged 83%. Nonspecific binding was determined in the presence of 1 μM unlabeled nifedipine. The K_d and B_{max} for [^{3}H]PN200-110 binding to both intact membranes and the digitonin-solubilized receptor were determined by a Scatchard transformation of the data. The K_d for [^{3}H]PN200-110 binding to intact T-tubule membranes ranged from 0.5 to 2.0 nM and was not significantly changed by solubilization of the binding site (see Fig. 1).

Specific DHP Binding Activity of Purified Receptor Preparations

The specific binding activity of the purified receptor was determined by two methods: (*a*) measuring reversible [^{3}H]PN200-110 binding to the soluble receptor directly, as described above, and (*b*) estimating the amount of receptor present in purified samples by measuring the amount of prelabeled receptor and correcting for [^{3}H]PN200-110 dissociation as described by Curtis and Catterall (12). The results of these two methods agree quite well for receptor purified through a single step, but for receptor purified through all four steps, the second method yields higher values for the specific activity than the direct assay of reversible binding (3,400 pmol/mg estimated from pre-bound [^{3}H]PN200-110 compared with 2,500 pmol/mg measured in the reversible binding assay). This result suggests that some of the receptor is denatured during purification. For the experiments described here, DHP receptor purified to a specific activity of 1,800–2,500 pmol/mg, determined by reversible binding assay, was used.

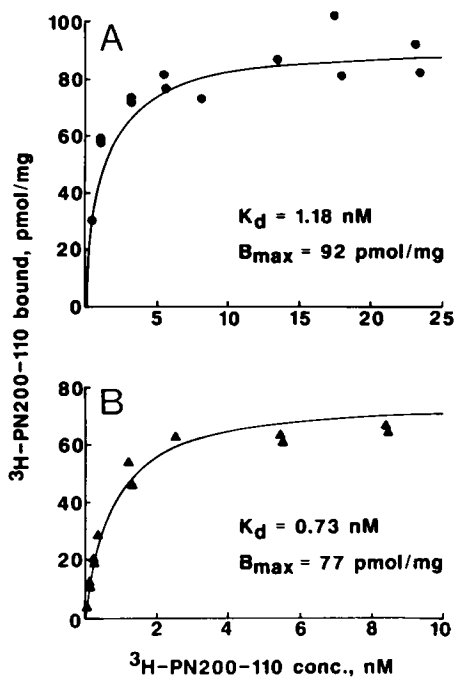

FIGURE 1 Specific [^{3}H]PN200-110 binding to intact T-tubule membranes and to digitonin-solubilized DHP receptor. (*A*) Intact T-tubule membranes (0.0025 mg protein in 0.5 ml final vol) were incubated for 90 min at 4°C in a solution containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, and the indicated final concentration of [^{3}H]PN200-110. Total binding was measured by filtration through Whatman GF/C filters. Specific binding was calculated by subtracting the nonspecific binding measured in the presence of 1 μM nifedipine from total binding. Data shown are duplicate samples from a single experiment. (*B*) Soluble DHP receptor was prepared as described in Methods from the same membranes used in *A*. The soluble DHP receptor (0.006 mg in 0.5 ml final vol) was incubated for 90 min at 4°C in a solution containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 0.05% (wt/vol) digitonin, and the indicated final concentration of [^{3}H]PN200-110. Total binding was measured by the Sephadex G-50 column centrifugation assay. Specific binding to duplicate samples are plotted for a single experiment. Curves were fit to a single binding site by computer analysis.

Protein Assay

Protein concentrations were measured using a Coomassie Blue dye binding assay (7).

SDS PAGE

Samples were lyophilized and analyzed on discontinuous polyacrylamide gels composed of a 4% stacking gel and a running gel containing a linear 5–15% acrylamide gradient (18). Gels were silver-stained according to Oakley et al. (24).

Reconstitution of the Purified DHP Receptor into Phospholipid Vesicles

Purified DHP receptor was incorporated into phospholipid vesicles by adding egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) and bovine brain phosphatidylethanolamine (Sigma Chemical Co.) dissolved in Triton X-100 to the purified soluble receptor. The final concentrations of lipid, protein, and detergents were: 0.2% (wt/vol) phosphatidylcholine, 0.13% (wt/vol) phosphatidylethanolamine, 0.005 mg/ml protein, 1.67% (wt/vol) Triton X-100, and 0.083% (wt/vol) digitonin in 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 0.4 M sucrose. To form lipid vesicles, the detergents were removed from the sample by adsorption to polystyrene beads (0.4 vol of Amberlite XAD-2 polystyrene beads [Sigma Chemical Co.] added to 1 vol of receptor/lipid sample), as described by Talvenheimo et al. (33).

