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SOLUBILIZATION AND AFFINITY LABELING OF A DIHYDROPYRIDINE BINDING SITE FROM SKELETAL MUSCLE: EFFECTS OF TEMPERATURE AND DILTIAZEM ON [^{3}H]DIHYDROPYRIDINE BINDING TO TRANSVERSE TUBULES

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Effects of temperature and d-cis-diltiazem (DTZ) on [^{3}H]nitrendipine (NTD) and [^{3}H]nimodipine (NIM) binding to skeletal muscle t-tubular membranes were studied. A decrease in temperature from 37°C to 10°C decreased K_D and increased B_{max} slightly. DTZ increased binding by increasing B_{max} under all conditions and also decreased K_D for NTD at 37°C.

The binding protein labeled with [^{3}H]isothiocyanate dihydropyridine revealed a molecular weight of 36,000. The binding site for NTD was solubilized by deoxycholate and dihydropyridine binding was still stimulated by DTZ in the solubilized form.

The slow inward calcium current is important in the regulation of contraction of muscle (1) and is thought to be mediated through an ion selective channel in membranes which can be blocked by the organic Ca^{2+} antagonists (2). The Ca^{2+} antagonists are a chemically diverse group including verapamil, diltiazem, and the dihydropyridines, of which class nimodipine and nitrendipine are representative. There may be an allosteric modulation of the binding of dihydropyridines by other Ca^{2+} antagonists including DTZ (4), although the nature of regulation is disputed.

Dihydropyridine binding has been used as a measure of Ca^{2+} channels, with the assumption that the interaction is exclusively at or in the calcium chan-

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The abbreviations used are: t-tubule(s), transverse tubule(s); NTD, nitrendipine; NIM, nimodipine; DTZ, d-cis-diltiazem; K_D, dissociation constant; B_{max}, maximal number of binding sites; DHP, dihydropyridine; DHP-N=C=S, isothiocyanate labeled dihydropyridine; PEG, polyethelene glycol; (Na) DOC, (sodium) deoxycholate; SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SM, surface membranes; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N'-Tetraacetic acid.

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nel. There is, however, a large discrepancy in the binding K_D and the pharmacological ED_{50} in heart (4) and skeletal muscle (5), although there is good agreement in vascular smooth muscle (6). A low affinity site has been described (7) which may explain the discrepancy in heart because it corresponds with the negative inotropic effect.

The richest source of dihydropyridine binding sites reported to date (50 pmole/mg) is the transverse tubule (t-tubule) fraction of rabbit skeletal muscle (8). In the present study, the putative "active" receptor or binding site in t-tubules (9) has been solubilized and the membrane bound receptor has been labeled with an isothiocyanate dihydropyridine derivative.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]NTD (70 Ci/mmole) was purchased from New England Nuclear, [^{3}H]NIM was obtained through the generosity of Dr. Alexander Scriabine, of Miles Laboratories, and d-cis-DTZ was obtained from Dr. Ronald K. Browne, of Marion Laboratories. The [^{3}H]dihydropyridine isothiocyanate (NET-819) was purchased from New England Nuclear. Sodium deoxycholate was obtained from Grand Island Biology Company and recrystallized from ethanol before use. Electrophoresis grade materials and molecular weight standards were purchased from BioRad, Polyethylene glycol 8000 from Sigma, [^{3}H]ouabain from New England Nuclear, and glass fiber filters (GF/C) from Whatman. All other chemicals were reagent grade or better.

Methods.

Isolation of transverse tubules. T-tubules were prepared from rabbit back skeletal muscle as described by Rosemblatt et al. (9). The final pellet (t-tubules) was homogenized and resuspended in a minimum volume of buffer, an aliquot taken for protein determination and the remainder quick frozen in dry ice/ethanol and stored at -70°C until used. Protein concentrations at various purification stages were assayed by the method of Lowry et al. (10).

Characterization of the t-tubule preparation. Ouabain binding was measured in the presence of 5 mM $MgCl_2$, 5 mM Triphosphate and 500 mM Tris-Cl, pH = 7.4, by incubation of membranes at 37°C in the presence of 10^{-7} M [^{3}H]ouabain for 45'. The maximal number of sites (B_{max}) was calculated from the amount bound, assuming a dissociation constant (K_D) of 53 nM (11). ATPase activity was monitored as described previously (12).

