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Developmental expression of voltage-sensitive K^+ channels in mouse skeletal muscle and C_2C_{12} cells

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The developmental expression of voltage-sensitive K^+ channels was analyzed by Northern blot in mouse skeletal muscle. Of nine *Shaker*-like genes studied, eight are expressed in this mammalian muscle. Their expression is differentially regulated during development. The mouse cell line C_2C_{12} has been used to study expression of voltage-sensitive K^+ channels during in vitro myotube differentiation. Different voltage-sensitive K^+ channel messages are also expressed in these cells which display a pattern of expression depending upon the differentiation stage. The message for the very peculiar K^+ channel of IsK type could only be detected by polymerase chain reaction on skeletal muscle mRNA.

Ionic channel; Myoblast; Myotube; Differentiation; Development

1. INTRODUCTION

Skeletal muscles are composed of different types of fibers with different contractile and electrical properties. In these fibers, voltage-activated K^+ channels determine fiber excitability by controlling the resting membrane potential and by shaping the action potential. Both the functional and the pharmacological properties of these channels have been described [1,2], but little is known about their structure or pattern of expression.

The successful cloning and expression of a wide variety of voltage-sensitive K^+ channels in the past few years have identified two very different types of gene families. The first one is the *Shaker*-like family, corresponding to voltage-sensitive channel subunits of 50–75 kDa with 6 transmembrane helices [3]. The second one is the IsK family, corresponding to a voltage-sensitive K^+ channel of 15 kDa with only one transmembrane helix. While only one gene was found to encode the IsK type of channel [4], at least 19 different genes have been described for the superfamily of the *Shaker*-related channels. *Shab*, *Shal* and *Shaw* are three *Drosophila* genes belonging to the *Shaker* superfamily. In the new nomenclature [5], the generic name, Kv1, corresponds to the genes which are very homologous to the *Drosophila Shaker* gene itself, Kv2 corresponding to *Shab*, Kv3 to *Shaw* and Kv4 to *Shal*.

Complementary DNA probes corresponding to different members of the two structural types of channels have been used to analyze channel expression during development of brain and cardiac muscle [6–9].

The purpose of this paper is to identify the multiple K^+ channel genes expressed in the developing mouse skeletal muscle and in the C_2C_{12} cell line, and to describe how these channels are differentially regulated and how their level of expression varies during muscle development. The investigated genes encode either for delayed rectifier K^+ channels (Kv1.1, Kv1.2, Kv1.3, Kv1.5, Kv1.6, Kv2.1 and Kv3.1) or for transient K^+ channels (Kv1.4 and Kv4.1) when expressed in *Xenopus* oocytes as homopolymers. The presence of the message for the very slowly activating K^+ channel (IsK) has also been investigated.

2. MATERIALS AND METHODS

Skeletal muscle was dissected from hindlimbs. Total RNA was extracted by the guanidium isothiocyanate method and poly(A)-rich RNA was isolated. For Northern blot analyses, aliquots of poly(A)-rich RNA were resolved through 1 M formaldehyde–1.2% agarose gels. Application of equal amounts of RNA to each lane was confirmed by ethidium bromide staining. RNA was blotted to Hybond-N membranes (Amersham). The blots were prehybridized for 5 h at 55°C in 50% formamide, 5 × SSPE (0.9 M sodium chloride, 80 mM sodium phosphate, pH 7.4, 5 mM EDTA), 0.1% SDS, 5 × Denhardt's solution, 20 mM potassium phosphate, pH 6.5, and 250 mg/ml denatured herring sperm DNA. The blots were hybridized overnight at 55°C with $2 \cdot 10^6$ cpm/ml of DNA probe. DNA fragments were labeled to a specific activity of 10^9 cpm/μg of DNA with [α-^{32}P]dCTP by random hexanucleotide primer extension.

