The expression of isoform-specific dihydropyridine receptor Ca$^{2+}$ channel (DHPR) $\alpha_1$-subunit genes in rat diaphragm, soleus and extensor digitorum longus muscles was investigated using RNase protection assays. As expected, mRNA expression levels for the DHPR skeletal muscle isoform were highest in extensor digitorum longus. Unexpectedly, both diaphragm and soleus expressed mRNA for the cardiac isoform at a significant level. Moreover, immunohistochemical experiments provided evidence of the cardiac DHPR isoform at the protein level in muscle fibres. The presence of the cardiac DHPR in the soleus and diaphragm is consistent with a degree of reported cardiac-like excitation-contraction coupling in these muscles, and may be an explanation for some of the therapeutic effects of theophylline in asthmatics, but is likely to serve some other role(s) as well.

**Key words** Cardiac muscle · Diaphragm · Dihydropyridine receptor · Extensor digitorum longus · Immunocytochemistry · Skeletal muscle · Soleus · L-Type Ca$^{2+}$ channels

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**Introduction**

Two Ca$^{2+}$ channels play a major role in skeletal muscle excitation-contraction (EC) coupling: the dihydropyridine receptor Ca$^{2+}$ channel (DHPR) present in the transverse (or $t$) tubule and the ryanodine receptor (RyR) of the sarcoplasmic reticulum terminal cisternae. The DHPR is an oligomeric complex composed of five protein subunits [6]. The 170-kDa $\alpha_1$-subunit serves dual functions, firstly as a voltage sensor that induces Ca$^{2+}$ release from the sarcoplasmic reticulum via activation of the RyR [31] and secondly as a slowly activating L-type Ca$^{2+}$ channel [2, 30, 34, 35].

Skeletal muscle contraction is not believed to be appreciably dependent upon the entry of extracellular Ca$^{2+}$ [22]. In contrast, the entry of extracellular Ca$^{2+}$ through L-type Ca$^{2+}$ channels is necessary for the initiation and maintenance of the heart contractile response, triggering a larger release of Ca$^{2+}$ ions from the sarcoplasmic reticulum [5]. Some of the discrepancies between skeletal muscle and heart EC coupling have been explained by structural differences of the DHPR at the molecular level. Phenotypic expression of cardiac- or skeletal-type EC coupling depends upon whether a cardiac or a skeletal isoform of the $\alpha_1$-subunit of the DHPR is present [37].

We have recently reported that chronic stimulation of skeletal muscle, which is responsible for fast- to slow-twitch transformation, affects expression of mRNA encoding the isoform-specific DHPR $\alpha_1$-subunit [26]. Diaphragm (DIA) muscle is an example of skeletal muscle that undergoes spontaneous chronic activity, and both DIA and soleus (SOL) twitch tensions have been reported to be reduced under conditions where Ca$^{2+}$ entry would be reduced [11, 12, 19, 21, 38, 39]. We investigated here the expression of genes encoding the $\alpha_1$-subunits of the skeletal and cardiac DHPR isoforms in DIA compared with that in SOL and extensor digitorum longus (EDL) muscles, which are typical slow- and fast-twitch muscles, respectively. We observed a reduced expression of skeletal DHPR mRNA in DIA and SOL versus EDL, but also significant expression of the cardiac DHPR gene in DIA and SOL. In addition, we used immunohistochemical techniques to demonstrate the presence of the cardiac DHPR isoform at the protein level in skeletal muscle. Some of these results have previously been reported in abstract form [27].
Materials and methods

RNA isolation and analysis

Experiments were carried out on DIA, EDL and SOL muscles from male adult rats weighing approximately 300 g. Animals were heavily anaesthetized by sodium pentobarbital (50 mg/kg ip, Abbott, Chicago, Ill., USA), and muscles were removed for RNA isolation. In certain animals, the heart was also removed for the same purpose. Total cellular RNA was isolated separately from each muscle sample using guanidinium isothiocyanate (RNAzol B kit, Biotex, Houston, Tex., USA). The RNase protection assay (RPA) was used in the present study to assess mRNA expression levels, as it provides a greater sensitivity than Northern blots. Antisense RNA probes were produced using T7 polymerase (Stratagene, La Jolla, Calif., USA) on linearized 1fb, C32, GAPDH or actin templates in the presence of [α-32P]rUTP (DuPont NEN, Boston, Mass., USA). RPAs were carried out using the RPA II Kit (Ambion, Austin, Tex., USA) and require Triton X-100 (1%) dissociation to unmask its epitope

Antibodies

Two Ca2+ channel primary antibodies directed against the α1-subunit of the channel were used for this study: anti-αTC (anti-CNC1 [10, 15]) and anti-pan α1 (anti-CP-[1382–1400]) [1]. Both antibodies were obtained from Alomone Labs, Jerusalem, Israel.