Incorporation of Calcium Channels into Planar Lipid Bilayers

Planar lipid bilayers were formed from a lipid solution in decane (33 mg/ml bovine brain phosphatidylethanolamine, 26 mg/ml bovine brain phosphatidylserine) (Avanti Polar Lipids, Birmingham, AL) across a 100–250-μm-diam hole in a Lexan partition (General Electric Co., Atlanta, GA) separating two experimental chambers, as described by Nelson et al. (20). Calcium channels from intact T-tubule membranes or reconstituted DHP receptor samples were incorporated into preformed planar lipid bilayers by adding the sample to one side of the bilayer to a final protein concentration of ~0.01 mg/ml (intact membranes) or 0.01 μg/ml solution (purified DHP receptor).

Electrical Recording

Current fluctuations across the bilayer were measured and command voltages were applied using a pair of Ag/AgCl electrodes, exactly as described previously (19, 20). The side of the bilayer to which protein is added is designated the *cis* side, the opposite side is defined as the *trans* side. The voltages in these experiments are defined as *cis* minus *trans*, with the *trans* side held at virtual ground. Currents through single calcium channels were recorded continuously on an FM or VCR tape recorder, then played back through an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) at 50–100 Hz for analysis on an AT&T PC6300 computer. The bilayer conductance before the addition of protein was always <10 pS.

Experimental Conditions

To compare single channel conductances of "purified" calcium channels in the bilayer to published work on single calcium channels in membrane patches or in bilayers, a large barium gradient was imposed across the bilayer using 80 mM $BaCl_2$ on the *cis* side and 50 mM NaCl on the *trans* side. At 0 mV divalent cation–selective channels are readily detected as positive current fluctuations carried by Ba ions moving from *cis* to *trans*. In some experiments (see figure legends for details) symmetrical $BaCl_2$ solutions were used. All solutions contained 10 mM Hepes, adjusted to pH 7 with $Ba(OH)_2$ or NaOH. In addition, the DHP agonist (29) Bay K 8644 (1–10 μM) was present in most experiments, unless noted otherwise, on both sides of the bilayer. Bay K 8644 was kindly provided by Dr. A. Scriabine (Miles Laboratories Inc., Naperville, IL).

RESULTS

Measurement of Reversible DHP Binding to the Digitonin-solubilized DHP Receptor

DHP binding to intact T-tubule membranes and to the unpurified soluble DHP receptor was remarkably similar. Fig. 1 compares specific [^{3}H]PN200-110 binding to intact T-tubule membranes (*A*) and to the unpurified solubilized receptor (*B*). The intact membranes bound 92 pmol [^{3}H]PN200-110 per mg protein with a dissociation constant (K_D) of 1.18 nM. The membrane preparation was solubilized and [^{3}H]PN200-110 binding to the soluble receptor was measured as described in Methods. The soluble receptor bound 77 pmol [^{3}H]PN200-110 per mg with a K_D of 0.73 nM. The similarity of the binding parameters for intact membranes and for the solubilized DHP receptor indicates that the initial solubilization step does not alter the affinity of the binding site for [^{3}H]PN200-110, nor does it selectivity solubilize DHP receptors from the membrane. Similar results were obtained for five separate experiments in which the K_D for [^{3}H]PN200-110 binding to the soluble receptor was compared with that of the starting membrane preparation. The average B_{max} and K_D for [^{3}H]PN200-110 binding to the intact T-tubule membranes used for receptor purification were 40 pmol/mg and 1.1 nM, respectively. The average B_{max} and K_D for the DHP receptor solubilized from these membranes were 42 pmol/mg and 1.4 nM, respectively.

Purification and Subunit Composition of the DHP Receptor

The DHP receptor was purified after solubilizing T-tubule membranes in digitonin. The soluble receptor was labeled with [^{3}H]PN200-110, chromatographed on WGA-Sepharose and DEAE-Sephadex, concentrated on WGA-Sepharose, and sedimented on a 5–20% sucrose gradient (see Methods for details). The specific [^{3}H]PN200-110 binding activity of the purified receptor, determined from measurement of reversible [^{3}H]PN200-110 binding, varied from 1,800 to 2,500 pmol/mg. The maximum theoretical specific activity of the skeletal muscle DHP receptor has been estimated to be 4,370 pmol DHP bound per mg protein, assuming that 1 mol of DHP binds to 1 mol of receptor, and that the molecular mass of the DHP binding site is 210 kD, as suggested by target size analysis (22). A specific activity of 2,500 pmol/mg therefore corresponds to a receptor preparation that is 60% pure with respect to DHP binding activity. The K_D for [^{3}H]PN200-110 binding to the soluble receptor did not change significantly after the first step of purification, but increased to 8–12 nM, corresponding to a 5–10-fold decrease in affinity for [^{3}H]PN200-110, after the second step of purification (data not shown). Flockerzi et al. (14, 15) report a similar K_D of 10–11 nM for [^{3}H]PN200-110 binding to the purified DHP receptor.

