Sodium Channel Development Changes the Conduction Velocity in Skeletal Muscle

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Abstract

Previous studies show that some of the properties of electrical signals in skeletal muscle sarcolemma change in the course of development. To discover the molecular basis of improvement of muscle performance, we analyzed the expression of sodium channels after birth. Using protein and immunoblot analysis, we found a 260 kDa component immunoreactive to the antibody against sodium channel α-subunit. The results showed an increase in the amount of sodium channel after birth. However, after 8 postnatal days the amount of channel protein started to decrease. At the age of 15 days, the channel expression was rising again. The pattern in the development of conduction velocity was surprisingly similar revealing a decrement in conduction speed. Taken together, the results show that both (1) the postnatal increase in the amount of voltage-gated sodium channels and (2) the down-regulation of channel expression have an impact on conduction velocity of sarcolemma thus changing the electrical properties of skeletal muscles during maturation.

Key words: conduction speed, excitation-contraction coupling, ion channel, maturation, sarcolemma.

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As well known, voltage-gated sodium channels are present in most excitable cell membranes and are responsible for the generation and the propagation of action potentials also along the membrane of muscle cells. In skeletal muscles, the sodium channels are located in the sarcolemma as well as in T-tubular membranes of the cell. The density of the sodium channels increases near the endplate region of the cell [3, 10]. Besides the voltage-dependent activation, also a rapid inactivation and selective ion conductance characterize the functional properties of the sodium channels. The opening probability of the channel increases as the membrane voltage turns into positive direction from the resting level. Thus the voltage sensitive sodium channels are activated as a result of a few mill volt depolarization of the cell membrane. Opening of the ion pore allows sodium ions to enter the cell. This increase in the sodium conductance of the membrane causes further depolarization and activation. The action potential spreads passively over the membrane from channel to channel starting at the postsynaptic membrane up to the transverse tubules (T-tubules) finally leading to the excitation-contraction (E-C) coupling [15, 19].

The sodium channels isolated from mammalian skeletal muscles contain a single large 260 kDa subunit (α) and a smaller, 38 kDa β1-like subunit. The α subunit is the major component comprising four pore forming domains with six transmembrane segments [6]. The domains are encoded by at least ten different genes [15]. The β subunit is needed for normal kinetics and voltage dependent gating [5].

There are two different types of sodium channels present in developing skeletal muscle, skeletal muscle type 1 (Nav1.4) and 2 (Nav1.5). The two isoforms are regulated differentially during development. Nav1.5 is expressed in the embryonic muscle but is down-regulated after birth whereas Nav1.4 is expressed in the postnatal muscle cells [26]. The channels are also named according to their binding affinity to the sodium channel toxin, tetrodotoxin. While the mRNA level of tetrodotoxin-resistant sodium channel (TTX-R) is largest before birth, the mRNA level of tetrodotoxin-sensitive sodium channel (TTX-S) is the opposite during the development of the muscle. The expression of adult form of sodium channel (TTX-S) protein begins after birth [17]. Embryonal and adult forms of the sodium channel protein are encoded by different genes and differ from each other in pharmacology and kinetics [2].

It has been shown by Järvilehto and Rissanen [11] that in the skeletal muscle of mouse the conduction velocity is increased by the age, but the correlation to sodium channels was only speculated. Now we have studied the

expression of voltage sensitive sodium channels in the developing striated muscle. There appears to be some visible contractions in mouse skeletal muscles right after birth. However, the movements of the animals are slow and uncoordinated. Adult mice, on the other hand, are moving fast and accurately. Based on these facts and earlier studies, our study hypothesis was that the number of sodium channels increases when the muscle develops. This statement was studied *in vivo* with two different analytical methods. Western blotting was used to identify the presence and location of the voltage dependent sodium channels and to quantitatively study the postnatal expression of the channel. To visualize the sodium channels at different stages of development, immunohistochemical methods were used.

