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Developmental regulation of expression of the α_1 and α_2 subunits mRNAs of the voltage-dependent calcium channel in a differentiating myogenic cell line

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The voltage-dependent calcium channel (VDCC) in skeletal muscle probably plays a key role in transducing membrane charge movement to the calcium release channel. We report here that the expression of VDCC α_1 and α_2 mRNAs is developmentally regulated in differentiating C2C12 myogenic cells. The α_1 mRNA is not detectable in the myoblast form of C2C12 cells while its expression is induced 20-fold in differentiated myotubes. In contrast, the α_2 mRNA is weakly expressed in myoblasts but is also induced upon myogenic differentiation.

Ca^{2+} channel, voltage-dependent; mRNA; Developmental expression; Subunit, α_2; Subunit, α_1; (C2C12 myogenic cell)

1. INTRODUCTION

The induction of cellular processes leading to myoblast differentiation is closely coupled to cessation of cell division [1]. The deprivation of serum mitogens such as PDGF, EGF, FGF or TGF-β [2,3] is a prerequisite for the terminal commitment. The differentiation process is well-characterized by the deregulation of myoblast type gene products, such as β-, γ-actin [2] and concomitantly by induction of muscle-specific genes (α-actin, ACh receptor, creatine kinase, Na,K-ATPase α_2 isoform [5] and components of the contractile apparatus (for review see [4]).

There is considerable evidence that excitation-contraction coupling in skeletal muscle T-tubule membranes, requires activation of a specific L-type voltage-dependent calcium channel (VDCC), which has been called a 'dihydropyridine receptor' [6–9]. The VDCC consists of a large 165 kDa subunit α_1, which contains the receptors for the calcium antagonists, a glycoprotein of about 150 kDa, α_2 and several smaller subunits [21]. Rios et al. [6] have demonstrated that depolarization of the T-tubule is converted into intramembrane charge movements by the VDCC, α_1 subunit which probably undergoes voltage-driven conformational changes that in turn causes calcium release channels to open. This sequence of events is also consistent with the absence of excitation–contraction coupling in skeletal muscle of dysgenic mice [10,11]. This developmental abnormality is associated with diminished expression of the α_1 subunit of the VDCC and an altered coupling between T-tubules and sarcoplasmic reticulum [12]. This defect can be reversed either by coculturing with spinal cord cells, which leads to formation of normal T-tubule and sarcoplasmic reticulum coupling [12], or by microinjecting these cells with an expression plasmid encoding the α_1 subunit [13].

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; TGF-β, tumor growth factor-β; ACh, acetylcholine; VDCC, voltage-dependent calcium channel; SSPE, 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4

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These observations taken together, clearly prove the essential role of the α_1 subunit of the VDCC in the excitation-contraction coupling of skeletal muscle and suggest that expression of this protein complex may be developmentally regulated in the muscle-differentiation process. This paper shows that differentiating murine C2C12 myogenic cells express mRNAs for α_1 and α_2 subunits of the voltage-gated calcium channel in a developmentally regulated manner.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Conditions for culturing of mouse C2C12 myoblast line and induction of myotube formation were essentially the same as described by Blau et al. [14]. Briefly, myoblasts were maintained in an undifferentiated form in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Gibco) and 0.5% chicken embryo extract (Gibco). In order to induce myotube formation and fusion to multinucleated fibers, confluent cultures of myoblasts were cultured in DMEM and 2% horse serum. 2–3 days were required to reach the formation of muscle fibers; the progress of the differentiation procedure was routinely followed by reverse phase microscopy.

2.2. *RNA preparation, electrophoresis and hybridization*

Total cellular RNA was isolated at different time points of the differentiation process according to the acid guanidium thiocyanate method of Chomczynski and Sacchi [15]. RNA samples (10 μg each) were denatured with formaldehyde, fractionated on 1.2% formaldehyde-agarose gel [16] and transferred onto Nytran membranes (Schleicher and Schuell) by capillary transfer, baked, prehybridized and hybridized according to the manufacturer's protocol. Following hybridization with rabbit skeletal muscle voltage dependent calcium channel α_1 and α_2 subunit-specific probes, filters were washed with 0.5 × SSPE and 0.1% SDS at 45°C for 1 h. For other probes washings were done at a stringency of 0.1 × SSPE and 0.1% SDS at 55°C.

2.3. *Probes and labeling of probes*

A 4.3 kb *Eco*RI/*Sac*I and a 3.4 kb *Eco*RI fragment from VDCC α_1 and α_2 cDNA clones, respectively [17,18], were used as subunit-specific probes for skeletal muscle VDCC mRNAs. A 1.1 kb *Pst*I fragment of rat β-actin cDNA which hybridizes to the cytoplasmic β- and γ-actin mRNAs as well as the muscle-specific α-actin mRNA, was used to monitor muscle cell differentiation [2]. To monitor the amount of RNA loaded on the Northern blots, the RNA samples were quantitated by hybridizing with a 30-mer synthetic oligodeoxynucleotide probe, complementary to the 18 S mouse ribosomal RNA. The probe was prepared with an automatic DNA synthesizer (Applied Biosystems). DNA probes were labeled by random priming (Pharmacia) according to the method of [19]. The oligodeoxynucleotide probe was end-labeled with T4 polynucleotide kinase [20] and purified with an Elu-tip (Schleicher and Schuell) according to manufacturers protocol.

