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# Monoclonal antibodies against the 1,4-dihydropyridine receptor associated with voltage-sensitive $Ca^{2+}$ channels detect similar polypeptides from a variety of tissues and species

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Four monoclonal antibodies have been raised against voltage-sensitive $Ca^{2+}$ channel dihydropyridine receptors from rabbit skeletal muscle. When tested by immunoblot assay of denatured transverse tubule membranes in reducing polyacrylamide gels, each recognised a single polypeptide of $M_r \sim 140000$ that co-migrated with the large glycoprotein subunit of the purified receptor. In blots of nonreducing gels, a larger protein of $M_r \sim 170000$ was seen and three of the antibodies recognised additional components at $M_r \sim 310000$ and $\sim 330000$. Crossreactive material of similar molecular mass was also seen in rabbit heart and brain, and in the skeletal muscle of other species.

Dihydropyridine; Dihydropyridine receptor; $Ca^{2+}$ channel; Monoclonal antibody

## 1. INTRODUCTION

Due to their high-affinity binding, the 1,4-dihydropyridine (DHP) $Ca^{2+}$ channel antagonists have proved most useful as biochemical probes for the voltage-sensitive $Ca^{2+}$ channel [1,2]. To date, most studies of the molecular properties of the DHP receptor have concentrated on the relatively abundant receptor of the skeletal muscle transverse tubule (T-tubule) membrane [3].

There is general agreement, both from purification [4–6] and affinity labelling studies [7,8], that a large glycoprotein of $M_r$ 130000–150000 is implicated in the skeletal muscle DHP receptor structure and several laboratories have provided evidence for additional components of $M_r$ 50000 and 32000–36000 [4–7,9]. Subunit analysis of pharmacologically more interesting DHP receptors such as those from heart [10] and smooth muscle has proved more difficult.

Using monoclonal antibodies raised against the skeletal muscle DHP receptor from rabbit, we demonstrate in this report that antigenic determinants on the skeletal muscle DHP-sensitive $Ca^{2+}$ channel are shared between putative $Ca^{2+}$ channel components in various tissues and species.

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## 2. MATERIALS AND METHODS

### 2.1. *Membrane preparations*

Skeletal muscle microsomal membrane fractions were prepared according to Fosset et al. [3]. Microsomes from other tissues were prepared according to Glossmann and Ferry [11] in 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Mops, pH 7.5. T-tubule membranes were prepared from rabbit white skeletal muscle as described by Rosemblatt et al. [12].

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2.2. *1,4-Dihydropyridine receptor purification*

Purification of the DHP receptor from T-tubule membranes was performed as described by Borsotto et al. [4] in medium A (0.1% CHAPS, 5% glycerol, 0.02% phosphatidylcholine, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM iodoacetamide, 0.1 mM PMSF, 1 $\mu$M pepstatin A, 20 mM Tris, pH 7.5) with a modification following the gel filtration step. Ultrogel A2 fractions containing DHP receptor activity were pooled and adjusted to contain 1% Triton X-100. Following incubation at 4°C for 15 min the mixture was applied to a 1.8 ml wheat germ agglutinin-Ultrogel (WGA-Ultrogel, LKB) column. The breakthrough was reapplied and the column washed sequentially with 10 ml medium A containing 0.1% Triton X-100 in place of CHAPS, 10 ml medium A containing 1% CHAPS and finally 5 ml medium A. The receptor was eluted with 200 mM *N*-acetyl-D-glucosamine in medium A.

2.3. *Preparation of monoclonal antibodies*

BALB/C mice were immunized by intraperitoneal injections of extensively purified DHP receptor (0.8–1.0 nmol [$^3$H]PN 200-110-binding sites per mg of protein; 10 $\mu$g protein per animal) prepared as an alum precipitate, followed after 6 weeks by a similar amount of protein injected in saline. 7 days before being killed one animal received about 5 $\mu$g purified receptor in Freund's incomplete adjuvant followed on each of the next 6 days by an intraperitoneal injection of a similar amount of receptor in saline. The spleen was excised 1 day later and the cells fused with $10^7$ NSO/1 myeloma cells as described by Galfre and Milstein [13] except that 50% polyethylene glycol 4000 (Merck, Cat. no.9727) was used. After selection of hybridoma cells, positive clones were replated at limiting dilution to ensure the monoclonal nature of the cell lines. Ascitic fluids were produced by injecting about $10^7$ monoclonal hybridoma cells into pristane-primed BALB/C mice.

