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PHYSIOLOGY

Abnormal Membrane Localization of α2 Isoform of Na,K-ATPase in *m. soleus* of Dysferlin-Deficient Mice

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Dysferlin protein plays a key role in the multimolecular complex responsible for the maintenance of sarcolemma integrity and skeletal muscle cell functioning. We studied the membrane distribution of nicotinic acetylcholine receptors and α2 isoform of Na,K-ATPase in motor endplates of *m. soleus* in dysferlin-deficient Bla/J mice (a dysferlinopathy model). Endplates of Bla/J mice were characterized by increased area (without changes in fragmentation degree) and reduced density of the membrane distribution of nicotinic acetylcholine receptors in comparison with the corresponding parameters in control C57Bl/6 mice. The density of the membrane distribution of α2 isoform of Na,K-ATPase was also reduced, but the level of the corresponding mRNA remained unchanged. It can be hypothesized that abnormal membrane localization of α2 isoform of Na,K-ATPase results from adaptive skeletal muscle remodeling under conditions of chronic motor dysfunction.

Key Words: *skeletal muscle; dysferlin; dysferlinopathy; Na,K-ATPase isoforms; nicotinic acetylcholine receptors*

Activity of Na,K-ATPase plays a crucial role in the maintenance of skeletal muscle electrogenesis and contractile function [3]. Na,K-ATPase is expressed in different molecular forms. In skeletal muscles, α1 and α2 isoforms of Na,K-ATPase are co-expressed, α2 isoform being dominating isoforms in this tissue (up to 87% of total Na,K-ATPase protein) [5]. It is also known that α1 isoform is functionally more stable compared to α2 isoform; adaptive plasticity of the latter is determined by specific localization and regulation of its different pools as well as by functional interactions with molecular environment [4,7,9].

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It is well known that increased motor activity is associated with an increase in Na,K-ATPase abundance in the sarcolemma of skeletal muscle fibers, while long-term decrease in motor activity leads to opposite changes [3]. The isoform specificity of these changes was mainly studied under conditions of chronic motor dysfunction in traumas [13] and during aging [10], *i.e.* the conditions that primarily affect α2 isoform of Na,K-ATPase. The study of initial changes occurring during muscle unloading also demonstrated that specific functional disturbances of the α2 isoform are among the earliest events preceding muscle atrophy caused by motor dysfunction [7,14].

Experimental models of various forms of motor activity disturbances can be promising for future studies of the function and regulation of different Na,K-ATPase isoforms in skeletal muscles. It was reported that skeletal muscle of mdx mice (a laboratory model of Duchenne muscular dystrophy) are characterized by

reduced electrogenic activity of Na,K-ATPase, but its isoform specificity was not studied yet [11].

We analyzed Na,K-ATPase localization in the endplates of *m. soleus* of Bla/J mice, a models for dysferlinopathy caused by deficiency of dysferlin, the key protein of multimolecular complex responsible for the maintenance of sarcolemma integrity during contractile activity [1,12].

MATERIALS AND METHODS

The experiments were performed on isolated neuromuscular preparations of *m. soleus* from 5-7-month-old male mice of C57Bl/6 (control) and Bla/J lines. The mice were kindly provided by Prof. V. M. Mikhailov and A. V. Sokolova, Cand. Biol. Sci., and were delivered from the vivarium of the Institute of Cytology, Russian Academy of Sciences. The animals were housed in the vivarium under standard conditions according to the international rules and Russian legislation.

Immediately after isolation, *m. soleus* with a nerve stump was placed in an experimental chamber with physiological solution containing (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 24 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose (pH 7.4) constantly aerated with carbogen (95% O_2 and 5% CO_2). The localization of nicotinic acetylcholine receptors (nAChR) and α2 isoform of Na,K-ATPase in the motor endplate membrane region was determined using α-bungarotoxin (α-BTX, 1 μM; Biotium) and ouabain (ouabain, 1 μM) fluorescently labeled with BODIPY (Ouabain-Bodipy, Invitrogen), respectively, as described previously [6]. The experiments were carried out at room temperature (~22°C). Images were obtained with a Leica TCS SP5 confocal system using a 63x lens with numerical aperture of 1.30. Image analysis was performed using ImageJ software.

The content of mRNA encoding α1 and α2 isoforms of Na,K-ATPase in muscle homogenate was determined by real-time PCR with Taq-Man probe (FAM) technology. RNA was isolated using Mini kit (Qiagen). The reaction was performed using reverse transcriptase III (Invitrogen), SUPERase (Ambion), and specific primers for α1 and α2 isoforms (Applied Biosystems) in an MX3000P system (Agilent Technologies). All the procedures were described in details previously [7].

