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Characterization of DHP binding protein in crayfish striated muscle

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The dihydropyridine calcium channel blocker, [^{3}H]PN 200-110, binds specifically also to crayfish muscle membranes, though with a binding capacity smaller than that measured with rabbit or human skeletal muscle membranes. [^{3}H]PN 200-110 binding proteins from the crayfish T-tubules were solubilized and purified on WGA Sepharose or extracted from gel. The purified protein has a molecular mass of approximately 190 kDa under nonreducing conditions and was able to transport calcium after reconstitution. Polyclonal antibodies against crayfish T-tubules enriched with purified DHP-binding protein were shown to bind to DHP-binding protein from both the crayfish and the rabbit skeletal muscle, although not with the same intensity. Electron microscopy showed the presence of ovoid particles. Our results suggest that a voltage-dependent calcium channel may be present in crayfish skeletal muscle, which is homological with the L-type calcium channel in rabbit skeletal muscle.

Crayfish; Calcium channel; Ca^{2+} antagonist

1. INTRODUCTION

The voltage-dependent DHP-sensitive Ca^{2+} channels found in vertebrate skeletal muscles, i.e. in rabbits [1], guinea pigs [2] and also in humans [3], play an important role as the voltage sensor for excitation-contraction (E-C) coupling [4]. Vertebrates are known to have sodium electrogenesis, in contrast to the Crustacea where the calcium electrogenesis is established. The calcium 'action potential' in invertebrates may play a role in direct activation of contraction [5]. The existence of voltage-dependent calcium channels in crayfish skeletal muscle was proved in a number of electrophysiological experiments (for review see [6]). The physiological evidence [7,8] has shown that the external calcium ions in the T-system of crayfish muscle fibres participate directly in the activation of contraction, in contrast with the situation in vertebrate striated muscle. The molecular mechanisms of the excitation-contraction coupling might thus be different in vertebrate and invertebrate muscle.

It was therefore of interest to examine the properties of the membrane molecular components of invertebrate skeletal muscle, which were suggested to play a crucial role in the transmission from T-tubules to the sarcoplasmic reticulum in vertebrate striated muscle [9]. Here we refer to the properties of dihydropyridine-sensitive Ca channels in crayfish striated muscle. Some of these results were communicated briefly at the XXXI Physiological Congress [10].

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Abbreviations: E-C, excitation-contraction; WGA, wheat germ agglutinin; Chaps, 3-(3-cholamidopropyl)-dimethyl-ammonio-1-propanesulfonate; DHP, dihydropyridine; SDS, sodium dodecyl sulphate

2. MATERIALS AND METHODS

T-tubule membranes were prepared according to Rosemblatt et al. [11]. Binding assays with [^{3}H]PN 200-110 were done as described [12]. The molecular weight of the DHP antagonist binding protein was estimated by the method of Ferry et al. [13] after covalent binding of [^{3}H]azidopine.

Polyclonal antibodies against crayfish T-tubules were prepared using the same procedure as that described for polyclonal antibodies against rabbit T-tubules [12]. All other methods used are described below.

2.1. *Purification of membrane glycoproteins on WGA-Sepharose*

Samples were prelabeled with [^{3}H]PN 200-110. The prelabeled membranes were centrifuged at 12000 × *g* to concentrate this and then solubilized in 50 mmol/l Hepes-NaOH, pH 7.4, 1 mmol/l $CaCl_2$, 1% Chaps for 30 min at room temperature. The solubilized samples were centrifuged repeatedly at 12000 × *g* to remove non-solubilized membranes, and the solubilized proteins were applied on a WGA-Sepharose column (Pharmacia, Sweden) (5 × 1 cm) equilibrated with 50 mmol/l Hepes-NaOH, pH 7.4, 1 mmol/l $CaCl_2$, 1% Chaps. Glycoproteins were eluted with the same buffer supplemented with 200 mmol/l *N*-acetyl-D-glucosamine (Serva, FRG). One ml fractions were collected, and radioactivity and protein content (by the method of Lowry et al. [14]) were determined in each fraction. The active fractions were subjected to electrophoresis in 8% polyacrylamide gels by the method of Laemmli [15]. The gels were silver stained according to Wray et al. [16].

