The Dihydropyridine Receptor: Expression of 190 kD α_1 Subunit in Crayfish Muscle

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Abstract. The existence of dihydropyridine receptor in crayfish striated muscle was proved by Northern blot analysis and ^{3}H PN 200—110 binding. The alphal subunit is encoded by a 8300 nt mRNA population and is expressed as 190 kD protein in crayfish T-tubular system, which binds ^{3}H PN 200—110 (B_{max} 1.5 ± 0.4 pmol/mg protein and K_D 6.2 ± 0.8 nmol/l). The purified protein is phosphorylated by cAMP-dependent protein kinase. The dihydropyridine receptor in crayfish striated muscle also contains alpha 2 subunit, which on Northern blot gives the same signal as the alpha 2 subunit from rabbit skeletal muscle.

Key words: Dihydropyridine receptor — Crayfish striated muscle — Alpha 1 subunit expression

Introduction

The dihydropyridine (DHP) receptor is considered to play an important role in excitation-contraction coupling. The missing of the receptor alpha1 subunit, which in the skeletal muscle forms the L-type of calcium channel, has been shown to result in destroyment of the coupling process (Tanabe et al. 1988; Knudson et al. 1989). The primary structure and function of the DHP receptor was recently well studied in skeletal muscle (Tanabe et al. 1987; Morton et al. 1988; Ruth et al. 1989; Ellis et al. 1988), heart (Mikami et al. 1989; Schneider and Hofmann 1988; Slish et al. 1989), smooth muscles (Somlyo and Himpens 1989) of mammals, and the DHP receptor was also found in neural and secretory cells (Reynolds et al. 1986; Armstrong and Matteson 1985).
The DHP receptor seems to be a fylogenetically old transmission system which has some common features with both chemical and voltage operated structures

in muscle cell membranes. L-type of calcium channel has been suggested to act as a voltage sensor in excitation-contraction coupling, through triggering the signal for calcium release from the sarcoplasmic recitulum (Rios and Brum 1987). The chemical mode of modulation of this receptor is not yet well established, because of little information about the functional meaning of the alpha2, beta and the gamma subunit. The only known facts are that the alpha1 subunit is phosphorylated by cAMP-dependent protein kinase (Tanabe et al. 1987); the beta subunit has phosphorylation sites for cGMP and cAMP-dependent protein kinases, and for casein kinase II (Ruth et al. 1989), and that channel functions can be modulated via G proteins.
Therefore, it is reasonable to study the subnit composition of DHP receptors from several types of tissues differing in the regulation mode. The present work deals with crayfish striated muscle. This muscle operates on the pure calcium electrogenesis principle, and the influx of calcium is unavoidable for contraction (Henček and Zachar 1977; Zahradník and Zachar 1983). It was of interest, whether the DHP receptor in this type of tissue is also reduced to only alpha1 subunit, whether it contains some other subunits.

Materials and Methods

The isolation and purification of alpha1 protein was done as described by Križanová et al. (1990a). Binding of ^{3}H PN 200—110 was done according to Križanová et al. (1990b), B_{max} and K_D values were estimated from Scatchard plots.

Phosphorylation: Protein (50—100 μg of purified alpha1 subunit) was phosphorylated by 2U of cAMP-dependent protein kinase (Sigma) in 40 nmol/l HEPES pH 6.87, 10 nmol/l $MgCl_2$, 100 nmol/l $CaCl_2$, 100 nmol/l DTT, and ^{32}P gamma ATP (1mCi/nmol) at 30°C for 30 minutes. The reaction was stopped by adding SDS to a final concentration of 1% and boiling the samples for 2 minutes. The samples were applied on 8 % SDS polyacrylamide gel. After the run was finished, the gel was cut into 5 mm tripes and the radioactivity of each stripe was measured.