K^+ channel DNA fragments used as probes were obtained by the polymerase chain reaction (PCR) and subcloned in Bluescript SK$^-$ (Stratagene). Their identity was verified by sequencing. PCR-amplified DNA fragments were as follows: Kv1.1 (MBKI) [11] nucleotide (nt) 1–1,501; Kv1.4 (HKI) [12] nt 2,138–3,116; Kv4.1 (m*Shal*) [13] nt 1,455–1,956; Kv3.1 (NGK2) [14] nt 1,035–1,459; Kv2.1 (m*Shab*) nt 1,751–2,224; L-type Ca^{2+} channel [16] nt 15–665. In all cases the

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number 1 was assigned to the adenosine of the initiation ATG codon. The Kv1.3 (HLK3) [17], mIsK [9], Kv1.2 (RCK5) [18] and Kv1.5 (mouse equivalent to Kv1 [6]) probes were cloned from cDNA libraries. The fragments used as probes were the entire coding sequences for Kv1.3, mIsK and Kv1.2 and nt 1,022–2,500 for Kv1.5. Filters were washed stepwise with 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7), 0.3% SDS and with 0.2 × SSC, 0.3% SDS at 65°C before autoradiography on X-OMAT-AR (Kodak) at −70°C.

Undifferentiated C_2Cl_{12} cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum. At 90% confluency, differentiation was induced for 1–18 days in DMEM containing 2% horse serum. At days indicated after serum withdrawal, poly(A)-rich mRNA was isolated and probed under the hybridization conditions described above.

3. RESULTS AND DISCUSSION

Fig. 1 shows comparative autoradiograms of mouse brain and skeletal muscle RNA blots probed with K^+ channel DNA probes. Poly(A)-rich RNAs were isolated from whole brain at 2 and 100 days postnatal and from hindlimb muscle at 2, 15 and 100 days postnatal. Hybridizations were performed under high stringency conditions to avoid cross-hybridization between the multiple K^+ channel isoforms, however, even under these conditions, each channel probe hybridized to multiple transcripts. This finding probably indicates the existence of alternatively processed mRNAs transcribed from a unique gene. Message heterogeneity for a given K^+ channel gene is not peculiar to mouse skeletal muscle: it has been observed in brain and heart (even with K^+ channel probes derived from poorly conserved non-coding sequences). This diversity arises from a different use of polyadenylation sites or from different transcription start sites, or from alternative splicing. Whether the splice sites are located in the coding or in the non-coding regions remains to be elucidated. The functional consequences of the K^+ channel message heterogeneity are still poorly understood.

IsK is a very peculiar voltage-sensitive K^+ channel found in epithelial and cardiac cells [9]. It is a relatively short polypeptide with no homology in sequence or structural organization with the *Shaker* family [19]. In *Xenopus* oocytes, it directs the expression of a very slowly activating, non-inactivating K^+ selective current. The IsK message was detected in mouse muscle by using the PCR technique but was undetectable by Northern blot analysis, indicating a very low level of transcripts at all developmental stages studied.

Probes hybridizing to *Shaker*-like transcripts revealed

Table 1

Expression of voltage-sensitive K^+ channels during mouse muscle and brain development

	Kv1.1		Kv1.2	Kv1.3	Kv1.4			Kv1.5		Kv1.6
	8.3 kb	2.8 kb	12 kb	10 kb	4.5 kb	3.6 kb	2.3 kb	6 kb	3 kb	6 kb
Muscle										
E16/E18	+	−	±	−	−	−	++	±	+	±
P2	++	±	±	−	−	−	++	±	+	±
P15	+++	++	±	−	−	−	++	+	++	±
P50	+	−	−	−	−	−	++	−	+	−
P100	+	−	−	−	−	−	++	−	+	−
Brain										
P2	++	−	+	+	++	+	−	+	+	±
P100	+++++	+++	+++	++	+++	++	−	++++	+++	+++

	Kv2.1				Kv3.1			Kv4.1	
	10.5 kb	8.5 kb	7.5 kb	4 kb	7.8 kb	4 kb	2.3 kb	5.2 kb	3.7 kb
Muscle									
E16/E18	+	+	−	+	+	−	−	++	+
P2	++	++	+	+++	+++	±	±	++	+
P15	++	++	+	+++	+++	+	++	++	+
P50	+	+	−	++	+++	++	−	−	−
P100	+	+	−	++	+++	++	+	−	−
Brain									
P2	+	+	−	++	+	+	−	++	+
P100	++++	++	++	+++	++++	+++++	++++	++++	++