3. RESULTS AND DISCUSSION

Cultures of proliferating C2C12 myoblasts were kept for 2 days in DME + 20% fetal bovine serum + 0.5% chicken embryo extract. When the cells reached about 90% confluence they were shifted to DME + 2% horse serum to induce the formation of differentiated myotubes. Myoblasts in control cultures were present as single, mononucleated cells, whereas myoblasts in differentiating medium started to fuse on day 1 and about 90% of nuclei were in myotubes by days 3–4.

To examine muscle-specific expression of VDCC α_1 and α_2 subunits during differentiation, total cellular RNA was isolated and size-separated on denaturing formaldehyde-agarose gels. The RNA blots were hybridized with α_1 and α_2 specific cDNA probes.

Fig.1a shows that in control myoblasts the VDCC α_1 subunit specific mRNA is not expressed at all or the expression is below the level of detection for this technique. When myoblasts start to fuse and form myotubes, the α_1 mRNA is induced (at 36 h) and accumulates to high levels (20-fold induction) between 60 and 96 h of myotube differentiation. In contrast to α_1 expression, a barely detectable level of α_2 mRNA was found in non-fused myoblasts, the amount increases upon myotube differentiation and reaches high levels (10-fold induction) between 36 and 96 h of myotube differentiation (fig.1b).

The myoblast differentiation process also was monitored by measuring the α-, β- and γ-actin gene transcripts with a cross-hybridizing β-actin cDNA probe. The results are presented in fig.1c. The cytoplasmic β/γ-actin mRNAs are prevalent in myoblasts and they were deinduced during differentiation, whereas α-actin transcripts are formed only after myotube formation begins. Moreover, the α-actin transcript is dominant in later stages of differentiation and in total RNA taken from mouse skeletal muscle tissue.

To monitor the amount of RNA samples loaded onto each slot, the blots were hybridized with a 30-mer oligonucleotide probe complementary to the 18 S mRNA (fig.1d). The results show similar levels of RNA for each sample analyzed.

Results of this study demonstrate that mRNAs for α_1 and α_2 subunits of voltage-gated calcium channel are expressed in a developmental fashion

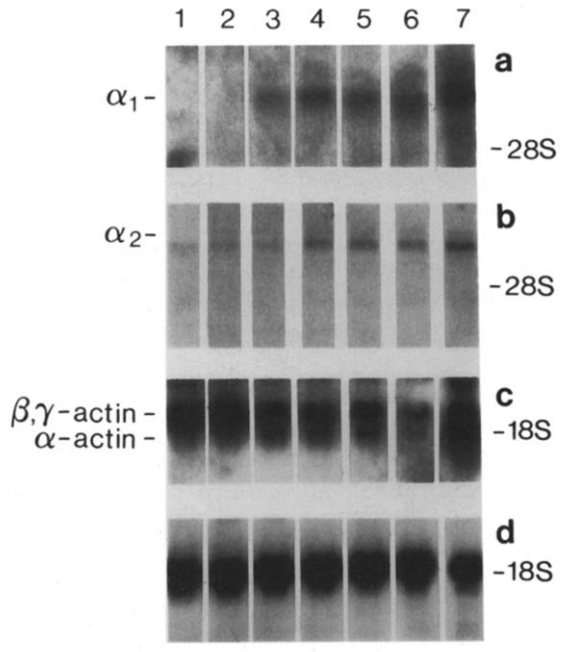

Fig.1. Expression of skeletal muscle voltage-dependent calcium channel α_1 and α_2 subunit mRNAs in myoblasts and differentiating myotubes of C2C12 cells. Lane 1: myoblasts at 48 h in growth medium; lanes 2–6: myotubes in differentiating medium at 12, 36, 60, 72 and 96 h, respectively; lane 7: mouse skeletal muscle RNA. Blots a and b were probed with rabbit skeletal muscle VDCC α_1 and α_2 subunit-specific cDNAs, respectively. The same blots were also hybridized with cross-hybridizing rat β-actin probe (c) or with 18 S rRNA oligonucleotide probe (d).

in differentiating C2C12 myogenic cells. The mRNA for VDCC α_1 subunit is not detectable in undifferentiated myoblasts, but is induced 20-fold during the terminal differentiation process. However, the non-committed myoblasts express a relatively low level of α_2 mRNA and upon differentiation the levels of this mRNA species were also increased. Thus, both of the α_1 and α_2 subunit mRNAs are coordinately induced during myocyte differentiation, albeit not to equimolar extents.

Our data are consistent with 2 recent observations [22,23]. Romey et al. [22] have demonstrated that the number of dihydropyridine binding sites are developmentally regulated in primary cultures of mouse skeletal muscle cells. Upon cessation of proliferation and during the fusion into myotubes they observed a dramatic increase in dihydropyridine binding sites, whereas the K_d value remained constant throughout the developmental process. Using immunochemical techniques, Morton et al. [23] have also shown that the expression of α_1 and α_2 subunits of dihydropyridine sensitive calcium channel are developmentally regulated.

A crucial question in understanding how the differential pattern of expression of VDCC α_1 and α_2 mRNAs are established and regulated can be answered by identification of *cis*-acting regulatory elements and *trans*-acting factors of the VDCC α_1 and α_2 subunit genes.

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