Isolation of mRNAs: Cytoplasmic RNAs were isolated from rabbit skeletal muscle, heart, lungs, brain and crayfisch striated muscle according to Chirgwin et al. (1979). The polyadenylated mRNAs were purified on the oligo (dT) cellulose column.

Northern blot analysis: The polyadenylated mRNAs (10 to 25 μg) were denatured with 1 mol/l glyoxal and 50 % dimethyl — sulphoxide, electrophorezed in 1.5 % agarose gels and transferred to Hybond N+ membranes (Amersham). Random prime-labeled hybridization probes were: 2134 nt Eco RI / Eco RV fragment from rabbit skeletal muscle cDNA (Tanabe et al. 1987) was used for alpha1 subunit; 3870 nt Eco RI / Eco RV fragment from pSPCa1 plasmid with cDNA for alpha2 subunit (sequence according to Ellis et al. 1988).
The hybridizations were performed in 50 % formamide at 42 °C, in the case of crayfish mRNA blots at 38 °C for 24 hours. The membranes were then washed twice in 2 × SSC and 0.1 % SDS at room

Table 1. Comparison of B_{max} and K_D values obtained for crayfish and mammalian tissues.

Tissue	Species	K_D (nmol)	B_{max} (pmol/mg prot.)	Antagonist	Citation
Heart	human	0.31 ± 0.0	161 ± 5.3	nitrendipine	(Finkell et al. 1988)
	guinea-pig	0.25	0.37	nimodipine	(Glossman et al. 1985)
Brain	rat	0.49 ± 0.1	0.56 ± 0.004	nitrendipine	(Finkell and Tallman 1987)
	guinea-pig	0.62 ± 0.08	0.570 ± 0.099	nimodipine	(Reynolds et al. 1986)
	rabbit	0.15	0.47	PN 200—110	(Desnuelle et al. 1985)
	rabbit	0.45	0.48	nitrendipine	–"–
	rabbit	1.04	1.53	nimodipine	–"–
Skeletal muscle	human	0.2	7.4	PN 200—110	(Romey et al. 1989)
	mouse	0.35	0.52	PN 200—110	(Dunn 1989)
	rabbit T	0.28 ± 0.04	62.6 ± 6	PN 200—110	(Higo et al. 1988)
	rabbit	0.46	3.4	PN 200—110	(Densuelle et al. 1985)
	rabbit	15.5	10.8	nimodipine	–"–
	quinea-pig	2.0	1.77	nimodipine	(Dacquet et al. 1988)
	rabbit P	11.2	2800	PN 200—110	(Wibo et al. 1988)
	crayfishCM	5.2 ± 1.3	0.34 ± 0.07	PN 200—110	present work +
	crayfish T	6.2 ± 0.8	1.5 ± 0.4	PN 200—110	present work +
Smooth	rat vas deferens	0.31 ± 0.02	0.097 ± 0.007	nimodipine	(Ferry et al. 1985)
	equine portal vein	0.09 ± 0.03	0.045 ± 0.015	PN 200—110	(Been 1989)

T — T-tubules
CM — crude membrane fraction
P — purified
\+ — mean values ± SEM of at least 3 measurements, each run in 3 parallel experiments, are shown