Materials and Methods

Electrophoresis and Western blotting

The Western blotting analysis of sodium channels was applied on muscle tissue from 13 developmental stages of mice. After cervical dislocation according to the conditions of Animal Ethics Committee of the University of Oulu (permit no. 056/01), *m. rectus femoris* (N = 6) from white laboratory mice (*Mus musculus*, random strain) at 3, 5, 7, 8, 9, 10, 13, 15, 18, 20, 25, 27 and 30 days after birth were excised and homogenized in cold 62.5 mM Tris-HCl containing protease inhibitors (leupeptin 1 µg/ml, pepstatin 1 µg/ml, 1 mM PMSF). The homogenate contained 0.5 mg/ml of protein (measured with Protein assay kit, Bio-Rad, USA). The sodium channel proteins were separated from sequentially centrifuged (1000g for 10 min, 4000 g for 5 min, 17 000 g for 30 min) tissue extracts by 5-8% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [14]. The electrophoresis was performed in a PROTEAN II Dual Slab Cell system (Bio-Rad, USA) at 4°C first at 50V for 4 h and then at 120 V for 24 h. One of the gels was stained with Coomassie Brilliant Blue. The other one was used for Western blot to evaluate the affinity of the antibody and to analyze the expression of postnatal development of sodium channels. Proteins were electrophoretically transferred to pure nitrocellulose membrane (Bio-Rad, USA) overnight at 30 V as described by Towbin et al. [23]. The blots were first incubated with polyclonal primary antibody (Anti-pan sodium channel, Alomone labs, Israel) for two hours, then with secondary antibody (Blotting Grade Affinity Purified Goat Anti-Rabbit IgG H+L Alkaline Phosphatase Conjugate, Bio-Rad, USA) also for two hours. Antibody detection was performed with BCIP/NBT substrate. The dilution for the primary antibody was 1:200. The densitograms of the protein bands resolved by SDS-gelelectrophoresis were measured with FluorS MultiImager program (Bio-Rad, USA).

Immunohistochemistry

The samples of leg muscles were fresh-frozen with liquid nitrogen and cut with a cryostat microtome into 10 µm thick sections perpendicular to the longitudinal axis of the muscle. The sections were quickly washed with phosphate buffer followed by a 0.3% TritonX-100/10% bovine serum albumin in 0.1 M phosphate buffer (PBTBSA) wash. The samples (N = 8 from each muscle) were incubated in the sodium channel monoclonal antibody (Anti-pan Sodium Channel, Sigma Aldrich, USA) overnight at room temperature. The concentration of the primary antibody was 0.7 µg/ml. Control sections were incubated in PBTBSA devoid of antibody. Sections were treated with 2.7% NaCl solution and washed with phosphate buffer. After the salt wash, samples were incubated in Alexa Fluor -conjugated antibody (Molecular Probes, Netherlands) for 60 min. Sections were rinsed again in PBTBSA and washed with 0.1 M and 0.05 M phosphate buffers. To preserve fluorescence, the sections were covered with Vectashield® mounting medium (Vector Laboratories, Inc., USA), viewed and photographed immediately with confocal laser scanning microscope (LSM 5 PASCAL, Leo, Germany) by using excitation at 488 nm.

In cell staining, also enzyme-label detection method was used in order to detect the antigen distribution more clearly. Frozen muscle sections (4 µm) were first incubated for 30 min with PBTBSA to reduce non-specific protein binding. Sections were then incubated with polyclonal antibody (Anti-pan Sodium Channel, Alomone Labs, Israel) diluted 1:50 in PBTBSA for 30 min. Sections were washed with PBTBSA, and incubated for another 30 min with alkaline phosphatase-conjucated goat anti-rabbit secondary antibody (Bio-Rad, USA) diluted 1:3000 in PBTBSA. BCIP/NBT substrate was added to the muscle sections and after 10 min the reaction was stopped with 20 mM EDTA in PBS.

Detection of motor endplates

The staining of motor endplates in the muscle cell sarcolemma was based on histochemical method for acetylcholinesterase according to Karnovsky and Roots [12] with the exception of substrate buffer used (sodium acetate buffer, pH 6.0).

Conduction velocity measurements

The conduction velocity in *m. sartorius* was measured as previously described [11] by recording the responses of electrical stimulation at two locations on the surface of the sarcolemma and calculating the time difference of the two corresponding recordings. The temperature was kept at +28°C as was the temperature of the muscle sarcolemma of newborn mice.

Statistical analysis

The relationships between the three variants (age, conduction speed, channel density) were investigated using Spearman's nonparametric correlation coefficient.

A P value of < 0.05 was accepted as indicative of a significant difference between the groups of observations. All the statistical analyses were performed with the SPSS for Windows software.

Results

Expression of sodium channels

In this study the evaluation of the density of sodium channels is based on the properties of the primary antibody. The used antibodies recognize the intracellular III-IV loop of the voltage sensitive sodium channel α-subunit. The epitope is identical in all known vertebrate adult type sodium channels [2, 7, 8, 21].