2.4. *Solid-phase antibody assay*

Microtitre plate wells were coated for 16 h at 4°C with 50 ng of purified DHP receptor in 50 $\mu$l of phosphate buffered saline (PBS; 50 mM sodium phosphate, pH 8.0, 0.9% NaCl). The CHAPS concentration in the diluted receptor was <0.003%. Each well was washed for 1 h with 0.5% casein, 10% calf serum in PBS. Wells were incubated for 2 h at room temperature with the hybridoma supernatant being tested, then sequentially with 50 $\mu$l of rabbit anti-mouse IgG (Miles; 1:1000 dilution) and goat anti-rabbit immunoglobulin coupled to peroxidase (Miles; 1:1000 dilution) in 1% calf serum, 0.05% Tween 20, PBS, each for 1 h at room temperature. Positive wells were identified by the development of blue colouration following the application of 50 $\mu$l of 10 mg/ml 3,3′,5,5′-tetramethylbenzidine (Miles) in 0.1 M sodium acetate/citrate, pH 6.0, 0.0045% $H_2O_2$. Between steps, wells were washed three times with 0.05% Tween 20 in PBS.

2.5. *Immunoblots*

Samples of T-tubule (60 $\mu$g) or microsomal membranes (0.25–3 mg protein) were denatured in 2% SDS, 9% glycerol, 75 mM Tris-HCl, pH 6.8, and either 0.6% dithiothreitol (disulphide reducing conditions) or 8 mM iodoacetamide (nonreducing conditions) and loaded on 4–12% linear polyacrylamide gradients according to Laemmli [14]. Following transfer of resolved proteins to nitrocellulose [15], the paper was incubated for 16 h at 4°C with 1% bovine serum albumin, 0.5% casein, 0.05% Tween 20, 0.9% NaCl, 10 mM Tris-HCl, pH 7.5. Antibody binding to nonreduced membrane components was assayed by incubating the nitrocellulose paper with diluted ascitic fluids (1:500 in 0.05% Tween 20, 0.9% NaCl, 10 mM Tris, pH 7.5) for 2 h at room temperature followed by sequential incubations with rabbit anti-mouse IgG (1:500 dilution, 1 h) and goat anti-rabbit IgG coupled to peroxidase (Miles; 1:500 dilution, 2 h). For reduced membrane samples, the rabbit anti-mouse IgG incubation was followed by sequential incubations with swine anti-rabbit IgG (Dakopatts; 1:500 dilution, 1 h) and rabbit peroxidase anti-peroxidase complex (Dakopatts; 1:500 dilution, 1 h). Immunoblots were then developed using 0.2 mg/ml diaminobenzidine (Sigma), 0.9% NaCl, 20 mM Tris-HCl, pH 7.5, 0.003% $H_2O_2$. Between steps the nitrocellulose paper was washed for 10 min in 0.25% sodium lauryl sarcosine, 0.25% Nonidet P-40, 1 M NaCl, 10 mM Tris-HCl, pH 7.5.

## 3. RESULTS AND DISCUSSION

The spleen of a hyperimmune BALB/C mouse was removed and monoclonal antibody producing hybridoma cells isolated as described in section 2. Cell supernatant from monoclonal cell lines was assayed in the solid-phase assay against extensively purified DHP receptor (0.8–1.0 nmol (+)-[$^3$H]PN 200-110-binding sites per mg of protein). From the receptor molecular size of $M_r$ ~210000 determined by radiation inactivation [16] and assuming one DHP binding site per receptor molecule, the specific activity of a homogeneous preparation would be 4.76 nmol/mg. The minimum purity of the preparations used for assay was estimated at 17% but it is likely that the degree of purity was substantially underestimated as a result of activity loss due to the half-life of the DHP receptor in CHAPS solution [17] and to the high detergent concentrations used during the WGA-Ultrogel purification step [4]. When analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions, these receptor preparations consisted of a major component of $M_r$ ~140000 with minor bands at $M_r$ ~100000 and 33000 (not shown). Out of 360 hybridoma supernatants tested, 33 were positive in the solid-phase assay against this extensively purified DHP receptor and 31 individual clones were isolated by plating at limiting dilution.

