

Expression of the potassium channel KV3.4 in mouse skeletal muscle parallels fiber type maturation and depends on excitation pattern

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Abstract We report the detailed expression pattern of the voltage-dependent potassium channel KV3.4 (rat homologue, Raw3) in mouse skeletal muscle. Using semi-quantitative RT-PCR, we show that its expression is detectable at embryonic day 17 and rises to adult levels within 2 weeks after birth. Expression is fiber type-dependent, with mRNA levels being 5–6-fold lower in the mixed slow/fast soleus muscle than in the fast tibialis anterior and extensor digitorum longus muscles. Fast muscles from myotonic mice exhibit low KV3.4 mRNA levels similar to those of wild-type soleus. In denervated extensor digitorum longus, KV3.4 expression declines to perinatal levels. We conclude that KV3.4 expression in mouse skeletal muscle is regulated by the pattern of excitation.

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Key words: KV3.4; Raw3; Denervation; Myotonia; Reverse transcription polymerase chain reaction

1. Introduction

In mammalian skeletal muscle, many genes drastically change their expression level during development and maturation, and respond to innervation and denervation. For example, genes for the myogenic factors MyoD, myogenin and myf-5 and for acetylcholine receptor subunits are down-regulated during synapse formation and up-regulated after surgical or physiological denervation [1,2]. In contrast, the genes for the Ca²⁺-binding protein parvalbumin (Pva) and for the muscular chloride channel 1 (ClC-1), which are highly expressed in mature fast-twitch muscle fibers, are down-regulated after denervation [2–4].

Neurogenic and muscle intrinsic factors interact during muscle development in order to establish and maintain the individual functional profiles of skeletal muscle fiber types and adapt it to changing conditions [5]. Expression of a slow phenotype is dependent on a tonic stimulation pattern [6,7], whereas the fast myofibrillar phenotype is to be considered the ‘default program’, which occurs even in the absence of innervation [8]. In the adult, neural activity plays an important role in the maintenance and plasticity of fiber type-specific profiles.

The myotonic mouse mutant ADR and its allelic forms are characterized by hyperexcitability due to the absence of a functional *Cle1* gene product [9,10]. As a consequence of repetitive action potentials and after-discharges of the sarcolemma, the fiber type profile is shifted towards fast-oxidative IIA

[11,12], comparable to the changes after chronic stimulation (cf. [5]).

The voltage-gated potassium channel KV3.4 (gene symbol: *Kcnc4*; chromosome 3 [13]) is a fast-inactivating member of the *Shaw* subfamily of *shaker*-type potassium channels [14]. Unlike other members of this subfamily, which are almost exclusively expressed in the central nervous system, KV3.4 mRNA is found mainly in skeletal muscle [15], where it might be involved in the modulation of Ca²⁺ inward currents [14].

Here we report the detailed expression pattern of KV3.4 in mouse skeletal muscle with respect to development, fiber type distribution and pathological activity patterns as found in the disease myotonia and after denervation.

2. Materials and methods

2.1. Animals and tissues

A2G and C57BL/6 wild-type and myotonic BALB/c mice carrying the *Cle1^{adr-mto2J}* allele [16] were used as a source of skeletal muscle RNA (M. extensor digitorum longus, EDL, M. tibialis anterior, TA and M. soleus); neonatal skeletal muscle RNAs were from total hindlimb muscles of 1–10 days old wild-type mice; embryonic RNA was from skinned total hindlimbs. Muscles from a minimum of three mice were pooled for each individual mRNA preparation.

2.2. Cell culture

M12 myogenic cells [17] were cultured as described [18]. Myotubes were harvested 4 days after induction of myoblast fusion.

2.3. Denervation experiments

Lower hindlimb muscles of wild-type mice were denervated by transection of the N. ischiadicus. Experiments were performed according to the German law for the protection of animals with an approved permit from the local authorities.