Fig. 2 shows a silver-stained polyacrylamide gel containing four consecutive sucrose gradient fractions from the final step of purification. Lanes *2* and *3* contained the peak of [^{3}H]PN200-110-labeled receptor (see figure legend for details). Two prominent polypeptides, with apparent molecular masses of 170/142 kD and 33 kD, co-migrate with the labeled receptor (and with the peak of reversible

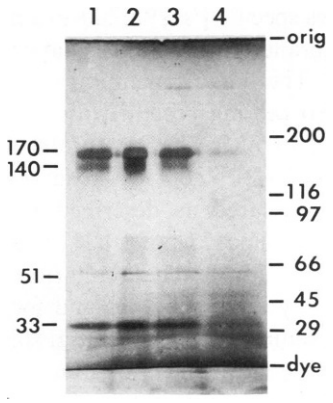

FIGURE 2 Purified DHP receptor analyzed by SDS PAGE. The four consecutive sucrose gradient fractions containing the peak of DHP binding activity from the final step of purification were individually lyophilized, dissolved in sample buffer containing 20 mM dithiothreitol, and boiled. A sample aliquot from each fraction was run in a separate lane of a 4–15% gradient gel, as described in Methods. Each lane contains 2 μg of protein, but the specific DHP binding activity varied as follows: lane *1*, 900 pmol/mg; lane *2*, 2,500 pmol/mg; lane *3*, 1,940 pmol/mg; lane *4*, 500 pmol/mg. The gel was silver-stained according to Oakley et al. (24). Molecular mass markers (in kilodaltons) were myosin, B-galactosidase, phosphorylase B, bovine serum albumin, egg albumin, and carbonic anhydrase.

binding activity). In many preparations, two smaller peptides with apparent molecular masses of 24 and 27 kD (visible in Fig. 2) also co-migrate with the peak of [^{3}H]PN200-110 binding activity. In the absence of disulfide reducing reagents, the large polypeptide migrates as a single band with an apparent molecular mass of 170–174 kD (not shown). In the presence of dithiothreitol, the large polypeptide(s) migrates as a doublet with apparent molecular masses of 142 and 170 kD. Similar results have been reported by other investigators (5, 12, 28). The 33-kD polypeptide is similar to the 32–33-kD peptide identified in purified receptor preparations by other laboratories (5, 12, 14, 28). A 51-kD peptide also co-migrates with DHP binding activity. However, the relative amount of this peptide present in purified DHP receptor samples is variable, possibly due to proteolytic degradation, as suggested by Curtis and Catterall (12). In the peak receptor fraction (Fig. 2, lane *2*), the 170/142 kD and 33 kD polypeptides account for 80% of the total silver-staining intensity.

Reconstitution of Functional Calcium Channels into Planar Lipid Bilayers from Purified DHP Receptor Preparations

DHP receptor purified to an estimated specific binding activity of 1,800–2,500 pmol/mg protein was reconstituted into phospholipid vesicles, then incorporated into preformed planar lipid bilayers by adding the vesicles to one side of the bilayer, as described in Methods. To optimize the detection of DHP-sensitive calcium channels, the calcium channel agonist Bay K 8644 was usually added to both sides of the bilayer.

Contrary to our expectations, two levels of unitary currents were detected from the purified DHP receptor preparation. Fig. 3 *A* shows the two levels of single channel current fluctuation recorded at two membrane potentials. Channel openings are indicated by upward deflections. Traces *a* and *b* in Fig. 3 *A* were selected to show the larger conductance level at the indicated potentials, while traces *c* and *d* show only the smaller conductance level. These records were obtained from a single DHP receptor preparation, purified to a specific activity of 1,800 pmol/mg.