Results shown correspond to three independent experiments. Transcript levels for each channel type are standardized to the maximum observed during development. Independently of the developmental stage, the relative abundances for the various transcripts are the following: Kv1.1 (8.3 kb) > Kv3.1 (7.8 kb) > Kv2.1 (4 kb) > Kv1.5 (3 kb) > Kv4.1 (5.2 kb) > Kv4.1 (3.7 kb) > Kv2.1 (10.5 kb) ≈ Kv2.1 (8.5 kb). The other transcripts indicated in the table (Kv1.1 (2.8 kb), Kv1.2 (12 kb), Kv1.4 (2.3 kb), Kv1.5 (6 kb), Kv1.6 (6 kb), Kv2.1 (7.5 kb) and Kv3.1 (2.3 and 4 kb)) are present at very low levels in muscle.

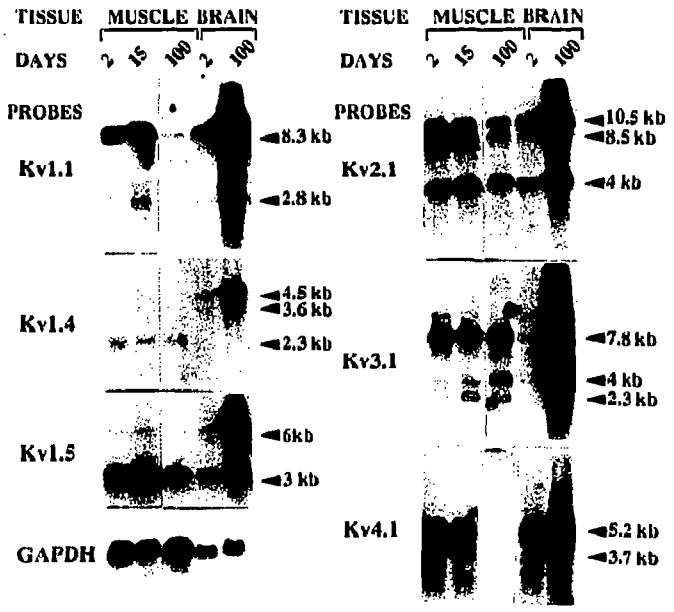

Fig. 1. Developmental expression of K^+ channels in muscle and brain. RNA was prepared from mice 2, 15 and 100 days after birth. 4 μg of poly(A)-rich RNA was loaded per lane. The filters probed with Kv1.1, Kv2.1, Kv3.1 and Kv1.4 cDNAs were exposed for 72 h. The filters probed with Kv1.5 and Kv4.1 cDNAs were exposed for 120 h. For control, blots were re-probed with the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

almost 19 different transcripts. Their sizes and relative amounts in muscle and brain are summarized in Table I. This table also includes results obtained with Kv1.2, Kv1.3 and Kv1.6 probes, as well as those obtained from tissues at embryonic stage E16–E18 and at day 50 postpartum which are not shown in Fig. 1. In whole brain, all channel isoforms were expressed at relatively low levels at day P2 and the expression of all of them markedly increased between day P2 and day P100. The situation was different in skeletal muscle. Levels of the different gene transcripts at day P2 were higher in muscle than in brain. Conversely, at day P100, levels of all transcripts were considerably higher in brain than in muscle. Moreover, the increase in K^+ channel gene transcripts was constant from the neonatal to the adult stage in the brain, whereas the different genes were maximally expressed at day P15 in muscle.

A detailed inspection of the results from 4 independent experiments indicates that K^+ channel subtypes develop according to 4 main types of pattern: (i) mRNA levels for Kv1.1, Kv1.5, Kv2.1 and 2.3 kb Kv3.1 increase more or less linearly from days E16–E18 to day P15 and then decline to a lower but consistent level at the adult stage; (ii) Kv1.4 mRNA is stably expressed at all stages examined. Approximately the same situation is observed for the 7.8 kb transcript of Kv1.3 except for the embryonic stage where its level is consistently found to be lower than after birth. Surprisingly the Kv1.4 probe hybridizes to a unique transcript of 2.3 kb in muscle as opposed to two transcripts of 3.6 and 4.5 kb in brain. The other probes examined in this study labeled the same size of transcripts in both tissues; (iii) mRNAs for Kv1.3, Kv1.6 and Kv4.1 are expressed before birth and remain detectable until day P15. Then, their expression level decreases and they become undetectable at day P50 and day P100; (iv) the expression of the 4 kb Kv3.1 transcript increases regularly from day P2 to day P100.