2.4. Reverse transcription and PCR

Total RNA from cells and tissues was purified according to Chomczynski and Sacchi [19]. 1 µg of total RNA in 20 µl reaction volume was subjected to reverse transcription (RT) according to Kawasaki [20] using 200 U MMLV reverse transcriptase (SuperScript, Life Technologies GmbH, Eggenstein, Germany). 1 µl RT was used for 20 µl duplex PCRs co-amplifying KV3.4 and L7 ribosomal protein cDNA as an internal standard [21]. The KV3.4 primers we used were derived from a partial genomic sequence [22]: KV3.4s (5′ TGG GCT GTG GTC ACC ATG AC-3′) and KV3.4a (5′-CTC TCG ACC ACA CCC TCT TCC-3′); L7 primers: L7s (5′-AGA TGT ACC GCA CTG AGA TTC-3′) and L7as (5′-ACT TAC CAA GAG ACC GAG CAA-3′). PCR was performed on a Trio-Thermoblock (Biometra, Göttingen, Germany) using AmpliTaq polymerase (Perkin Elmer, Überlingen, Germany) and 0.5 µM of each primer under the following conditions: 4 min initial denaturation at 94°C, 10 cycles with 30 s denaturation at 92°C, 1 min annealing at 62°C and 30 s elongation at 72°C, seven cycles with the same parameter except for an additional 10 s stepwise increment of elongation time and finally a 5 min last extension step. For control experiments, a total number of 24 amplification cycles were run. The PCR products were separated on a 1.5% TBE agarose gel and visualized by ethidium bromide staining.

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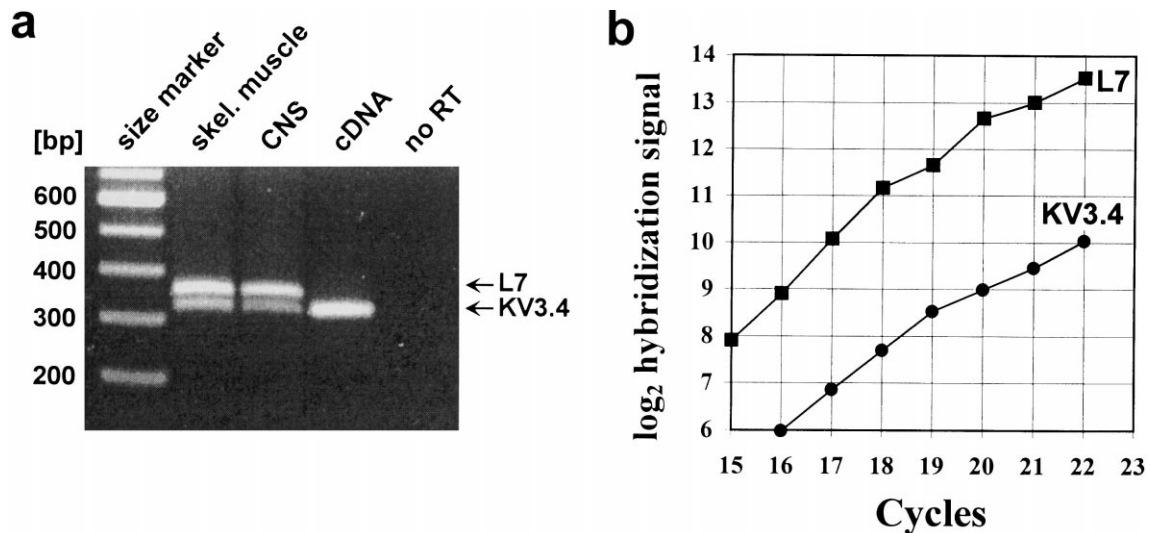


Fig. 1. Duplex RT-PCR to determine KV3.4 mRNA levels in mouse. a: Samples from adult skeletal muscle and brain separated on a 1.5% TBE agarose gel. The cDNA of the rat KV3.4 homologue Raw3 served as a positive control for KV3.4 PCR, RNA without reverse transcription was used to test for the amplification of genomic background in RNA samples. This genomic contamination was below 1% of the total PCR product, as calculated from the ratio of corresponding radioactive hybridization signals of non-reverse transcribed to those of cDNA-converted samples (data not shown). However, all quantitated data were corrected for genomic background amplification. b: Increment of KV3.4 and L7 RT-PCR products from murine adult skeletal muscle RNA with total cycle number. Quantitation was done by liquid scintillation counting of radioactive hybridization. Values are given as binary logarithms of counts per minute. From these data, 18 cycles were chosen for subsequent experiments to ensure maximal sensitivity and linearity of the PCR reaction.