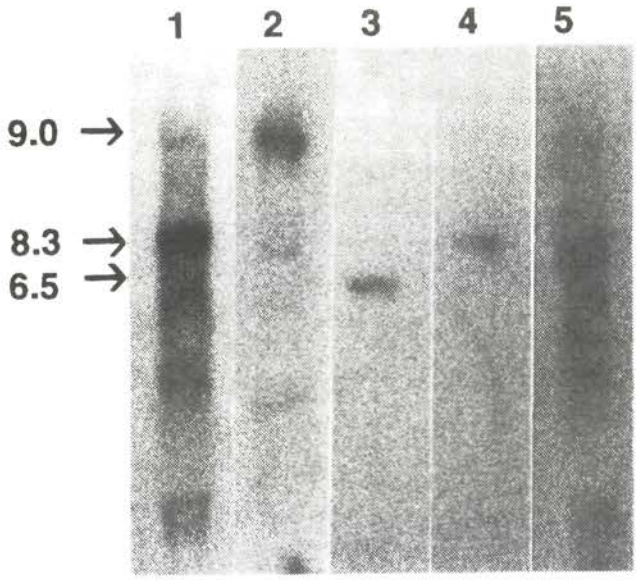

Figure 1: Northern blot analysis of mRNAs from various tissues with alpha1 cDNA probe from rabbit skeletal muscle.
Lanes: 1-rabbit lungs, 2-rabbit brain, 3-rabbit skeletal muscle, 4-crayfish striated muscle, 5-rabbit heart. The lenght was estimated according to positions of 28S and 18S ribosomal RNAs in the gel.

temperature, twice in 1 × SSC and 0.1 % SDS at room temperature. The last wash was done in 0.1 × SSC and 0.1 % SDS at 50 °C for 10 minutes. In the case of crayfisch mRNA blots, the last wash was performed at 42 °C for 10 minutes. The membranes were then autoradiographed from 24 hours to one week.

Results and Discussion

Available information about DHP receptors does not allow full evaluation of their role in the muscle, neuronal and sectory cells. The best studied functions are connected with L-type calcium channel, represented by alpha1 subunit (Catterall 1988; Catterall et al. 1988; Flockerzi et al. 1986). Nevertheless, it was shown that only 5 % of DHP binding sites in rabbit skeletal muscles act as calcium channels (Schwartz et al. 1985); the functional meaning of another sites and other subunits can only be assumed. The possibility that what is commonly known as DHP receptor in reality represents a spectrum of homologic proteins associated in various tissues in a different way, has recently been supported by several works. The alpha1 subunits from skeletal muscle, heart and smooth muscle are highly homologous, though not identical proteins. It was also shown,

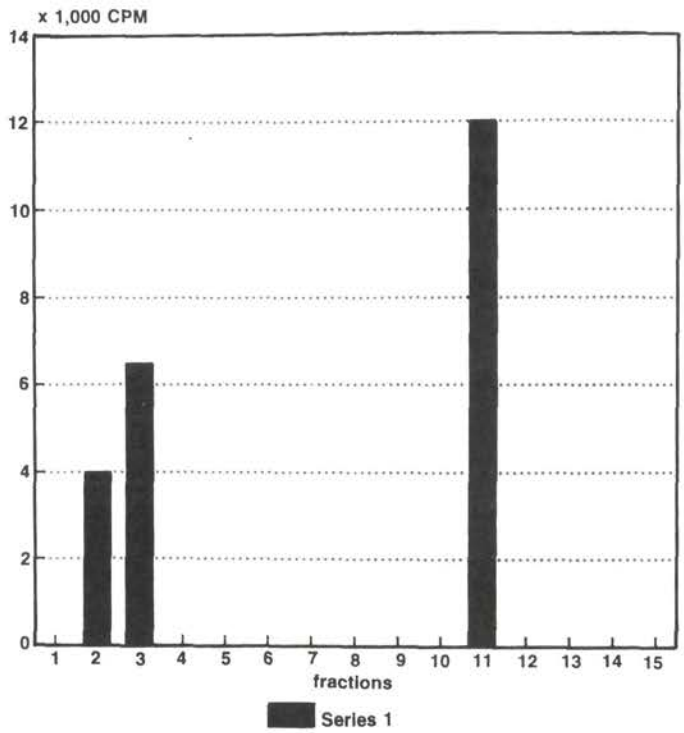

Figure 2: cAMP-dependent phosphorylaltion of crayfisch alpha1 subunit. The protein with molecular weight of approximately 190 kD was phosphorylated specifically with cAMP-dependent protein kinase (fractions 2 and 3). Autophosphorylation of cAMP protein kinase was observed in fraction 11.