Both purification [4–6] and affinity labelling studies [7,8] have identified a polypeptide chain of $M_r$ 140000 as a component of the DHP receptor of the skeletal muscle voltage-sensitive $Ca^{2+}$ channel. Four antibodies ($\alpha$DHP-R 11, 13, 14 and 15), out of the panel of 31, produced detectable reaction on immunoblots of skeletal muscle T-tubule membranes. Under reducing conditions all four an-

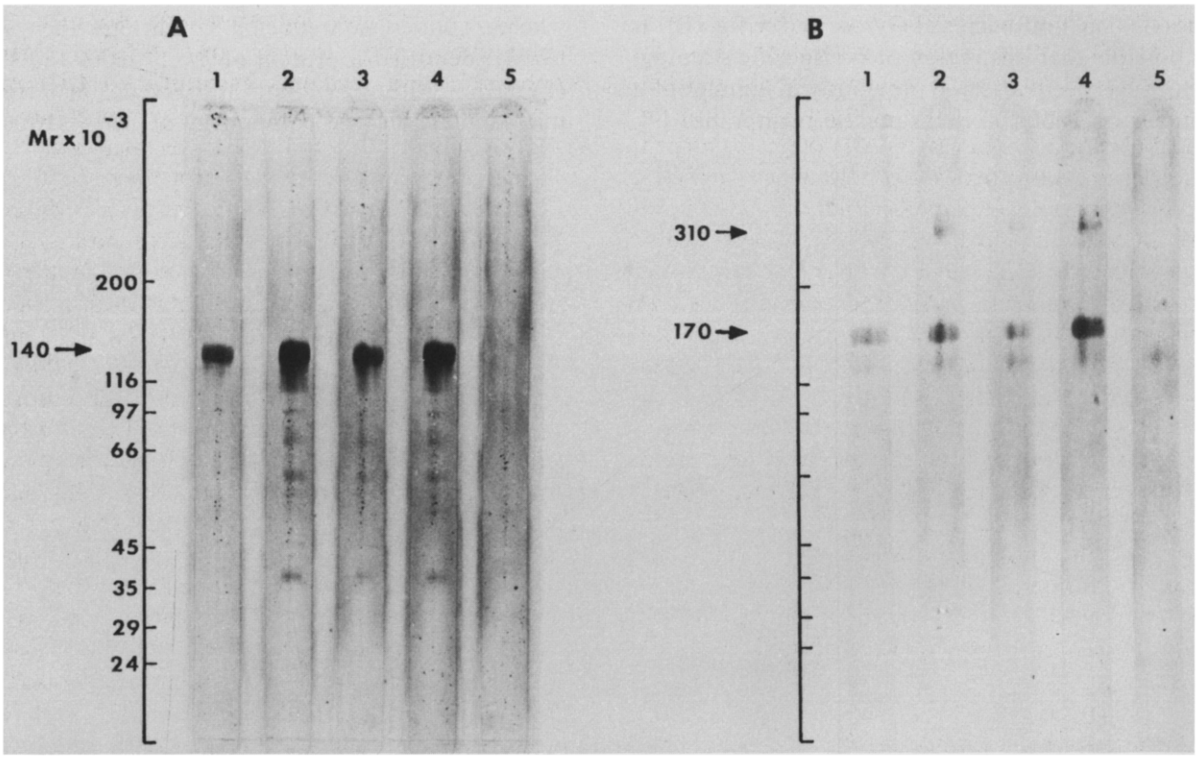


Fig.1. Immunoblots of rabbit skeletal muscle transverse tubule membranes after electrophoresis under reducing (A) and nonreducing conditions (B). Lanes: 1, $\alpha$DHP-R 11; 2, $\alpha$DHP-R 13; 3, $\alpha$DHP-R 14; 4, $\alpha$DHP-R 15; 5, anti-gyrase B control ascitic fluid (Harrison, T.M., unpublished). $M_r$ markers are from Sigma: myosin (200000), $\beta$-galactosidase (116000), phosphorylase b (97000), bovine serum albumin (66000), ovalbumin (45000), pepsin (35000), carbonic anhydrase (29000) and trypsinogen (24000).

tibodies bound specifically to a single polypeptide of $M_r$ ~140000 (fig.1A). This polypeptide co-migrated with the 140 kDa polypeptide present in the extensively purified DHP receptor preparations (not shown) indicating that these monoclonal antibodies detect specifically the large component of the $Ca^{2+}$ channel protein.