Calcium transport after 10 minutes was measured by incubation at 30°C in 30 mM histidine, 100 mM KCl, 6 mM $MgCl_2$, 5 mM NaN_3, 2.5 mM Tris-oxalate, 0.5 mM EGTA, 10 µM free ^{45}Ca, pH = 7.0. Aliquots for filtration on 0.45 µm Gelman filters were taken at 0.5, 1.0, 1.5, and 10 minutes.

Total cholesterol was measured using the kit (350 A) supplied by Sigma. After 30 minutes incubation at 37°C in plastic cuvettes, the absorbance was read at 500 nm and the µmoles cholesterol in the assay calculated by using the appropriate blanks and standards.

SDS-PAGE was done according to Laemmli (13), using 10% acrylamide gels (either slabs or tubes). Fixing/staining was carried out by the Coomassie blue method of Weber and Osborn (14).

Standard [^{3}H]dihydropyridine binding assay. These assays were done as described by Fosset et al. (8). D-cis-DTZ at 10 µM was included in some

experiments. All experiments were carried out under dim yellow light. All data were analyzed by the method of Scatchard (15).

Solubilization of dihydropyridine receptors. Basically, the technique used is a variation of sarcoplasmic reticulum solubilization as described by MacLennan (16). In a total volume of 5.8 ml in centrifuge tubes, sarcoplasmic reticulum microsomes were incubated for 10-15' at 0°C with varying concentrations of sodium deoxycholate (DOC) and potassium chloride (KCl) in 50 mM Tris-Cl, pH = 7.4, and centrifuged at 150,000 ×g for 1 hr (45K rpm, 50.3 Ti rotor). The supernatant was removed and assayed for [^{3}H]NTD binding by the polyethylene glycol precipitation method as described by Curtis and Catterall (17).

Affinity labeling of t-tubules with an isothiocyanate-labeled dihydropyridine. This was done at 37°C in 50 mM $Na_2B_4O_7$, pH = 9.32, buffer containing 100 mM KCl and 1 mM $CaCl_2$. Nonspecific labeling was done in the presence of 2 μM unlabeled nimodipine.

RESULTS

Characteristics of the t-tubule prep from rabbit skeletal muscle. The starting microsomes ("SR") appear to be nearly pure sarcoplasmic reticulum as judged by very low ouabain binding, very low cholesterol content, very high Ca^{2+} uptake, very high Ca^{2+}-ATPase (see Table I), and a protein SDS-PAGE electrophoretic pattern dominated by the Ca^{2+}-ATPase. The "surface membranes" have an intermediate composition as judged by a higher ouabain binding, higher cholesterol content, and higher Mg^{2+}-ATPase level, while still having a very high Ca^{2+} loading rate and level. The t-tubule preparation is distinct in having a very high Mg^{2+}-ATPase, a very high cholesterol content, and a very low Ca^{2+} uptake, as well as a moderate number of ouabain binding sites. The

TABLE I

Transverse Tubule Characterization

Level of Membrane Purification	Ouabain Binding (maximal) (pmole/mg)	Total Cholesterol (μmole/mg)	$^{45}Ca^{2+}$ uptake (in the presence of oxalate) Total (nmole/mg)	Rate (nmole/mg/min)	ATPase activity (μmole/mg/hr) Total	Na^+/K^+ ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Starting Microsomes (SR)	0.2±0.08	0.028±0.014	1700	2500	370±12	0	340±10	30±4
Surface Membranes (SM)	2.3±0.61	0.099±0.048	1100	3100	440±38	0	280±37	160±7
Transverse Tubules (t-tubules)	8.6±0.40	0.940±0.071	57	110	870±20	48±17	140±28	760±40