It is clear that the regulation of expression of K^+ channel genes in developing skeletal muscle is complex and specific for each channel subtype. Whether the observed changes in mRNA levels result from a transcriptional regulation or/and from a differential message stability remains an open question (also different amounts of mRNAs may not necessarily reflect differences in K^+ channel protein levels). Functional studies have shown that other muscle channels are developmentally regulated, such as voltage-sensitive Na^+ [20], Ca^{2+} [21], Cl^- [22] channels or acetylcholine receptors [23]. Developmental changes of some of the K^+ channels in mouse skeletal muscle are similar to those observed for other membrane proteins in charge of ion transport in rodent muscle. For example, the regulation for Kv1.1 and Kv1.5 K^+ channels is similar to that found for the ε subunit of the acetylcholine receptor [23]. In addition, during muscle development, the 4 kb transcript of the Kv3.1 channel is expressed in a pattern similar to that of a subtype of the voltage-sensitive Na^+ channel (SKM1) [24] and of the CLC-1 Cl^- channel [22] transcripts. This suggests that there might be very similar or identical mechanisms of regulation for different types of ionic channels.

K^+ channel expression was also analyzed during differentiation of the muscle C_2C_{12} cell line in vitro. Differ-

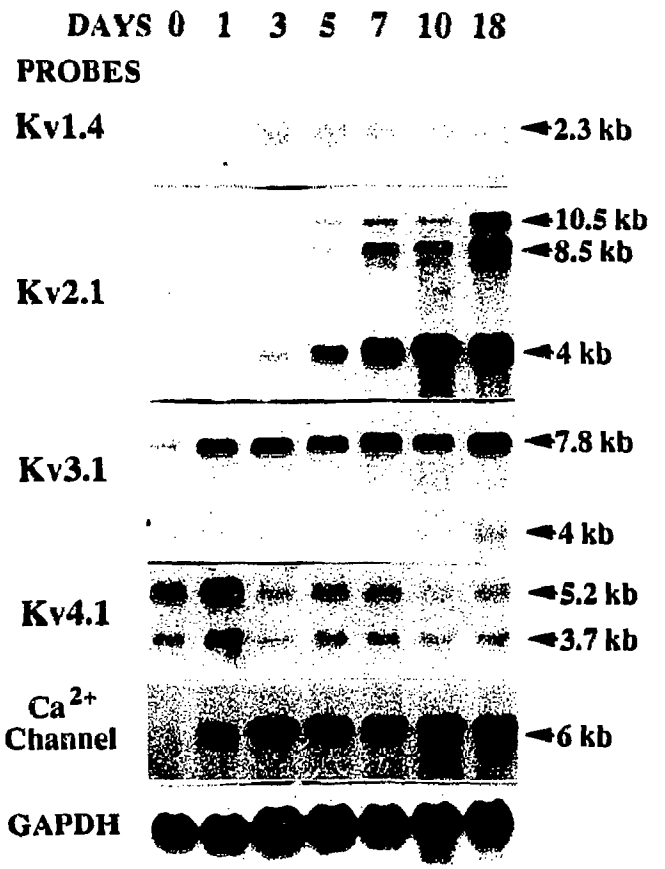

Fig. 2. Expression of K^+ channels in C_2C_{12} cells during differentiation. RNA was isolated from proliferating cells (day 0) and from differentiated cells at 1, 3, 5, 7, 10 and 18 days. 5 μg of poly(A)-rich RNA was loaded per lane. The filters probed with K^+ channels cDNAs were exposed for 72 h and the filter probed with L-type Ca^{2+} channel cDNA was exposed for 24 h. For control, blots were reprobed with GAPDH cDNA.