that probably due to posttranslational modifications two different forms of alpha1 subunit exist in mammalian skeletal muscle (DeJongh et al. 1989). First, we tried to identify, whether the DHP binding protein (Križanová et al. 1990a) is homologous to alpha1 subunit from mammalian tissues. Fig. 1 shows a Northern blot analysis with signals for alpha1 subunit in various tissues. The 1324 nt probe derived from the coding sequence of the skeletal muscle alpha1 subunit hybridized to one poly $(A)^+$ mRNA species of skeletal muscle sized 6500 nt. At least two positive populations of RNAs are in the heart, lungs and brain at about 8300 nt and 9000 nt. The polyadenylated RNAs from crayfish muscle showed only one band at 8300 nt. This signal was obtained from 25 μg of mRNAs, whereas from skeletal muscle, heart and smooth muscles the signal

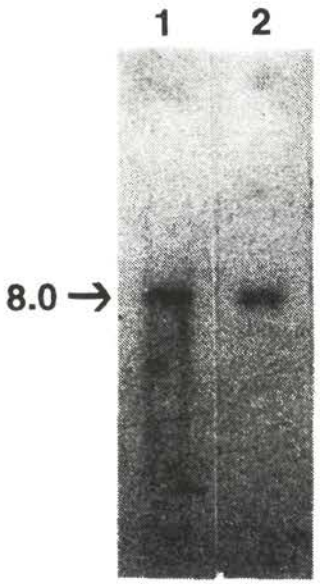

Figure 3: Northern blot analysis of crayfish striated muscle mRNAs with a probe derived from rabbit muscle alpha2 cDNA.
Lanes: 1-rabbit skeletal muscle mRNAs, 2-crayfish striated muscle. In both cases the same 8000 nt band appeared.

was readable from 5 and 10 μg of material. The low density of DHP receptor alphal subunits in crayfish muscle is also documented by low B_{max} values for PN 200—110, which are ten times smaller for T-tubular preparation (1.5 pmol/mg prot.) and also for crude membranes (0.34 $\pm$ 0.07 pmol/mg prot.) as compared with rabbit skeletal muscle. The DHP binding protein from the crayfish T-tubular system is phosphorylated by cAMP-dependent protein kinase (Figure 2), and has a M_r of 190 kD in SDS polyacrylamide gel under non-reducing conditions, which remains unchanged during purification steps with high salt solution. This value is intermediate between those reported for the two forms (170 kD and 212 kD) in rabbit skeletal muscle (DeJongh et al. 1989), and is closer to the cardiac alpha1 subunit (Schneider and Hofmann 1988). The role of alpha2 subunit in the DHP receptor has recently been discussed with respect to its primary structure which is not homologic with any other protein. Also, no information is available about its physical and functional connection with other receptor subunits. First evidence about the possible role of the alpha2 subunit has come from Mikami et al. (1989). Coexpression of cardiac alphal subunit with alphal subunit in *Xenopus leavis* oocytes resuled in an increase of the peak

inward current for barium ions. The Northern blot analysis of crayfisch muscle mRNAs (Fig. 3) shows a signal for the alpha2 subunit, identical with that for the rabbit skeletal muscle (8000 nt).

This signal was also obtained from 25 μg of mRNAs under less stringent hybridisation conditions than those used for the alpha1 subunit (see Materials and Methods).

The results show that crayfish striated muscle contains at least alpha1 and alpha 2 subunits homologous to the respective proteins from mamalian muscles. Nevertheless, the binding properties for dihydropyridines and the relative molecular weights are closer to respective values reported for the cardiac DHP receptor than for the skeletal alpha1 subunit. The low density of DHP receptors in crayfish T-tubular system and the existence of the calcium release channel in this tissue (Zachar and Zacharová 1989) indicate that, also in muscles with pure calcium electrogenesis, the primary function of the L-type of calcium channel is not to transport calcium for muscle contraction but to trigger the signal for calcium release from sarcoplasmic reticulum.

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