2.2. *Purification of the α_1 subunit by extraction from gel*

The T-tubule fraction was first subjected to electrophoresis under nonreducing conditions in 8% gels. Some of the gels were silver stained to show the protein profile. The α_1 subunit was cut out of the re-

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maining gels and eluted on an Extraphor LKB apparatus. The running buffer was 50 mmol/l Hepes-NaOH, 50 mmol/l glycine, 0.1% SDS, pH 8.9, and the protein was eluted with 1 mol/l sodium chloride. The extraction was done for 1 h at 100 V. After extraction the protein was desalted by centrifugation through a Whatman ultrafilter and diluted in 100 μl of 50 mmol/l Hepes-NaOH and 1 mmol/l $CaCl_2$ and used either for electrophoresis or for the binding studies.

2.3. *Immunoblotting*

After SDS polyacrylamide gel electrophoresis, the gel was blotted on Hybond C nitrocellulose membrane (Amersham) for 24 h in buffer BT (12 mmol/l Tris-HCl, pH 7.0, 192 mmol/l glycine and 20% methanol). Blot was fixed in 5% dried milk in TBS buffer (20 mmol/l Tris base, 137 mmol sodium chloride and 1 mol/l hydrochloric acid, pH 7.6). After blocking and washing the membrane was incubated 1 h with polyclonal antibody prepared as described in [12]. All other operations (binding of biotinylated antibody, reaction with streptavidine alkaline phosphatase and staining with Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) were done with blotting detection kit for mouse antibodies from Amersham (UK).

2.4. *Reconstitution of extracted protein and calcium transport*

The reconstitution was done with an azolectin-protein ratio of 40:1. The mixture was solubilized in 1% Chaps. After solubilization, Chaps was removed on a Sephadex G-50 column. The vesicles were eluted with 20 mmol/l Tris-HCl, pH 7.4. The proteoliposomes were sonicated 2 × 30 s to obtain unilamellar particles.

The proteoliposomes were incubated with $^{45}Ca^{2+}$ in 0.5 mol/l K^+ and/or Na^+ solution for defined periods of time (1, 5, 10, 30 and 60 min). Then, free calcium was removed on CM-Sepharose. The radioactivity was subsequently measured on a Rackbeta counter (LKB).

2.5. *Electron microscopy*

One drop of the purified dihydropyridine receptor suspension was applied to the grid coated with a formvar film, stabilized by a carbon layer. After 5 min the drop was removed by touching the grid with a strip of filter paper.

Double staining. The sample was stained with 1% aqueous uranyl acetate for 30 min. The grid was then washed with several drops of the distilled water and then treated with 1% lead citrate for 3 min. The grid was washed again with distilled water. Excess solution was removed by touching with filter paper and the grid was dried under vacuum in the electron microscope. The grid was examined in the electron microscope JEM 1200 EX at 80 kV.

3. RESULTS

[^{3}H]PN 200-110 was observed to bind specifically to crayfish striated muscle T-tubule membranes. In the crude membrane fraction the specific activity was only 12.6 fmol/mg protein (Table I). This low binding is closer to that measured in heart muscle rather than to that in skeletal muscle. We tried to purify the DHP binding protein and to estimate its molecular weight. Affinity chromatography on WGA-Sepharose column has proved useful for the purification of the calcium channel protein from rabbit skeletal muscle [17] and was therefore used in the present work. After elution of the unbound proteins the glycoproteins were eluted with *N*-acetyl-D-glucosamine. The presence of the DHP binding protein in this eluate was demonstrated by determining the radioactive peak (Fig. 1). Similar elution profiles were obtained from rabbit skeletal muscle [1].