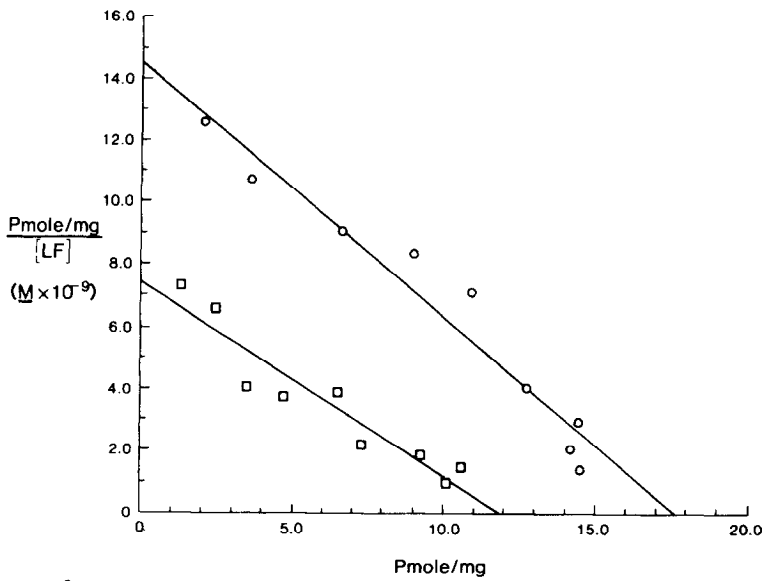

Figure 1: ^{3}H-NTD binding to t-tubules at 10°C
The Scatchard plots shown are obtained under conditions in Experimental Procedures in the absence (□) and the presence (○) of 10 μM d-cis-diltiazem.

yield of t-tubules obtained here is generally less than that reported by Rosemblatt et al. (9). From an average starting muscle weight of 175 g, an average of 3.4 mg of t-tubules was obtained (t-tubules were 9.0 ± 2.7% of the SM and 1.3 ± 0.4% of the starting microsomes).

[^{3}H]NTD and [^{3}H]NIM binding to transverse tubules at 10 and 37°C. D-cis-DTZ potentiated the binding of [^{3}H]NTD and [^{3}H]NIM mainly by increasing B_{max} (from ~12 to 18 pmole/mg) (Figure 1). This is contrary to the report of a lack of an effect of DTZ on presumably the same preparation of rabbit skeletal t-tubule membranes under the same ionic assay conditions (8).

For a given membrane preparation, B_{max} for nimodipine and nitrendipine was the same (data not shown). Under all conditions, [^{3}H]NTD revealed a slightly higher affinity (lower K_D) for the receptor (Table 2). DTZ also has an effect on K_D for [^{3}H]NTD binding at 37°C but not at 10°C, and although DTZ increased B_{max} under all conditions studied, the effect was greater at 37°C and also greater for [^{3}H]NIM than for [^{3}H]NTD at the same temperature (Table 2). [^{3}H]Nimodipine binding capacity was more temperature dependent, both with and without DTZ present (Table 2). In general, the highest number of

TABLE II

Effect of Temperature and D-cis-diltiazem on Dihydropyridine Binding to Transverse Tubules

A. Dissociation Constants (K_D)

	^{3}H-Nimodipine		^{3}H-Nitrendipine	
	- DTZ	+ DTZ	- DTZ	+ DTZ
10°C	2.35 ± 0.49	2.65 ± 0.74	1.70 ± 0.59	1.44 ± 0.47
37°C	3.18 ± 0.85	2.91 ± 0.69	4.34 ± 0.74	2.41 ± 0.70

B. Maximal Binding (B_{max}), expressed as a ratio.

^{3}H-Nimodipine				^{3}H-Nitrendipine			
$\frac{B_{max}\ (+\ DTZ)}{B_{max}\ (-\ DTZ)}$		$\frac{B_{max}\ (10°C)}{B_{max}\ (37°C)}$		$\frac{B_{max}\ (+\ DTZ)}{B_{max}\ (-\ DTZ)}$		$\frac{B_{max}\ (10°C)}{B_{max}\ (37°C)}$	
10°C	37°C	- DTZ	+ DTZ	10°C	37°C	- DTZ	+ DTZ
1.60 ± 0.24	2.02 ± 0.46	1.36 ± 0.27	1.15 ± 0.05	1.47 ± 0.22	1.72 ± 0.24	1.21 ± 0.11	1.02 ± 0.07

The numbers given represent the averages ± the standard deviations for 4 or 5 preparations based on Scatchard plots of data obtained as described in Experimental Procedures.

[^{3}H]dihydropyridine sites labeled seemed to be approached at 10°C with DTZ present. The reason for the slight temperature dependence of B_{max} is not clear, but it is interesting that lowering the temperature appears to have the same effect as adding DTZ, i.e., both seem to either make "new sites" available or convert putative "low affinity" sites to "high affinity" sites.

Solubilization of the high affinity dihydropyridine receptor. The material solubilized by NaDOC was checked for specific binding by both equilibrium dialysis and the polyethylene glycol precipitation. The total (± DTZ) and the non-specific binding increased with increasing DOC (Figure 2). Presumably, the non-specific binding increase is due to a large increase in the total protein in the supernatant (and, therefore, in the assay). Also, specific binding was observed upon equilibrium dialysis (3 days, 4°C, [^{3}H]NTD; results not shown).