entiated C_2C_{12} myocytes express Na^+ and Ca^{2+} currents which closely resemble those observed in rat muscle [25]. In order to analyze the expression of K^+ channel genes during differentiation, Northern blot analysis was performed both at the myoblast and myotube stages. Poly(A)-rich RNAs were prepared from exponentially growing cells and from differentiated cells taken at 1, 3, 5, 7, 10 and 18 days after serum withdrawal. mRNAs were probed with the same K^+ channel probes as for the muscle tissue. An L-type Ca^{2+} channel-specific probe was added for comparison (Fig. 2). The IsK message was not detected even with the PCR method. Of the nine *Shaker*-type K^+ channel probes used, only 4 gave positive hybridizing signals with C_2C_{12} mRNAs, corresponding to Kv1.4, Kv2.1, Kv3.1 and Kv4.1. All of the four gene types related to the *Drosophila* K^+ channel genes, *Shaker* (Kv1), *Shab* (Kv2), *Shaw* (Kv3) and *Shal* (Kv4) were revealed, but from 6 different Kv1-related probes, only one (Kv1.4) detected a transcript. This is interesting since it has been suggested that only subunits belonging to the same gene family (*Shaker* or *Shab*, or *Shal* or *Shaw*) can assemble into heteropolymeric associations to form tetramers which are the functional units of the K^+ channels [3]. One should then expect that only homotetramers of the 4 detected genes are expressed in C_2C_{12} cells. Moreover, one should emphasize that the absence of signal with Kv1.1 and Kv1.5 probes is surprising since it contrast with the very strong signals they produce in muscle in vivo. These differences between mouse muscle in vivo and the muscular cell line in vitro could be due to the fact that a variety of specialized fiber types are present in total muscles, each of them expressing a different pattern of voltage-sensitive K^+ currents [3]. The C_2C_{12} cell line could derive from one type of stem cell that does not express Kv1 channels except for Kv1.4. Another hypothesis may be even more attractive, in that Kv1.1, Kv1.3, Kv1.5 and Kv1.6 expression is influenced by innervation, as described for other ionic channels and receptors [21,26,27].

Kv4.1 transcripts are present at day 0 and their level does not increase during differentiation. Conversely, levels of transcripts for other K^+ channels increase continuously during myotube formation. This increase in K^+ channel mRNAs after serum withdrawal is in line with the rise in K^+ channel density recorded in developing myocytes [28]. The maximum of transcript accumulation was reached at day 18 after serum withdrawal. The two Kv4.1 transcripts showed a transient peak at day 1 and then decreased to a level lower than that found at confluency. The message for the *Shaker*-related Kv1.4 channel was the least abundant in C_2C_{12} cells. This observation was also made with muscle tissue (Fig. 1). As in muscle, the size of the transcript in C_2C_{12} cells revealed by this Kv1.4 probe was 2.3 kb. Generally speaking, sizes of transcripts found for C_2C_{12} cell mRNAs are the same as those observed for mouse skeletal muscle. In order to compare the ontogenesis of voltage-sensitive K^+ channels with that of other ionic channels important for excitation–contraction coupling in skeletal muscle, we have analyzed in Fig. 2 the hybridization pattern of L-type Ca^{2+} channels. A 6 kb message was stably expressed after 3 days of differentiation corresponding to myotube formation. This appearance of Ca^{2+} channels shown by molecular biology techniques is in perfect agreement with the appearance of L-type Ca^{2+} currents recorded in differentiating C_2C_{12} cells [25]. L-Type Ca^{2+} channel ontogenesis has the same time–course as the appearance of the 2.3 kb message for the Kv1.4 channel. It is possible that there is a common regulation for L-type Ca^{2+} channels and for at least one class of K^+ channels. Myogenic factors of the MyoD family play an important role in muscle development [29]. They may be in charge of the control of expression of the large repertoire of muscle ionic channel genes and particularly of the K^+ channel genes identified in this paper.

In summary, this work has identified the expression of several genes encoding voltage-sensitive K^+ channels

in mammalian skeletal muscle. This observation strongly suggests that these genes will lead to the expression of different types of subunits which, since they can form heteropolymeric assemblies, will lead to families of voltage-sensitive K^+ channels with different biophysical and regulation properties. Since the expression of each gene is developmentally regulated, one should expect different compositions of voltage-sensitive K^+ channels at different stages of muscle development leading, of course, to different states of excitability.

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