2.5. Slot blot hybridization and quantitation

Amplification products were heat-denatured for 10 min and subsequently cooled on ice. After addition of $20\times$ SSC to a final volume of 500 μ l, PCR samples were immediately applied to a Minifold II Slot Blotter (Schleicher und Schüll, Dassel, Germany) using Nylonbind B membrane (Serva, Heidelberg, Germany). One series was probed with a 32 P-labeled 576 bp *Hind*III-*Eco*RV fragment of rat Raw3-cDNA (nt 1861–2437), another was hybridized with the L7 PCR product described above. After stringent washing in $0.1\times$ SSC for 15 min at 65°C, membranes were autoradiographed. For quantitation, slot dots were cut out of the membrane and subjected to liquid scintillation counting using a TriCarb 300 (Packard-Canberra, Frankfurt/M., Germany).

3. Results and discussion

Although in rodents KV3.4 expression is highest in skeletal muscle, absolute mRNA concentrations are low and therefore difficult to demonstrate by Northern blot analysis. To overcome this limitation, we have developed a semi-quantitative duplex RT-PCR, amplifying a 322 bp fragment from an internal exon of the murine KV3.4 gene together with a 352 bp fragment of the small ribosomal protein L7. The L7 mRNA served as an internal standard, as its level is not affected by the physiological and pathological conditions investigated in this work (Fig. 1).

KV3.4 expression is low but detectable in hindlimb muscles of the 17dE mouse embryo and rises postnatally within about 2 weeks to the adult level (Fig. 2). In cultured myoblasts and myotubes, virtually no KV3.4 mRNA could be demonstrated. Thus KV3.4 expression is up-regulated late during muscle ontogeny and parallels the maturation of muscle fibers.

We further investigated whether KV3.4 mRNA is differentially expressed with respect to fiber type composition. KV3.4 is highly expressed in the mixed glycolytic-oxidative fast EDL and TA muscles, which are composed mainly of IIB and IID/X fibers [23]. The mixed slow-fast soleus muscle, which is

composed of type I slow oxidative and type IIA fast oxidative-glycolytic fibers, showed 5–6-fold lower expression levels (Fig. 3). A similar result has been found by Northern blotting on rat EDL and soleus muscles [24].

To elucidate the influence of activity pattern of the skeletal muscle fiber on KV3.4 expression, mRNA levels of EDL, TA and soleus muscles from the myotonic mouse mutant ADR were determined. Expression of KV3.4 in the myotonic EDL is greatly reduced and comparable to that of the wild-type

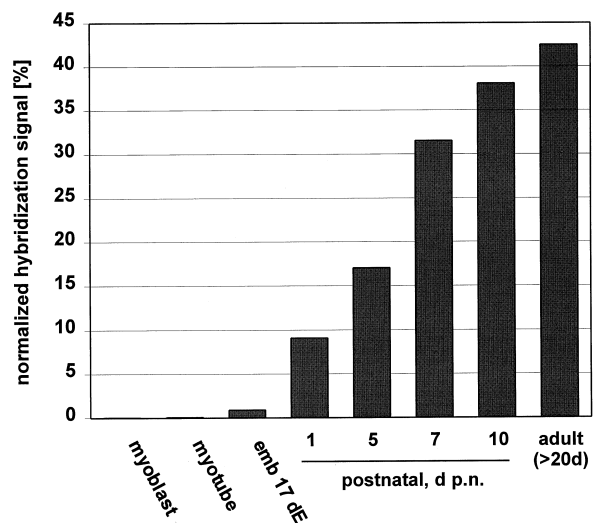


Fig. 2. Expression of KV3.4 during mouse muscle development. Myoblasts and myotubes were from the secondary mouse myogenic cell strain M12. Embryonic total RNA was purified from total skinned hindlimbs. RNAs were prepared from lower hindlimb muscles of mice of the indicated ages. KV3.4 mRNA levels were quantitated as described in Fig. 1b and normalized for the expression of the ribosomal protein L7. Values are given as percentage of L7 hybridization signal.