Table I

Purification table of α_1 subunit of calcium channel from crayfish striated muscle

Fraction	Protein (mg)	PN 200-110 binding		Yield (%)	Purification (-fold)
		Specific activity (fmol/mg)	Total activity (fmol)		
Crude membrane fraction	5.2	12.6	65.52	100	1
T-tubules	2.1	22.2	46.62	71.2	1.76
WGA-Sepharose	0.3	107.2	32.16	49.9	8.5
Extraction	0.003	2609.1	7.82	11.9	207.0

The protein fractions obtained from WGA-Sepharose column were prelabelled with azidopine and applied on 8% polyacrylamide gels. Electrophoresis and radioactivity measurements showed a band at M_w 180–195 kDa (Fig. 2). The specific activity of PN 200-110 increased to 107.2 fmol/mg protein (Table I), which corresponded to 8.5-fold purification. A more exact purification was done by the extraction of the [^{3}H]PN 200-110 binding protein from gel. The protein could bind PN 200-110; the specific activity was 2.6 pmol/mg protein (Table I). The molecular mass of this protein, as determined by SDS polyacrylamide gel electrophoresis after desalting, was 185–190 kDa (Fig. 3). This molecular mass is closer to that of cardiac α_1 subunit [18] than to that determined for the skeletal muscle α_1 subunit of the DHP-receptor. The protein purified in this way was able to transport calcium after reconstitution (Fig. 4). The transport was 1.5-times higher when BAY K8644 was used (not shown).

The homology of the α_1 subunits from rabbit skeletal and crayfish muscles was checked with polyclonal antibodies, although some previous works [19] showed

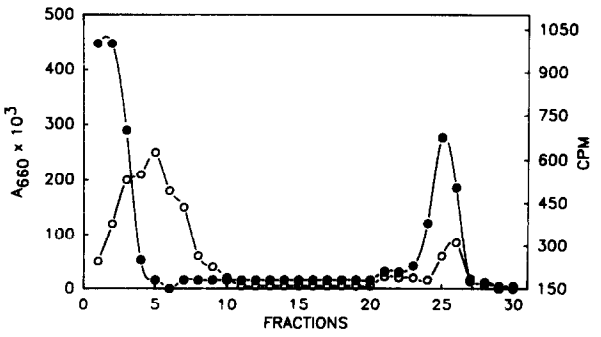

Fig. 1. Protein and radioactivity elution profiles of crayfish membrane proteins during purification on WGA-Sepharose. For details see section 2. Elution of glycoproteins with *N*-acetyl-D-glucosamine started from fraction 20. The filled circles represent the radioactivity profile, and the empty ones the protein elution profile. The first radioactivity peak corresponds to unbound radioactivity, the other one to specific radioactivity bound to proteins from crayfish muscle membranes.

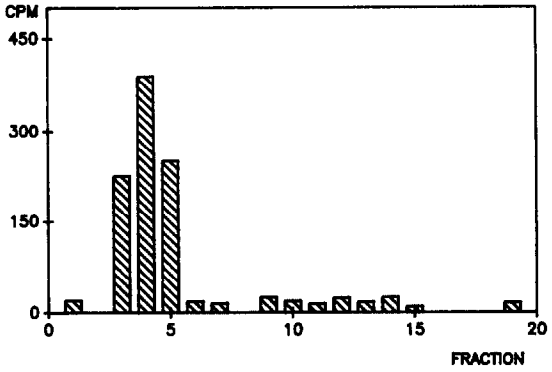

Fig. 2. Determination of the molecular mass of the DHP binding protein in crayfish striated muscle. Specific binding of the dihydropyridine arylazidoderivate, azidopine, appeared in fractions 3, 4 and 5 after cutting the gel into 0.5 cm slices. These fractions correspond to molecular masses of 185–195 kDa, as determined with the high molecular mass calibration kit (Pharmacia).