In order to analyze the expression of sodium channels after birth, muscle tissue samples from mice of different ages were collected and ran in SDS-PAGE. Comparison with standards revealed proteins of 260 kDa in the samples of young mice. The optical densities of protein bands are shown in Figure 1. This 260 kDa protein seems to disappear at the age of 10 days to appear again at the age of 15 days. Nevertheless, there is an increase in the density of the bands when moving towards the samples from older mice.

As shown also in Figure 1, the antibody specifically reacted in Western blot against sodium channel protein from *m. rectus femoris*. When compared with the polyacrylamide gel, the immunoblots were somewhat weaker stained. With the exception of 260 kDa band which was missing in the 1 to 13 day-old mice, the increase in the relative amount of protein was similar to that noted in the SDS-PAGE.

The immunohistochemically stained sodium channels are seen as brilliant green marks in the cross sections of leg muscle fibers (Fig. 2). The qualitative evaluation of the density of Na^+-channels was based on the intensity of fluorescence. Though weak, the staining is first detectable in the 8 d sample. Since then the staining is intensified to attain a further increase at the 25 and 30-day-samples. Staining in the surface membrane is not homogenous and sodium channels seem to aggregate in repeated areas in the membrane.

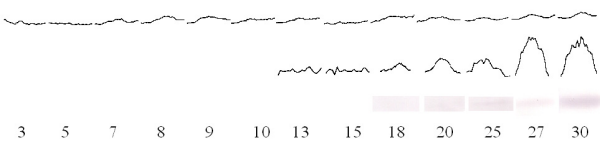

Figure 1. Densitometrical analysis of sodium channel expression. The skeletal muscle proteins were electrophoresed on gradient (5-8%) sodium dodecyl sulphate/polyacrylamide gel, visualised, and analysed densitometrically. The upper densitograms correspond to the 260 kDa bands in the Coomassie brilliant blue stained gel, the lower are densitograms from Western blot (bands below the densitograms).

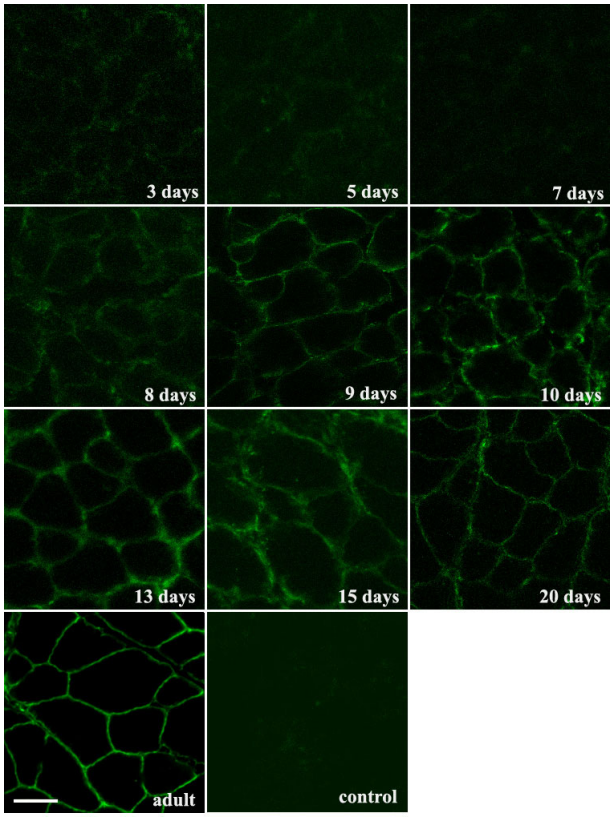

Figure 2. Immunohistologically identified sodium channels in cross-sections from mouse skeletal muscle of different developmental stages. The sections are from leg muscle m. rectus femoris *at 3-30 days of age treated with monoclonal Anti-pan sodium channel antibody. Bar 20 μm.*

Aggregation of sodium channels

The aggregation of antigens in the cell membrane is more clearly seen in the samples stained with alkaline peroxidase-conjucated secondary antibody. Comparison to the samples revealing motor endplates shows higher sodium channel density near the neuromuscular junction (Fig. 3a, 3b).

Conduction velocity

The recordings from conduction velocity studies showed a clear increase in the propagation of action potential measured as compound action potential (Fig. 4 upper graph). However there appeared to be a slight decrease in the conduction velocity at the age of 8 to 13 days. The pattern of the conduction velocity was similar to that of optical densities of the 260 kDa bands corresponding sodium channel α_1-subunit proteins from mice of different age (Fig. 4 lower graph).