To show that the presence of two sizes of divalent cation–selective event in the purified receptor preparation is not an unusual property of the detergent-treated, reconstituted protein, channels were also incorporated into bilayers from intact T-tubule membranes. Fig. 3 *B* shows a continuous record of current fluctuations obtained from intact T-tubule membranes at +95 mV in symmetrical 125 mM $BaCl_2$ solution. This record, which is typical of records obtained from the intact unpurified membranes, contains at least two sizes of current fluctuation. Under these conditions, the larger event has a conductance of ~20 pS, and the smaller event has a conductance of ~10 pS. Both types of event are selective for barium over either sodium or chloride. Thus, at least two divalent cation–selective conductance levels are detectable in the membrane preparation used as the starting material for purification of the DHP receptor.

Current–Voltage Relationships of Purified Calcium Channels

Fig. 3 *C* shows the current–voltage relationships obtained for the two conductance levels incorporated from the purified DHP receptor preparation. Under the asymmetric ionic conditions used for these experiments, the I-V relationships are nonlinear. The positive current measured at 0 mV must be carried by barium ions, and the extrapolated reversal potentials for both unitary current events is clearly more negative than −50 mV, indicating that both types of event are highly selective for barium over sodium or chloride. The slope conductance for each channel type was estimated from the portion of the curve between +20 and +100 mV. The larger unitary current event has a conductance of 16 ± 3 pS (80 mM barium), while the smaller event has a conductance of 7 ± 1 pS (mean ±95% confidence limit, n = 4 experiments). These conductance levels are reasonably close to the conductance levels of the divalent cation–selective channels from the intact T-tubule membranes (see also references 1 and 27).

Most of the data plotted in Fig. 3 *C* were obtained in the presence of 6 μM Bay K 8644 (*solid symbols*). However, the same two unitary current levels were observed in the absence of Bay K 8644 (*open symbols*), indicating that Bay K 8644 is not required for the observation of either type of event and does not affect the observed current levels. Furthermore, the presence of two single channel conductance levels is clearly not induced by Bay K 8644.