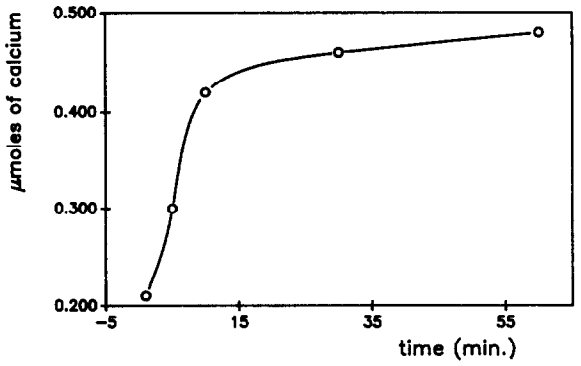

Fig. 4. The protein extracted from gel was reconstituted into azolectin vesicles and the $^{45}Ca^{2+}$ transport was measured. The curve represents the time dependence of μmoles of calcium transported into proteoliposomes. This transport can be stimulated approximately 1.5-times by addition of Bay K8644 (not shown).

that monoclonal antibodies against Ca^{2+} antagonist receptor from rabbit skeletal muscle did not bind to membranes from crayfish muscle. Polyclonal antibodies against crayfish muscle T-tubules enriched with purified α_1 subunit were prepared and tested for crossreactivity with the DHP binding protein from rabbit skeletal muscle. The immunoblot showed that mouse polyclonal antibodies against crayfish T-tubule membranes bound both to the crayfish and the rabbit protein (Fig. 5), although not in the same manner. The polyclonal antibodies reacted with the protein spectrum of crayfish T-tubules, while only with a few proteins of rabbit skeletal T-tubules. However, these antibodies bound to the 165 kDa protein from rabbit skeletal muscle and to the 190 kDa protein from crayfish muscle.

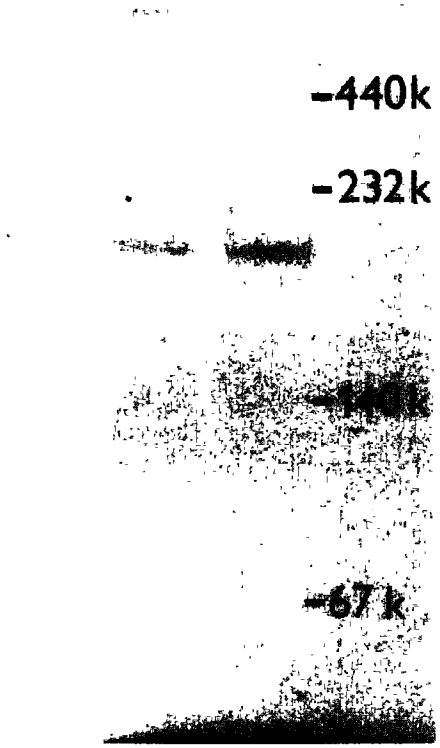

Fig. 3. Electrophoresis of the DHP-binding protein after extraction from gel. The protein was desalted and electrophoresis was done according to Laemmli (1971) in 8% gel. After silver staining the 190 kDa protein appeared. This protein could bind PN 200-110 (Table I).

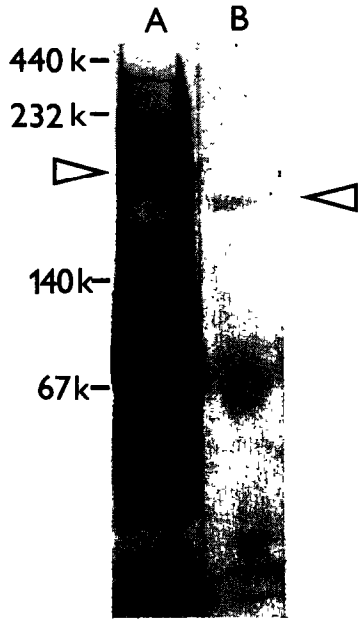

Fig. 5. Anti-crayfish polyclonal antibodies were used for immunoblot with crayfish (A) and rabbit (B) T-tubules. The antibodies bound to a wide range of proteins from crayfish T-tubules (A), while only to some few proteins from rabbit T-tubules (B). However, they bound to the 190 kDa crayfish muscle protein and to the 165 kDa rabbit muscle protein, which correspond to the α_1 subunits of the crayfish and rabbit skeletal muscle, respectively.