Statistical analysis was performed using by ANOVA and Student's *t* test using Origin Pro 8 software. Cumulative curves were analyzed using the Kolmogorov—Smirnov test (GraphPad Prism 7 software). The data are presented as *M*±*SEM*.

RESULTS

The distribution of α-BTX (nAChR) in the endplates region of mouse soleus muscles is presented in Figure 1. It has been previously shown that nAChR are fragmentary distributed within the endplate in the form of branches and islets [2,15]. The number of these fragments in one endplate in control C57Bl/6 mice was 3.3±0.2 (123 endplates, 12 muscles, 9 mice); in Bla/J mice, the degree of fragmentation did not differ from the control and was 3.4±0.2 (140 endplates, 13 muscles, 10 mice). Cumulative curves illustrating the distribution of the number and areas of endplate fragments were also similar for C57Bl/6 and Bla/J mice (data not shown).

In experiments with double labeling of nAChR and α2 isoform of Na,K-ATPase, 47 endplates in 5 muscles from 3 C57Bl/6 mice and 65 endplates in 6

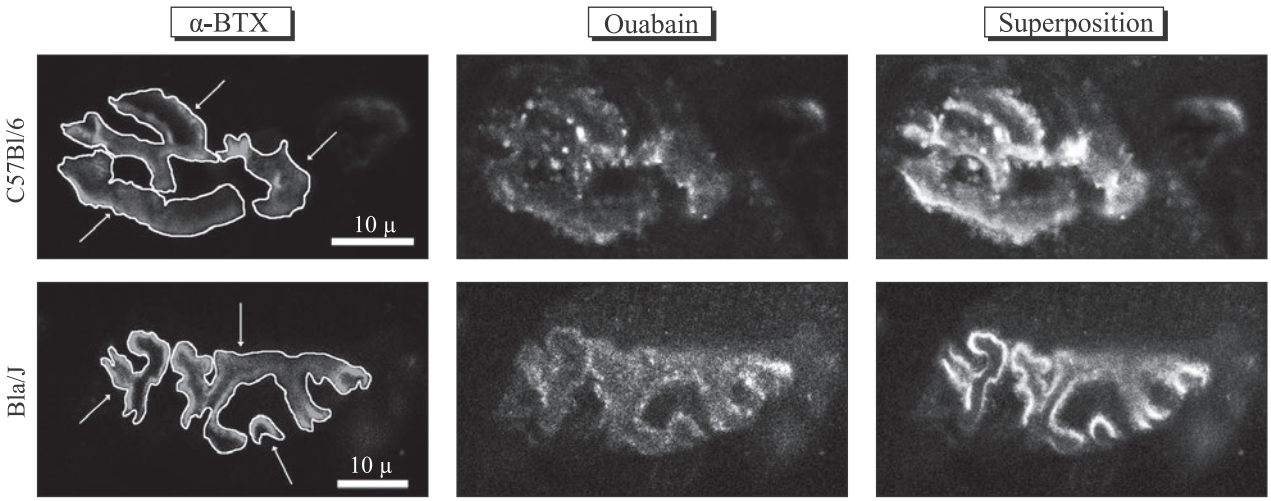

Fig. 1. Representative images of α-BTX-labeled nAChR and ouabain-labeled α2 Na,K-ATPase distribution in endplates of *m. soleus* in C57Bl/6 and Bla/J mice. Arrows show individual fragments of the endplates in the form of branches and islets.