Under nonreducing conditions the four antibodies detected larger protein bands (fig.1B). All four recognized a protein of $M_r$ 165000–170000 and $\alpha$DHP-Rs 13, 14 and 15 detected two larger proteins of $M_r$ 310000 and 330000. It has been suggested that the transition between $M_r$ 170000 and $M_r$ 140000 on disulphide reduction is due to the release of a small disulphide linked component of $M_r$ ~32000 [9]. Specific labelling of a 140 kDa polypeptide could not be detected under nonreducing conditions. A diffuse region of nonspecific staining in the 130–140 kDa region was also present in the control track of nonreduced material in which the $\alpha$DHP-R antibody was replaced by a monoclonal anti-bacterial Gyrase B (see fig.1B). It is possible that this region of nonspecific staining, which has been observed previously in immunoblot studies on rabbit skeletal muscle membranes [9], was due to endogenous immunoglobulin molecules associated with the membrane preparation.

The observation of specific anti-DHP receptor antibody binding to large protein bands of $M_r$ >300000 indicates that additional polypeptides may be required for the complete native structure of the DHP-receptor $Ca^{2+}$ channel protein. Although there are no indications of their identity in this study, possible polypeptide candidates of $M_r$ 50000–55000 and ~99000 have been implicated in other studies of the DHP receptor protein [6,7].

Possible immunochemical similarities between putative DHP-sensitive $Ca^{2+}$ channels in different species and tissues were investigated by immunoblot assay using $\alpha$DHP-R 13. Immunocrossreactivity was detected between the rabbit skeletal muscle DHP receptor and a component in skeletal muscle microsome preparations from mouse, rat and frog. In each case a single polypeptide of $M_r$ 140000–145000 was detected by $\alpha$DHP-R 13 under reducing conditions (fig.2A), whereas under nonreducing conditions the antibody identified a protein of $M_r$ 170000–180000 (fig.2B). Although not observed in frog microsomes, a large component of $M_r$ ~310000

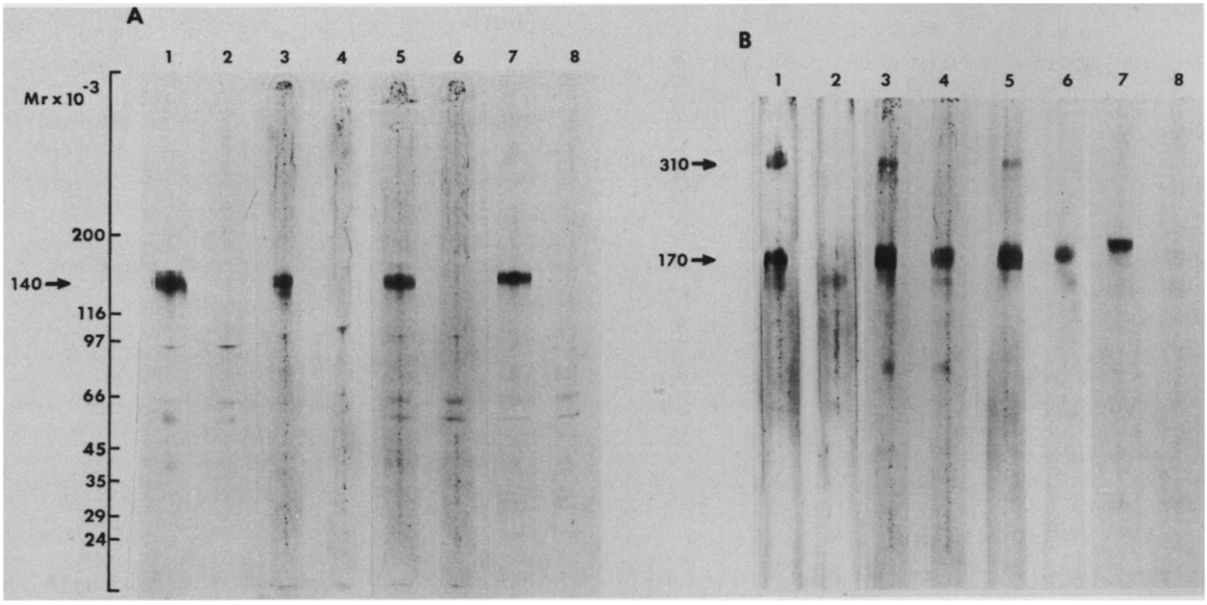


Fig.2. Immunoblots of skeletal muscle microsomes after electrophoresis under reducing (A) and nonreducing conditions (B). Lanes: 1 and 2, rabbit (0.25 mg); 3 and 4, rat (0.75 mg); 5 and 6, mouse (0.3 mg); 7 and 8, frog (0.33 mg). Lanes 1, 3, 5 and 7 with $\alpha$DHP-R 13, lanes 2, 4, 6 and 8 with anti-gyrase B control ascitic fluid. $M_r$ markers as in fig.1.