Western blots

In order to ascertain whether the anti-αTC antibody cross-reacts with the skeletal isoform of the DHPR or is specific for the cardiac isoform of the DHPR, Western blots were performed with crude membrane preparations from rat heart and rat skeletal muscle.

Crude cardiac membranes were prepared by homogenizing 0.65 g of minced rat thigh skeletal muscle in 1 vol of 0.3 M sucrose, 10 mM imidazole, pH 7.4 with a Tissue-Tearor handheld homogenizer. One such preparation was prepared in the absence of protease inhibitors, and a second was prepared in the presence of 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM leupeptin, 100 nM aprotonin, and 1 μM pepstatin during homogenization and through all subsequent steps, including resuspension and storage. Additional homogenization medium was added to the suspension and it was centrifuged at 4000 g for 10 min to remove debris. The supernatant was then centrifuged at 300,000 g for 60 min to yield crude rat cardiac membranes.

Crude skeletal muscle membranes were prepared by homogenizing 1.5–6 g of minced rat thigh skeletal muscle in 5 vols of 0.3 M sucrose, 10 mM imidazole, pH 7.4 with a Tissue-Tearor homogenizer. As with cardiac membranes, separate preparations were made with and without the protease inhibitors listed above present in all solutions. The homogenate was centrifuged at 11,000 g for 10 min, and the supernatant set aside. The pellet was rehomogenized in 5 vols of homogenization medium and centrifuged at 14,500 g for 15 min. The supernatants from the two separate homogenizations were then pooled and centrifuged at 300,000 g for 60 min to yield crude rat skeletal muscle membranes.

Protein concentrations were determined using a Coomassie Blue G-250 protein reagent (Pierce no. 22300; Rockford, Ill., USA).

Western blots were performed by applying 25 μg of each crude membrane preparation to a 7.5% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel. The fractionated proteins were then transferred to a PVDF membrane and probed with primary antibodies (anti-αTC at 1:200 dilution or anti-pan α1 at 1:300 dilution) followed by a secondary antibody [goat anti-rabbit immunoglobulin G (IgG) at 1:2500 dilution], and finally detected by enhanced chemiluminescence using an ECL detection kit (Amersham). To strip the blot for reprobing, the blot was incubated at 50°C in stripping buffer (62.5 mM Tris/HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS) for 25 min and washed in TBS 2×10 min at room temperature. The blot was then used for reprobing.

Immunohistochemistry

To corroborate cardiac DHPR expression at the protein level and to attempt to localize the cell types involved in its production in skeletal muscle, indirect immunohistochemistry was carried out with the two Ca2+ channel primary antibodies directed against the α1-subunit of the channel on sections of the DIA, SOL and EDL muscles. After deep anaesthesia was induced by sodium pentobarbital, rats were intracardially perfused with 200 ml saline at 37°C followed by 1 l of ice-cold 4% buffered paraformaldehyde. The EDL, SOL and DIA muscles, as well as heart were removed and postfixed at 4°C for 2 h in the same fixative, followed by storage in 30% sucrose in 0.1 M phosphate buffer (PB) solution overnight at 4°C for cryoprotection. Subsequently, tissue samples from each muscle type were prepared for paraffin sections. The tissue was washed several times in 0.1 M PB followed by dehydration with graded alcohol solutions, clearing in xylene, immersion in paraffin by an automatic processor, and then embedding in paraffin. Two sets of paraffin sections at 4 μm thickness were cut and mounted on coated slides. Cross and longitudinal sections from the muscles of EDL, SOL and DIA and random sections from the heart were cut and mounted directly onto gelatin-coated glass slides. After air-drying, the sections were deparaffinized with xylene and hydrated with graded alcohol solutions and PBS. Before routine immunocytochemical staining, the sections were pretreated with 10 mM citrate, pH 6.0 in a 98°C oven for 20 min, followed by washing with 0.1 M PB for 10 min (6 changes), then incubated at room temperature overnight in polyclonal rabbit anti-αTC (1:200) for one set of slides. A second set of slides was incubated in polyclonal rabbit anti-pan-α1 (1:200) to serve as a negative control stain. This antibody was not specifically recommended for immunocytochemistry because its epitope is masked by α2 and δ chains in situ and requires Triton X-100 (1%) dissociation to unmask its epitope.