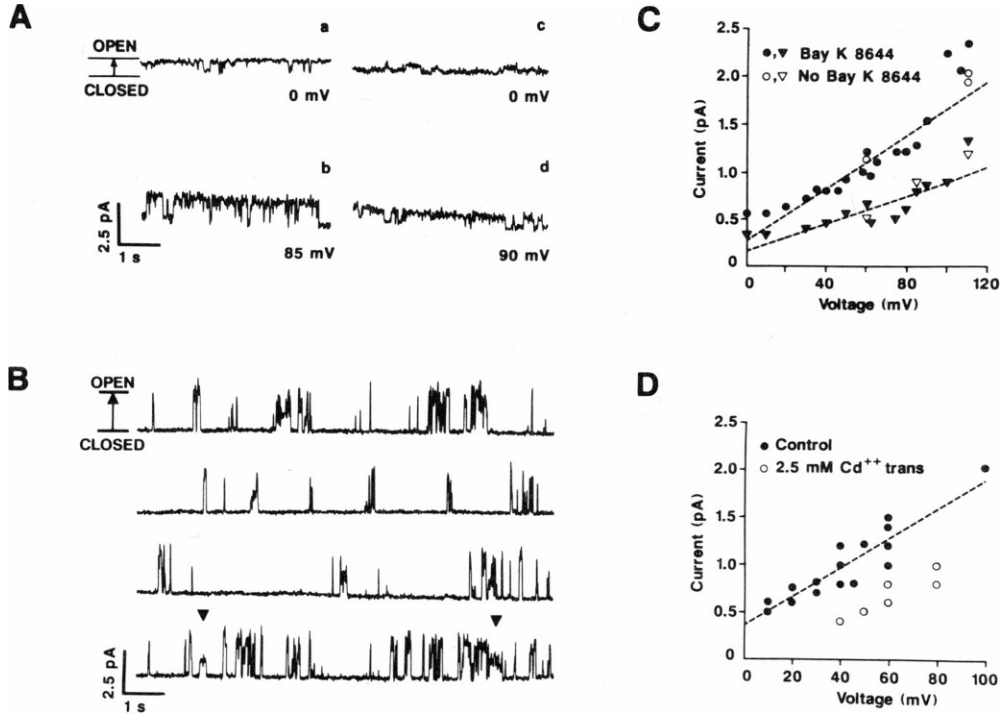

FIGURE 3 (*A*) Purified DHP receptor incorporated into planar lipid bilayers exhibits two conductance levels. DHP receptor purified to a specific binding activity of 1,800 pmol/mg was incorporated into planar lipid bilayers as described in Methods. The *cis* compartment contained 80 mM $BaCl_2$, the *trans* compartment contained 50 mM NaCl. Bay K 8644 (6 μM final concentration) was added to both sides of the bilayer. Single channel currents were recorded at the indicated holding potentials. Records *a* and *b* were selected to show the larger conductance level, records *c* and *d*, obtained from the same DHP receptor preparation, were selected to show the smaller conductance level. All records were filtered at 50 Hz. (*B*) Single channel currents from intact T-tubule membranes incorporated into planar lipid bilayers. Single channels were incorporated into planar lipid bilayers from intact T-tubule membranes containing 40 pmol/mg DHP receptor as described in Methods. Symmetric ionic conditions (125 mM $BaCl_2$ *cis* and *trans*) were used and Bay K 8644 (6 μM final concentration) was added to both sides of the bilayer. Small conductance events are indicated by arrows. (*C*) Current-voltage relationships for purified DHP receptor. Single channel current amplitudes are plotted versus holding potential for both the large conductance level (*circles*) and the small conductance level (*triangles*) exhibited by the purified DHP receptor preparation (1,800 pmol/mg). Ionic conditions were exactly as described for *A*. Open symbols (○, ▽) indicate current levels measured in the absence of Bay K 9644, closed symbols (●, ▼) indicate current levels measured in the presence of 6 μM Bay K 8644 (both sides of the bilayer). The slope conductances, calculated by least squares fit to the data between +20 mV and +100 mV, are 16 ± 3 pS for the larger current level and 7 ± 1 pS for the smaller current level. (*D*) Cadmium block of the 16 pS conductance level from purified DHP receptor. Single channel current amplitudes were measured as a function of membrane potential in the absence (●) and presence (○) of 2.5 mM cadmium (*trans* side only). Ionic conditions and DHP receptor preparation were identical to those used in *A*. 6 μM Bay K 8644 was present on both sides of the bilayer.

Cadmium Block of the 16 pS Purified Calcium Channel

The sensitivity of the 16 pS conductance level to cadmium was tested by adding 2.5 mM cadmium to the *trans* side of the bilayer. Cadmium reduced the unitary current amplitude by 40%, as shown by the open symbols in Fig. 3 *D*. Since the mechanism of cadmium block is proposed to involve very rapid movement of cadmium on and off a channel binding site (too rapid to be resolved into discrete block and unblock events), the predicted effect of cadmium block is an apparent reduction in the single channel current amplitude (20). Previous work (3) has shown that 0.4 mM cadmium reduces the macroscopic calcium current (with 10 mM calcium as the charge carrier) in intact skeletal muscle by 50%, in accordance with our observation. That cadmium blocks the 16 pS current fluctuations from purified DHP receptor preparations supports the identification of this channel as a calcium channel.

Nifedipine Inhibition of the 16 pS Purified Calcium Channel

The effect of the DHP antagonist nifedipine on the probability of the 16 pS channel being open was measured (Fig. 4, *A–C*). Symmetrical 100 mM barium solutions containing 6 μM Bay K 8644 were present on both sides of the bilayer for this experiment. The left panel of Fig. 4 *A* shows a continuous record of channel activity at a holding potential of −40 mV before the addition of nifedipine. After 30 min of recording the activity of this channel, 2 μM nifedipine was added to both sides of the bilayer. Within 5 min channel activity decreased dramatically, as indicated