Correlation analysis

Spearman's correlation analysis demonstrated a significant correlation between age and conduction velocity ($p = 0.037$) as well as age and sodium channel density ($p = 0.012$). However, no significant relationship

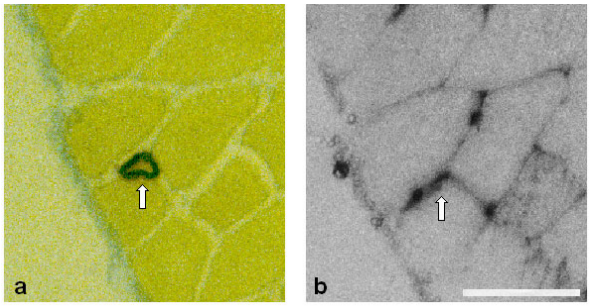

Figure 3. The end plate regions (a) and distribution of sodium channels (b) in mature skeletal muscle of mouse. The distribution of sodium channels labelled with polyclonal Na^+ antibody is seen as dark precipitations closely related to end-plate regions (arrows indicate the same area of the two subsequent sections). Bar 20 µm.

was found between the changes in conduction velocity and sodium channel density (p = 0.285).

Discussion

From the point of view of motor activity, the primary task of skeletal muscle development is to create contractile machinery, which is activated in response to signals coming from the motor nerve. In order to connect and synchronize different nerve impulses to muscular contraction, the presence of electrically excitable membranes is necessary. The ionic basis of the propagated action potential and thus the improvement of movements after birth are partly, but crucially, due to the development of sodium channels. Despite of their importance in muscle function, especially the immunohistochemical studies focusing on sodium channels in the developing muscle are rather few in number.

Although the sodium channel protein from membranes of skeletal muscle is very susceptible to proteolytic cleavage during purification [1], we managed to preserve the 260 kDa subunit by using sequential centrifugation with protease inhibitors. The results from immunoblotting reveal the first detectable sodium channels in muscle not earlier than 15 days after birth. On the other hand, there is a band corresponding to the 260 kDa protein (sodium channel) in the electrophoresis gel in the samples from newborn mice already. The lack of immunoreactivity with the primary antibody in the samples of young mice is probably caused by low concentration of the protein. The bands in the gel could also correspond to the embryonal isoform of sodium channel since there are some structural differences between the adult and embryonal isoforms of sodium channel. However, the epitope recognized by the antibody used is expressed in both isoforms.

According to our results, the number of sodium channels starts to decrease 8 days after birth. On the other hand, the amount increases again after 15 postnatal days. According to Goldin [9], the Nav1.4 isoform is expressed at high levels in adult rat skeletal muscle

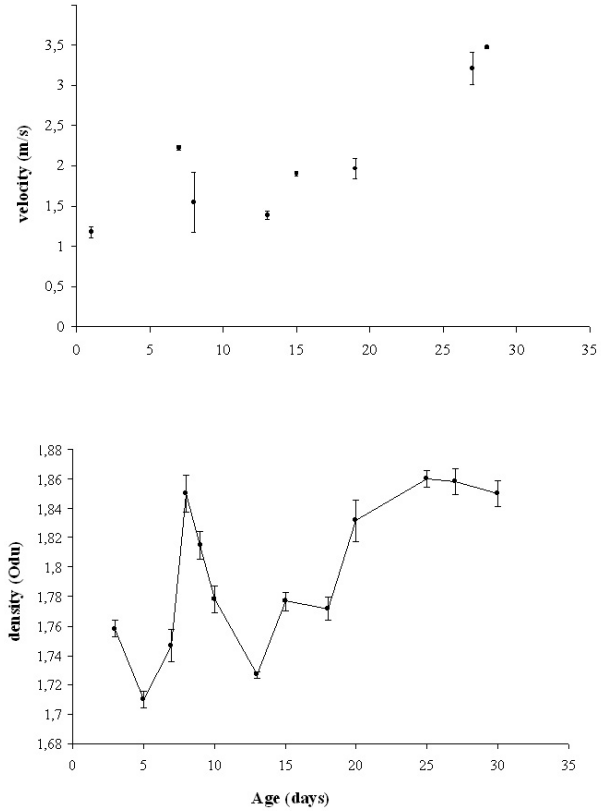

Figure 4. The conduction velocities of skeletal muscle cell sarkolemma of mice at different ages (upper graph) and the densities of the 260 kDa bands from SDS-PAGE (lower graph). A conduction velocity increase from 1.18 to 3.47 m/s is seen during the maturation of skeletal muscle. Compare the pattern of (A) to that of (B). Values expressed