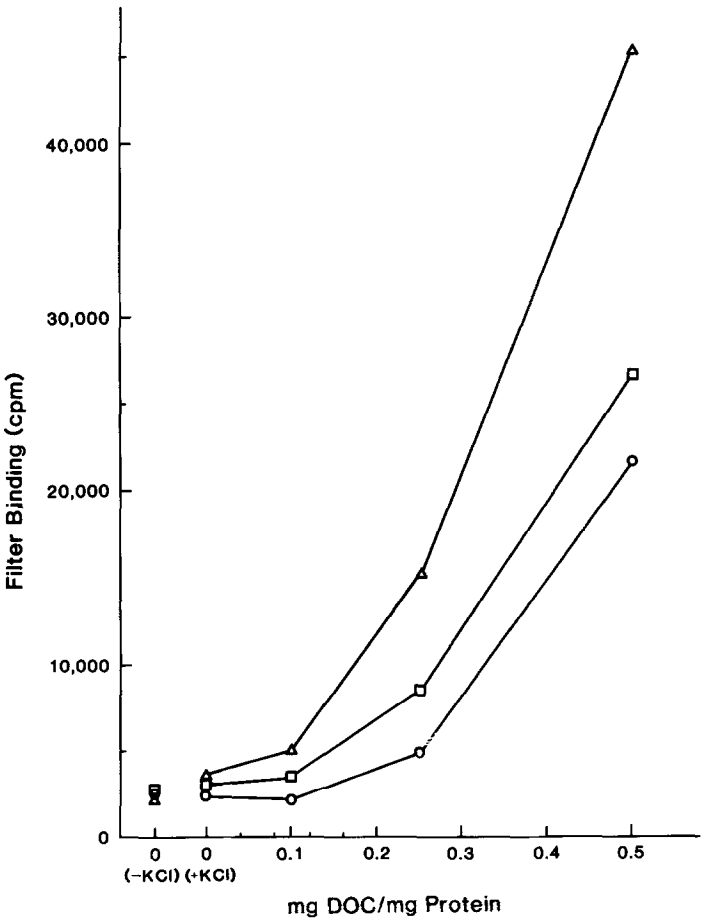

Figure 2: ^{3}H-NTD binding to supernatants of DOC solubilized rabbit skeletal muscle microsomes
^{3}H-NTD binding was determined by the polyethylene glycol precipitation method described in Experimental Procedures. Non-specific binding (○), total binding (□) and total binding in the presence of 10 μM d-cis-diltiazem (Δ) was measured in 50 mM Tris-Cl, 100 mM KCl, 1 mM $CaCl_2$ pH = 7.4.

Affinity labeling of the dihydropyridine receptor. Proteins of 36,000 and 115,000 Da were labeled (Fig. 3). The labeling of the 115,000 MW protein was not decreased by inclusion of unlabeled DHP. Attempts to get more of the radioactivity into the gel by further heating of the samples before electrophoresis resulted in the creation of smaller, labeled, presumably proteolytic fragments (<30,000 MW). It thus appears that the 36,000 MW binding protein is both susceptible to aggregation and thermally-induced proteolysis.