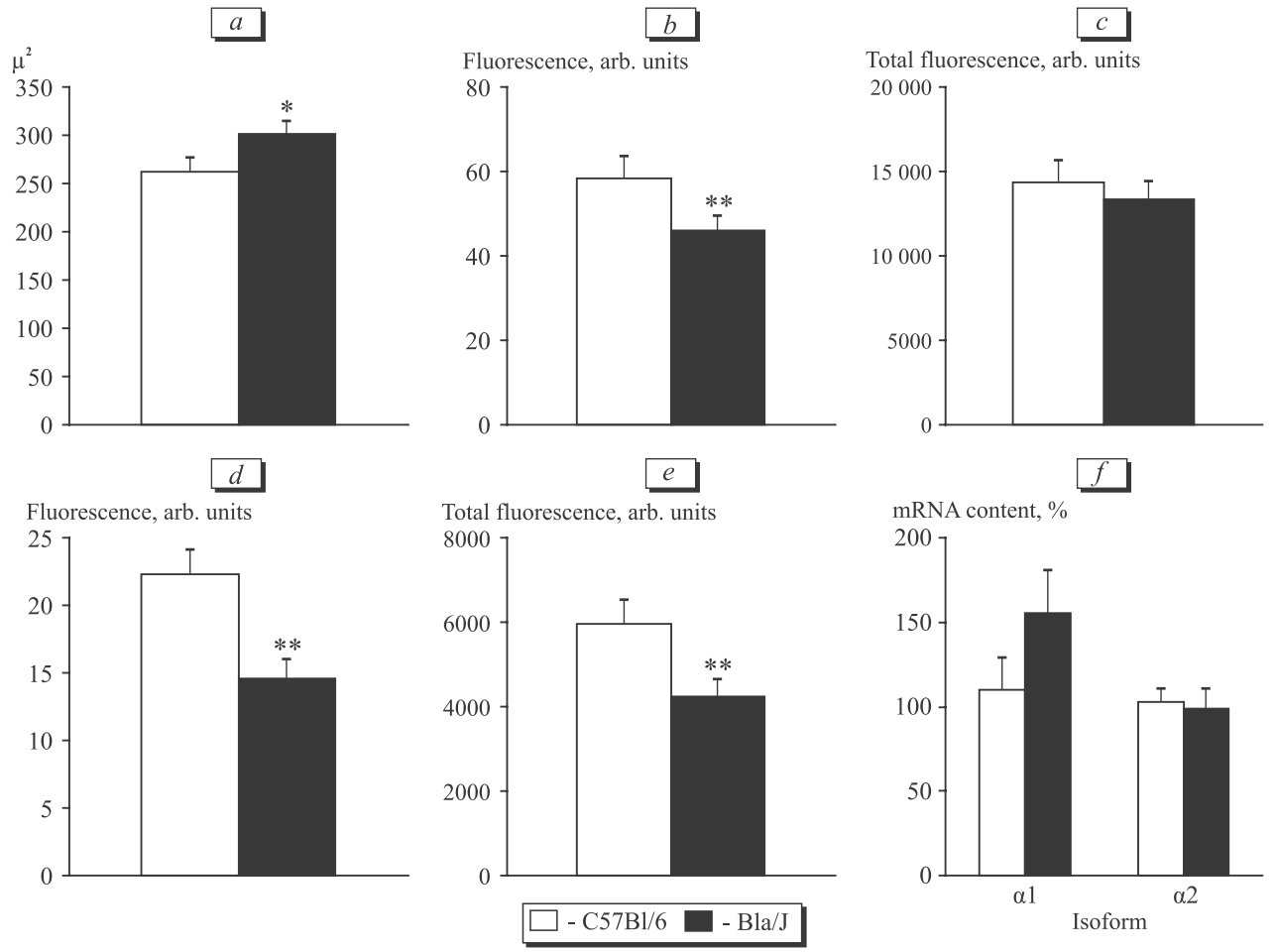

Fig. 2. Total endplate area (*a*), relative and total fluorescence intensity of α-BTX (*b*, *c*) and ouabain (*d*, *e*) over the entire area of the endplate, and Na,K-ATPase α1 and α2 mRNA content in total homogenate of *m. soleus* (mean for 6-8 muscles) (*f*). **p*<0.05, ***p*<0.01 in comparison with the control (C57Bl/6).

muscles from 5 Bla/J mice were analyzed. The total area of each endplate was determined as the sum of areas of all its fragments. In Bla/J mice, the total endplate area was greater by 15% than in C57Bl/6 mice (302±13 μ² *vs*. 262±15 μ², $p<0.05$, Fig. 2, *a*). Due to significant decrease in the relative α-BTX fluorescence intensity per endplate area unit (by 21%, $p<0.01$, Fig. 2, *b*), the relative level of α-BTX fluorescence intensity for the whole endplate area in Bla/J and C57Bl/6 mice was similar (Fig. 2, *c*).