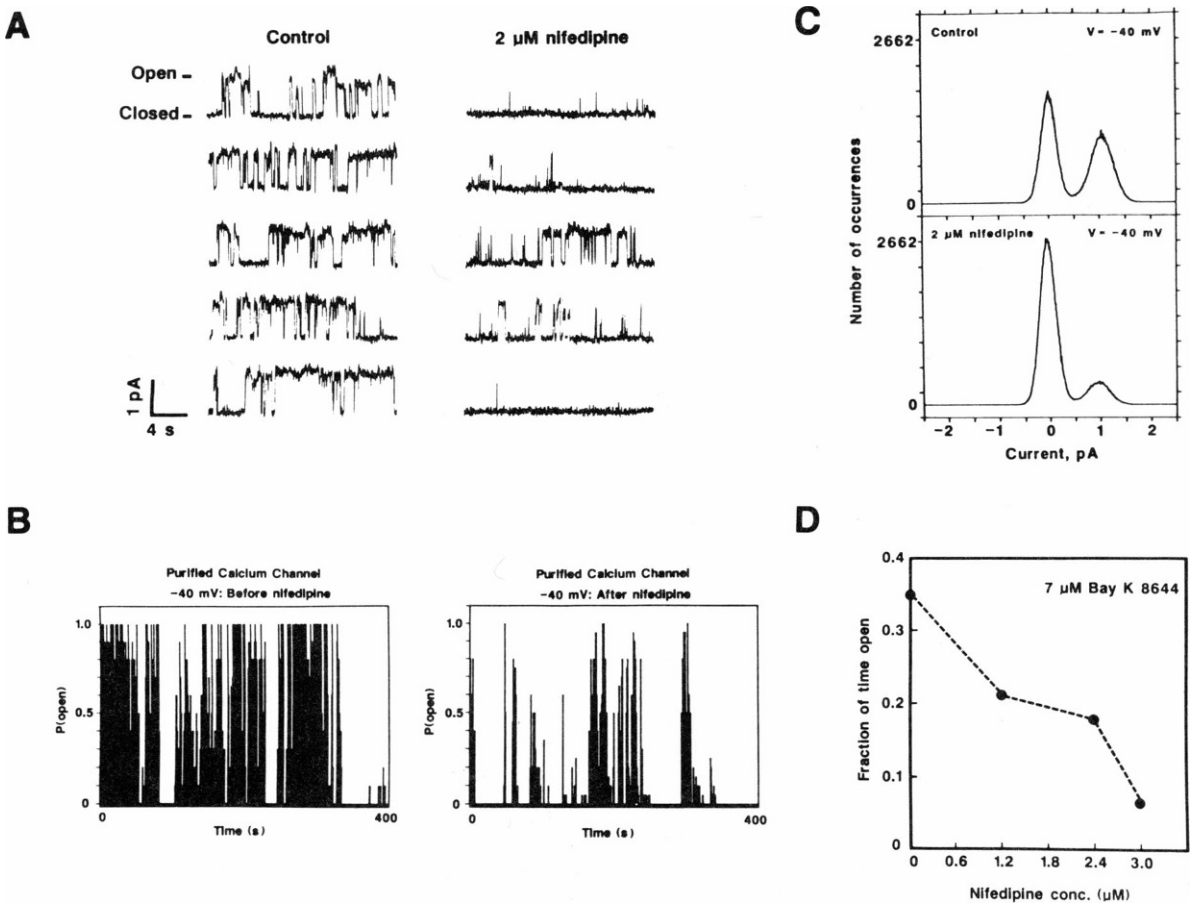

FIGURE 4 Inhibition of the 16 pS purified calcium channel by nifedipine. (*A*) Single channel records before and after the addition of 2 μM nifedipine. DHP receptor purified to a specific binding activity of 2,500 pmol/mg was reconstituted into a planar lipid bilayer. The *cis* and *trans* compartments contained 100 mM $BaCl_2$, and 7 μM Bay K 8644 was present on both sides of the bilayer. The membrane potential was held at −40 mV. After control records were obtained, nifedipine was added to both sides of the bilayer to a final concentration of 2 μM, and recording was resumed 5 min after the drug addition. The records shown were filtered at 100 Hz. (*B*) Reduction of P(open) by nifedipine. 5 min segments of single channel activity from the experiment shown in *A* were divided into 100-ms time bins. P(open) was measured and is plotted for each consecutive bin. (*C*) Current amplitude histograms before and after the addition of nifedipine. Current amplitudes from the experiment in *A* were measured and are shown plotted in a frequency histogram. (*D*) Increasing concentrations of nifedipine reduce P(open) of the purified 15–20 pS calcium channel. DHP receptor purified to a specific binding activity of 1,935 pmol/mg was reconstituted into a planar lipid bilayer. Asymmetric ionic conditions were used: the *cis* compartment contained 80 mM $BaCl_2$, the *trans* compartment contained 50 mM NaCl, and Bay K 8644 (7 μM) was present on both sides of the bilayer. The membrane potential was held at 0 mV. Nifedipine was increased from 0 to 3 μM by adding the drug to both sides of the bilayer. Recording was resumed 5 min after each drug addition. The fraction of time a channel was open was then measured during a 1-min period after each nifedipine addition and is shown plotted versus nifedipine concentration.

by the record shown in the right panel of Fig. 4 *A*. Although the channel continued to open in bursts of activity, longer periods without activity were observed. This is illustrated graphically in Fig. 4 *B*. The experimental records taken before (*left*) and after (*right*) the addition of nifedipine were divided into sequential 100-ms bins, and the probability of the channel being open was determined for each bin, and the results plotted as shown. Nifedipine, even in the presence of 6 μM Bay K 8644, substantially decreases the probability of the channel being open.