whereas Nav1.5 is not observed in adult skeletal muscle but is detectable in neonatal muscle. Based on the gene expression studies, the Nav1.5 mRNA reaches peak level before birth. The mRNA level of the adult isoform starts to increase at the age of 15 days [17]. Kraner et al. [13] showed that the expression is regulated by many specific developmental factors similar with cis-regulatory elements of the acetylcholine receptor δ-subunit. There are also many other similarities in the development of sodium channels and acetylcholine receptors. The acetylcholine receptors (adult type) are also expressed postnatally (after 5 days) whereas the embryonal type is down-regulated at the same time [24].

Our results indicate that the number of sodium channels is further increased between postnatal days 20 and 25. This is understandable if we consider the E-C coupling and the increasing conduction velocity of the cell membrane in developing muscle. A clear increase in conduction velocity was detected during the first three postnatal weeks in mouse striated muscle. On the other hand, there was a similar kind of decrease in the conduction velocity as was in the amount of sodium chan-

nels detected by SDS-PAGE. This could implicate the downregulation of embryonal sodium channel isoform.

One of the restrictive structures having an effect on the development and differentiation of skeletal muscle is the motoneuron. Neuronal excitation in neonatal skeletal muscle differs remarkably from that in adults. At birth in mammals the excitation is based on polyneuronal innervation. In mouse skeletal muscle the polyneuronal innervation disappears during the second week after birth [20], at the same time as the embryonal form of sodium channel is downregulated and the adult form of sodium channel appears. Also our previous study [18] concerning the expression of L-type calcium channels shows a similar kind of pattern in appearance of the channel protein. Based on the findings, the Ca^{2+}-channel is expressed to the T-tubules of skeletal muscle cell after birth and the density of the channel protein increases during the postnatal development thus improving the E-C coupling.

According to the results from immunohistochemical study, the density of sodium channels in the plasmalemma, as judged by the intensity of fluorescence, seems to rise after 8 postnatal days (Fig. 2). Lupa et al. [17] investigated striated muscle of the rat. In their immunofluorescence study the appearance of sodium channels occurred during maturation of the neuromuscular junction at the age of 17 days. Here one should keep in mind the differences in developmental pattern of mouse and rat. In the loose-patch voltage-clamp studies, however, sodium channels were detected earlier and the results were thus in accordance with our results.

Synaptic region in developing muscle is a complex structure and most of the studies about the neuromuscular junction have been concentrated on research of acetylcholine receptor. As described earlier [16], an extracellular matrix protein called agrin is involved in signaling acetylcholine receptor clustering near the end plate region. It has also been suggested that agrin is involved in sodium channel distribution in skeletal muscle development [22]. Previous “loose patch clamp”-studies about sodium channel distribution in postnatal striated muscle denote the highest density around the endplate region [3, 4]. In prior immunohistochemical studies, Haimovitch et al. [10] have also found dense antibody binding (brighter fluorescence) in short, 10-15 µm regions. With the exception of those endplate regions, the binding was homogenous in other parts of the surface membrane. Wood et al. [25] demonstrated the accumulation of sodium channels during development. According to the results, the density of the channels was more pronounced at the neuromuscular junction already at birth in rat skeletal muscle. Our results support the fact, that the binding of the antibody is more pronounced in some parts of the membrane. Stronger binding in muscle surface membrane indicates the areas near the endplate region.

In conclusion, our findings show that sodium channel is expressed in skeletal muscle sarcolemma of mouse already at birth. The results also suggest that the number as well as the density of sodium channels increases markedly during the postnatal development of mouse skeletal muscle. However, at the age of 8 the amount of sodium channels starts to decrease. After 15 postnatal days the amount of sodium channel protein is increasing again. The measurements of conduction velocity reveal similar kind of pattern in the electrical properties of cell membrane. This implicates that the increasing amount of sodium channels enhances the propagation of action potential along the plasmalemma of skeletal muscle cell.

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List of abbreviations:

E-C coupling; excitation-contraction coupling
SDS-PAGE; sodium dodecyl sulphate polyacrylamide gel electrophoresis
T-tubules; transverse tubules
TTS; tetrodotoxin sensitive
TTX; tetrodotoxin resistant

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