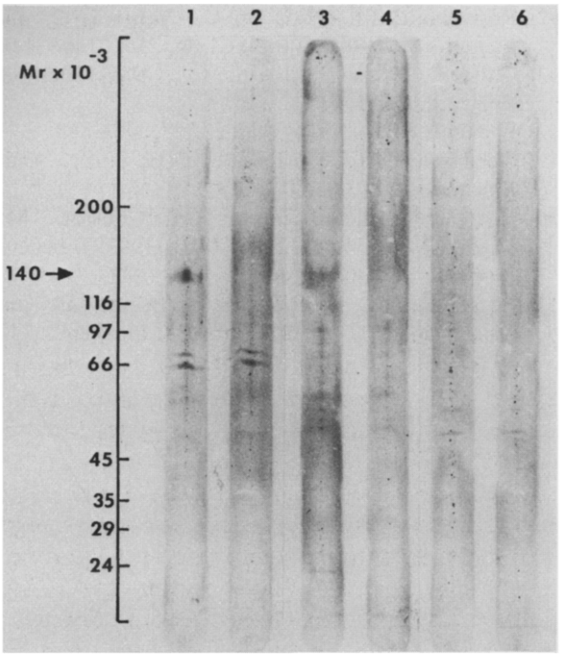


Fig.3. Immunoblots of brain, heart and liver microsomes after electrophoresis under reducing conditions. Lanes: 1 and 2, brain (3.0 mg); 3 and 4, heart (1.85 mg); 5 and 6, liver (3.0 mg). Lanes 1, 3 and 5 with $\alpha$DHP-R 13, lanes 2, 4 and 6 with anti-gyrase B control ascitic fluid. $M_r$ markers as in fig.1.

was recognised by $\alpha$DHP-R 13 in both mouse and rat preparations as well as rabbit under nonreducing conditions. This similarity in reducing/nonreducing immunoblot profiles indicates that, although minor differences in subunit molecular mass exist (fig.2 and [5]), the structure of the skeletal muscle DHP receptor is very similar in each of the species investigated. As with the immunoblots of rabbit T-tubule membranes (fig.1) a diffuse region of nonspecific staining was present under nonreducing conditions on blots of all three mammalian microsomal preparations but absent from those of frog (see fig.2B). Due to this nonspecific staining, the presence of specific staining at $M_r$ 170000 in mouse and rat samples was not as clearly identified as in rabbit and frog samples. In these cases specific staining appeared as darker and broader bands in the 170 kDa region compared to control tracks (fig.2B).

Immunocrossreactivity between the rabbit skeletal muscle DHP receptor and microsomal components from a number of other rabbit tissues was also investigated. Heart and brain microsomes both contained a component of $M_r$ ~140000 under reducing conditions which was recognised specifically by $\alpha$DHP-R 13 (fig.3). It is noteworthy that in these two tissues the presence of DHP-sensitive $Ca^{2+}$ channels has been established by extensive ligand binding studies [1,2]. We were unable to detect this component in any other tissue examined, including liver (fig.3), kidney, lung and small intestine, which is not surprising in view of the low levels of specific DHP binding which have been reported in these cases [2].

In conclusion, this work provides evidence that the structures of the voltage-sensitive $Ca^{2+}$ channel DHP receptors, both in different species and in different tissues, are immunologically related. Our results are consistent with the hypothesis that the large 140 kDa polypeptide of the native DHP receptor is disulphide linked to a second small polypeptide component [9]. Furthermore, data obtained under nonreducing conditions indicate that additional components of the $Ca^{2+}$ channel protein remain to be resolved unequivocably.

The series of monoclonal antibodies described in this paper will provide useful tools, not only for future immunological studies of the DHP-sensitive $Ca^{2+}$ channel but also in purification studies of pharmacologically important $Ca^{2+}$ channels from heart and smooth muscle and for the isolation of $Ca^{2+}$ channel cDNA for primary sequence determination.

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