Fig. 4 *C* shows the current amplitude histograms obtained before (*top*) and after (*bottom*) the addition of nifedipine. Nifedipine causes a small reduction in unitary current amplitude and reduces the fraction of time the channel spends in the open state from 0.46 to 0.15 (the relative fraction of time spent in the open and closed states is proportional to the area under the amplitude histograms). Affolter and Coronado (1) have reported similar effects of nitrendipine on the kinetics of single calcium channels from intact T-tubule membranes incorporated into bilayers. Although the significance of the nifedipine-induced reduction in single channel conductance was not analyzed further, this result suggests the interesting possibility that nifedipine modifies the conformation of the open channel in addition to reducing P(open).

In a separate experiment the effect of increasing concentrations of nifedipine on the fractional open time of the 16 pS events was measured at 0 mV in the presence of 7 μM

Bay K 8644. For this experiment nifedipine was added to both sides of a bilayer containing a single channel. 5 min after each nifedipine addition, the fraction of time the channel was open was measured for 1 min and this is plotted against nifedipine concentration in Fig. 4 *D*. The fractional open time was reduced to 50% of the control by 2 μM nifedipine. This agrees well with the nitrendipine inhibition of single calcium channels observed by Affolter and Coronado (1) and the nifedipine inhibition of calcium currents in intact muscle fibers (K_i = 0.9 μM) measured by Almers and McCleskey (2).

DISCUSSION

The Molecular Composition of the DHP-binding Protein

We have purified the T-tubule DHP receptor using a method similar to that used by Curtis and Catterall (12) and subsequently by Flockerzi et al. (14, 15). The purified receptor is at least 50% pure with respect to DHP binding activity, a level comparable to that obtained by Curtis and Catterall (12) and Flockerzi et al. (15). Two predominant polypeptides, with molecular masses of 170/142 kD and 33 kD, co-purify with DHP binding activity. The 170/142 kD polypeptide may in fact represent two distinct polypeptides. Three additional peptides, with molecular masses of 51, 27, and 24 kD, are also detectable in the isolated receptor preparation and appear to co-migrate with the DHP binding activity. It is not yet clear which of these peptides are absolutely required for either drug binding activity or divalent cation transport activity.

The DHP receptor has now been purified from T-tubule membranes by several laboratories (5, 6, 12, 14, 15). Although there is substantial agreement on the presence of at least two polypeptides in the purifed DHP receptor preparation (170/142 kD and 33 kD), the exact subunit composition of the receptor has not been conclusively defined. Curtis and Catterall (12) report that the purified DHP receptor consists of three polypeptide subunits with molecular masses of 135, 50, and 33 kD. Borsotto et al. (5, 6) identified polypeptides with molecular masses of 142 and 32–33 kD in purified DHP receptor preparations, but did not observe a 50-kD polypeptide. Under some conditions, 29- and 25-kD polypeptides have also been identified in this preparation (28). Flockerzi et al. (14, 15) recently reported that the purifed receptor, in addition to 142-, 56-, and 31-kD polypeptides, may also contain 122-, 26-, 22-kD peptides.

The purified DHP receptor polypeptides retain reversible high affinity DHP binding activity (this paper, and references 14 and 15), which is allosterically modulated by verapamil and diltiazem (14), indicating that the purified polypeptide contains the binding sites for diltiazem and verapamil as well as for DHPs. Curtis and Catterall (13) reconstituted the purified receptor, consisting of polypeptides with molecular masses of 135, 50, and 33 kD, into lipid vesicles and demonstrated that this preparation could promote divalent cation movements. Divalent cation transport was inhibited by PN200-110, D600, verapamil, and inorganic calcium channel antagonists.

Reconstitution of Calcium Channels from the Purified DHP Preparation into Planar Lipid Bilayers

We have incorporated purified DHP receptor into planar lipid bilayers using an approach that was previously used to incorporate purified brain sodium channels into planar lipid bilayers (17). We identified divalent cation–selective channels with conductance levels of 7 and 16 pS (80 mM barium as the charge carrier) in preparations of purified DHP binding proteins. Flockerzi et al. (15) have incorporated purified DHP receptors into bilayers formed on the tips of patch pipettes and identified a 20 pS divalent cation–selective channel (in symmetrical 90 mM barium) from this preparation. The probability of this channel being open was increased by the DHP agonist Bay K 8644 and was decreased by the phenylalkylamine D600.