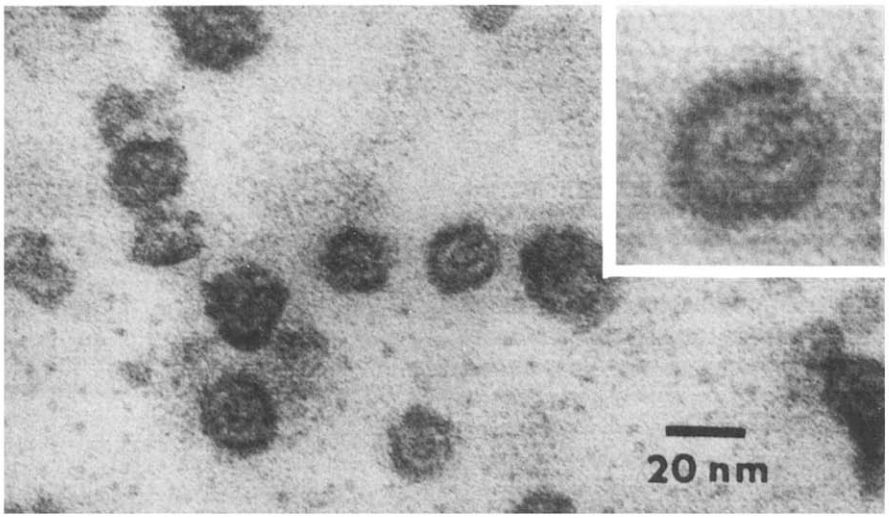

Fig. 5. Electron micrograph of the crayfish DHP receptor purified on WGA-Sepharose. The positive image shows the structure of the receptor, probably the ovoid particle (16 × 23 nm). The bar is 20 nm.

From all these experiments it is clear that a receptor/protein is present in crayfish muscle which can bind the dihydropyridine antagonist PN 200-110; this protein is homological with the Ca^{2+} antagonist receptor from rabbit skeletal muscle. Electron microscopy (Fig. 6) of the purified fraction revealed ovoid particles similar to those observed by Campbell et al. [20].

4. DISCUSSION

The existence of voltage-dependent calcium channels in crayfish striated muscle was described previously in several physiological works [21,22]. The presence of L-type of calcium channel was proved on internally perfused muscle fibre segments [22,23]. Verapamil partially inhibited calcium currents [23] while the effect of nifedipine was dependent on its concentration [24]. The calcium channels in this species have not been characterized biochemically as yet.

Based on the data obtained, we can conclude that crayfish muscle contains the L-type of calcium channel which differs from that in rabbit skeletal muscle by the binding capacity of dihydropyridine antagonists, by the molecular mass of α_1 subunit and also by the reactivity with polyclonal antibodies against crayfish T-tubules.

Many of these characteristics (molecular mass of approx. 190 kDa, binding capacity of PN 200-110, a.o.) are closer to those of the α_1 subunit from heart muscle [18]. It is to be noted in this connection, that the invertebrate striated muscle and the heart muscle of vertebrates are both using the external calcium ions in the activation of contraction.

Crayfish has been the species most distant from vertebrates, in which the presence of the DHP receptor has been proved. This receptor was not found in protozoans (*Paramaecium*) [25] or in insects (*Drosophila melanogaster*) [26,27]. An analysis of the species which confirmed presence of the DHP receptor suggests that this receptor is mostly associated with the T-tubular system. The existence of the α_1 subunit in smooth muscles [28,29] introduced uncertainty into the issue, although it has been known [30] that the two proteins are not identical despite their homology.

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