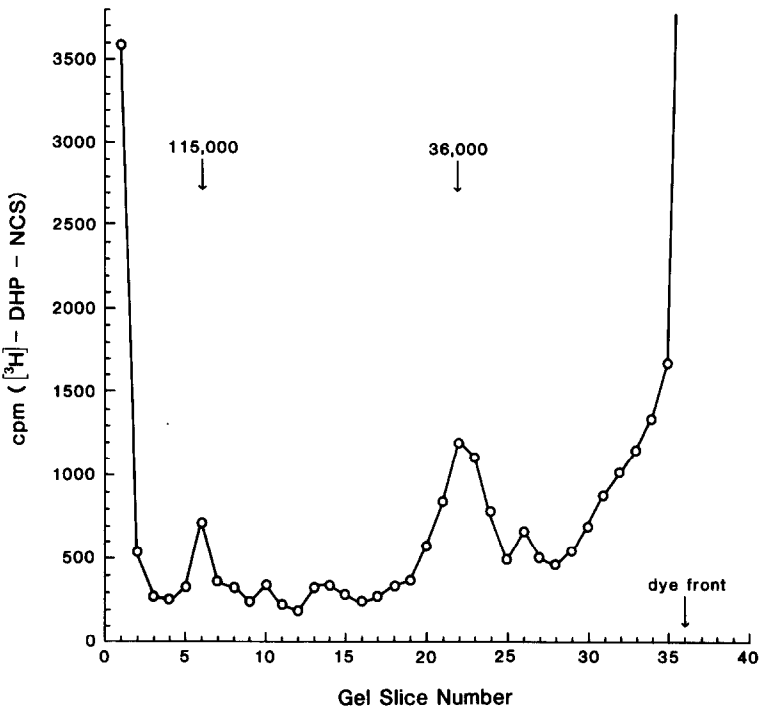

Figure 3: ^{3}H-DHP-isothiocyanate affinity labeling of t-tubular membranes
Membranes were incubated at 37°C in pH = 9.32 $Na_2B_4D_7$ buffer containing 100 mM KCl and 1 mM $CaCl_2$ for 30 minutes, centrifuged down, solubilized in SDS-PAGE sample buffer containing 5% β-mercaptoethanol, heated for 5 minutes at 100°C and loaded onto a 10% gel. After electrophoresis, the gel was sliced (2 mm/slice), digested with H_2O_2 and counted. BioRad molecular weight standards and unlabeled t-tubules were run on parallel gels.

DISCUSSION

The t-tubules prepared here have physical and enzymatic properties very similar to other preparations described in the literature. However, there are several discrepancies between this work and other published work. The number of ouabain sites is lower than the total reported by Lau et al. (11) of 37 pmol/mg using t-tubules derived by a French press treatment. We attempted to use deoxycholate as was reported (11) to expose latent sites but found only a decrease in ouabain binding. Our [$^{45}Ca^{2+}$] uptake data, cholesterol data, and SDS-PAGE protein band pattern agree well with Rosemblatt et al. (9). Therefore, the preparation we describe is enriched in t-tubules and contains only slight (5-10%) contamination of SR or SL (Hidalgo et al. [18]).

The total number of [^{3}H]DHP binding sites in this preparation is about 1/3 that reported by Fosset et al. (8). Other investigators have not been

able to obtain these very high binding densities, including the most recent study of Curtis and Catterall (19), in which the authors report only 5.9 pmole/mg. The binding of both DHPs used is indeed stimulated by d-cis-DTZ at 10°C and 37°C, contrary to Fosset et al. (9). A possible explanation is a change in the configuration of the membrane/proteins in some purification step which would either "open up" new sites or convert "low affinity sites" into "high affinity sites." It is possible that the state of the channel could be changed by some seemingly trivial, overlooked detail in a purification step.

The main effect of DTZ on binding is an increase in B_{max}, and this effect was more pronounced on NIM than NTD binding. The K_D reported here is in excellent agreement with Fosset et al. (8) for [^{3}H]NTD binding at 10°C (1.7 nM compared to 1.8 nM). The molecular mechanism of the stimulation of binding by DTZ is not known although radiation inactivation analysis data (20) suggest that DTZ causes dissociation of a $M_r \approx 60,000$ subunit from the channel labeled by DHPs.

After completion of our work, a report appeared describing the solubilization of prelabeled t-tubule membranes and the subsequent partial purification of the [^{3}H]NTD binding complex (19). The authors reported a subunit structure of α (M_r = 130,000), β (50,000) and γ (33,000). [^{3}H]DHP binding to our NaDOC solubilized receptor is still stimulated by DTZ, which probably indicates that the putative coupling of proteins making up the channel is still intact on the solubilized form as was the solubilized channel described by Glossman and Ferry (21).

Labeling of t-tubules with [^{3}H]isothiocyanate DHP resulted in proteins of apparent molecular weight of 115,000 and 36,000. Venter et al. (22) reported a M_r 45,000 protein labeled with the same compound in guinea pig smooth muscle. The 36,000 MW protein labeled in this work was very reproducible and is probably identical to the γ subunit reported very recently by Curtis and Catterall (19). The protein labeled at Mr $\approx$115,000 was also fairly reproducible but less specific, i.e., it was labeled in the presence of unlabeled

DHP almost to the same level. The apparent molecular weight of this protein also varied from 110,000 - 150,000. This could be the α subunit reported by Curtis and Catterall (19). A model of the putative Ca^{2+} channel consistent with all the current data is as follows. The channel would consist of 3 subunits as reported (19): α (M_r = 130,000); β (M_r = 60,000), possibly the diltiazem binding protein implicated by Goll et al. (20); γ (M_r = 33-36,000), the dihydropyridine binding protein labeled in this work.

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