We have extended the observations of Flockerzi et al. (15) in at least two significant ways. In addition to identifying two divalent cation–selective conductance levels of 7 and 16 pS from the purified DHP receptor preparation, we demonstrate that the 16 pS calcium channel can be inhibited by the DHP antagonist nifedipine, and we show that the 16 pS channel is blocked by the inorganic antagonist cadmium. Moreover, the concentrations of nifedipine and cadmium required to inhibit the channel are in accordance with both the single channel data from the intact T-tubules (1) and with macroscopic calcium current measurements in intact skeletal muscle (3, 25). These results provide additional support for the idea that the calcium channels in the bilayer from the purified preparation are indeed related to the calcium channels in the intact tissue.

Although the 16 pS calcium channel that we have identified is similar to the channel identified by Flockerzi et al. (15) and by Affolter and Coronado (1), it has a single channel conductance nearly twice that of the DHP-sensitive calcium channel from skeletal muscle identified by Rosenberg et al. (27), which had a conductance of 10.6 pS in 110 mM barium. Our results provide a possible explanation for this apparent discrepancy. We consistently observe two conductance levels of 7 and 16 pS, either singly, or together in the same bilayer, from purified DHP receptor preparations. Skeletal muscle may contain two distinct types of calcium channel, or alternatively, the DHP-sensitive calcium channel in skeletal muscle may exhibit two primary conductance levels. We do not yet have experimental evidence which would allow us to distinguish between these alternatives.

In some experiments we observe only one of the two conductance levels. The experiment shown in Fig. 3, for

example, lasted for more than 2 h, and during this time we observed only the 16 pS conductance level. This observation argues against the conclusion that the two conductance levels arise from the same molecule. However, because we do not know what factors might control the frequency of appearance of different conductance states, we cannot completely rule out this alternative. It is possible, for example, that a covalent modification of the channel molecule determines its conductance level. In this case, the presence of modified channels in our preparation would determine which conductance levels we observe, and these conductance levels would not necessarily be interconvertible under our experimental conditions. Such a possibility is not without precedent; gramicidin channels exhibit multiple conductance levels. The relative frequencies of these levels exhibit substantial variation, for reasons that are not fully understood (8).

We observe single 7 pS channels less frequently than 16 pS channels. There are several possible explanations for the infrequent appearance of this channel: the 7 pS channel may not be as abundant as the 16 pS channel in our preparations, it may not incorporate into bilayers as readily, or it may open only rarely under our experimental conditions. The infrequent appearance of this channel and its small conductance level have made it difficult to test the effects of antagonists such as cadmium and nifedipine. An important goal of future experiments is to determine whether this channel is a DHP-insensitive contaminant in the DHP receptor preparation. If the 7 and 16 pS channels are two distinct molecules, the two types of calcium channel are likely to contain sufficient structural homology to explain the presence of both channel types in DHP receptor samples.

Two calcium channel conductance levels in T-tubule membrane preparations have not been previously described at the single channel level. However, multiple conductance levels of single calcium channels have been described in a variety of other cell types (21, 23, 35). Moreover, Beam et al. (4) and Cognard et al. (10), using intracellular recording, have described two components of macroscopic calcium current in cultured skeletal muscle cells, a slow DHP-sensitive component, and a fast, transient DHP-insensitive component. It is possible that the two conductance levels we have observed from intact T-tubule membranes underlie the two components of voltage-dependent calcium current in skeletal muscle.

The presence of two calcium channel conductance levels in purified DHP receptor preparations raises two important questions regarding the properties of DHP-sensitive calcium channel. First, as suggested above, a single DHP-sensitive calcium channel molecule may exhibit multiple conductance levels, a possibility that has not been adequately explored. Alternatively, the purified DHP receptor preparation may contain two very closely related, but distinct, calcium channel molecules. Our results and those of Flockerzi et al. (15) firmly establish the presence of functional calcium channels in the purified DHP receptor preparation. It should be stressed, however, that the single channels examined in bilayers need not be representative of the functional state of the purified protein. In fact, the recent appreciation of the similarities between the pharmacology of the "voltage sensors" that couple excitation to contraction in skeletal muscle and calcium channels has led to the suggestion that the DHP-binding protein is in fact this "voltage sensor" (26). Thus, it is possible that some of the polypeptides that we have purified are in fact the voltage sensor and that calcium channels have co-purified with these molecules. The resolution of this problem depends on the development of more specific biochemical probes for the DHP-sensitive calcium channel polypeptides as well as on experiments designed to determine whether this channel exhibits multiple conductance levels.

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