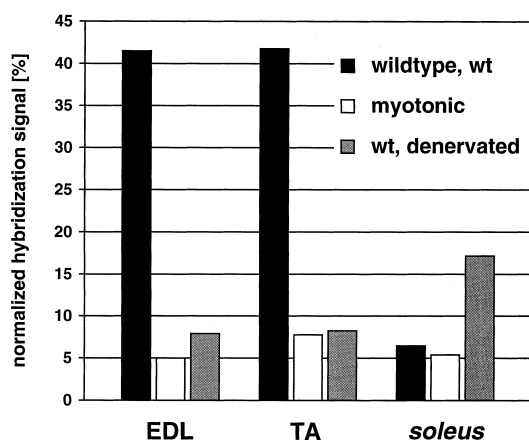


Fig. 3. Role of excitability and innervation on KV3.4 expression in murine fast and mixed fast-slow twitch muscles. KV3.4 mRNA levels in muscles of the myotonic mouse mutant ADR*2J and in denervated wild-type muscles are compared to the corresponding untreated wild-type muscles. KV3.4 expression in surgically denervated wild-type muscles was determined 7 days after transection. The effect of denervation was validated by Northern blot analysis of nicotinic acetylcholine receptor α subunit (nAChR α) expression, which is steeply up-regulated upon denervation (data not shown). Quantitation and normalization as in Fig. 2.

soleus, whereas the myotonic soleus remains essentially unaffected (Fig. 3). This secondary reduction of KV3.4 expression in the myotonic mouse is restricted to skeletal muscle, as mRNA levels in the CNS of mutant mice were not reduced (data not shown). These data suggest a strong influence of the muscular activity pattern on KV3.4 mRNA levels, most likely on transcription.

To test whether motoneural activity is necessary for expression, the effect of denervation on fast and mixed slow-fast twitch muscles was investigated. KV3.4 expression was reduced in denervated EDL and TA, but slightly elevated in the soleus. Possibly the tonic excitation pattern of the soleus muscle suppresses KV3.4 expression.

Table 1 summarizes the data of KV3.4 expression in skeletal muscle of rodents and compares them to those of the muscular chloride channel CIC-1 and the Ca²⁺-binding protein parvalbumin. These messages have in common that they are not significantly expressed in myotubes, and are up-regulated perinatally during the process of innervation. Highest expression levels correlate with the abundance of glycolytic

Table 1

Comparison of expression of three activity-regulated genes in murine skeletal muscle

	KV3.4	CIC-1	Pva
Brain	+ [15]	0 [28]	+ [29]
Skeletal muscle (adult)	+	+ [28]	+ [25]
Postnatal development	↗	↗ [30]	↗ [3]
EDL/soleus	6	5 [4]	20 [4]
Myotonia			
EDL	↓	≈ [4]	↓↓ [4]
soleus	≈	↑ [4]	↓ [31]
Denervation			
EDL	↓	↓ [4]	↓ [3]

Arrows pointing up, mRNA levels were increased compared to control wild-type; arrows pointing down, mRNA levels were decreased. Factors: two arrows, > 20-fold, one arrow, 2–20-fold, ≈, no significant increase. KV3.4 and Pva mRNA levels were not changed in the brain of myotonic mice [26].

fast-twitch fibers [4,25]. All three genes are down-regulated in the denervated muscle, which suggests that innervation is the major stimulus of expression during maturation. The pathological condition myotonia leads to a down-regulation of KV3.4 mRNA levels in fast-twitch muscles like TA and EDL, as was shown for parvalbumin [26]. However, regulation of CIC-1 expression in myotonic muscles differs from that of KV3.4 and parvalbumin mRNAs (Table 1). Comparison of the promoter regions of these genes may help to delineate the *cis*- and *trans*-acting regulatory elements, or modules of them [27], which are responsible for the expression of maturation- and activity-dependent genes in the skeletal muscle.

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