It is known that α2 isoform of Na,K-ATPase is specifically localized in the endplate membrane region [6,7]. In our experiments, the fluorescent signal from α-BTX (nAChR) and ouabain (α2 Na,K-ATPase) in both mouse lines were localized in the endplate membrane and coincided when superimposed (Fig. 1). However, the intensity of ouabain fluorescence in Bla/J mice was significantly lower than in C57Bl/6 mice (by 34%; $p<0.01$; Fig. 2, *d*). The total level of ouabain fluorescence per endplate was reduced in Bla/J mice in comparison with C57Bl/6 mice ($p<0.01$, Fig. 2, *e*), However, the expression of mRNA encoding α1 and α2 isoforms of Na,K-ATPase was similar in Bla/J and C57Bl/6 mice (Fig. 2, *f*).

A strong positive correlation between the intensities of ouabain (α2 Na,K-ATPase) and α-BTX (nAChR) fluorescence was observed in both C57Bl/6 and Bla/J mice (Fig. 3, *b*).

We demonstrate a slight increase in the total area of endplates in *m. soleus* of Bla/J mice without changes in the degree of their fragmentation. At the same time, the relative fluorescence intensity of α-BTX per endplate unit area was decreased, which can reflect a compensatory decrease in the density of nAChR distribution in the membrane. This resulted in unchanged total fluorescence intensity of α-BTX over the entire area of the endplate in Bla/J mice in comparison with that in the control C57Bl/6 mice. Thus, changes in the structure of the endplates in *m. soleus* of Bla/J mice are less pronounced than in mdx mice [15], a laboratory model of Duchenne muscle dystrophy caused by loss of interaction between the cytoskeleton, dystro-

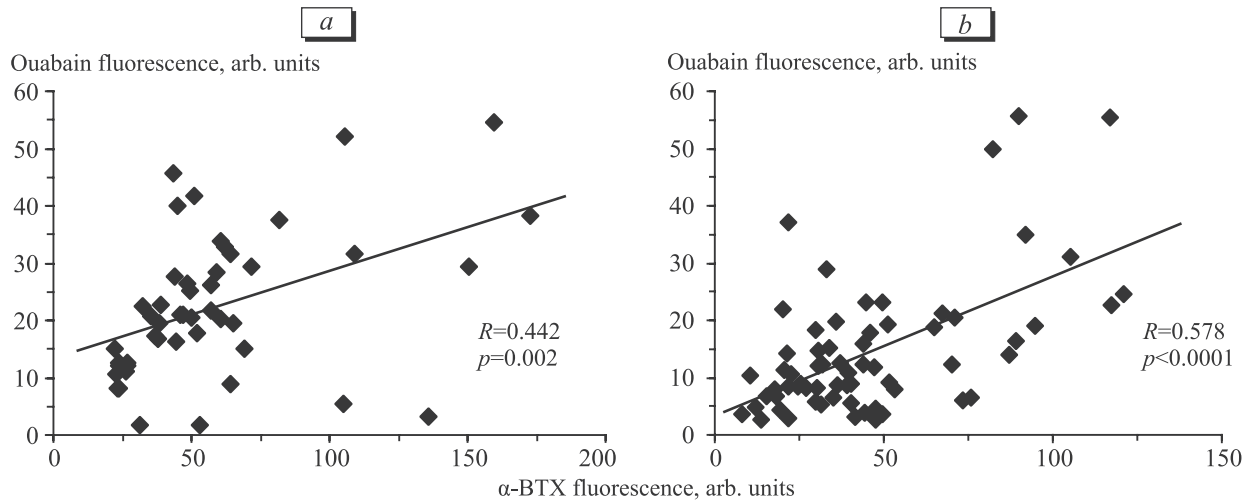

Fig. 3. Correlation between the intensities of ouabain (α2 Na,K-ATPase) and α-BTX (nAChR) fluorescence in individual endplates of *m. soleus* from C57Bl/6 (*a*) and Bla/J mice (*b*).

phin-glycoprotein complex, and extracellular matrix proteins due to impaired dystrophin synthesis.

In Bla/J mice, the relative fluorescence intensity of ouabain was significantly reduced in comparison with that in C57Bl/6 mice, which can reflect a decrease in the distribution density of α2 isoform of Na,K-ATPase in the endplate membrane region. This pool of α2 isoform interacts with nAChR [6,9] and cholesterol [8,14] thereby participating in the maintenance of postsynaptic electrogenesis. If the positive correlation between fluorescence intensities of ouabain (α2 isoform) and α-BTX (nAChR) reflects the interaction between these proteins, the presence of this correlation in Bla/J mice suggests that dysferlin deficiency does not significantly affect this interaction.

The mechanism for reduced density of the distribution of α2 isoform of Na,K-ATPase in the membrane of *m. soleus* in Bla/J mice under conditions of unchanged expression of its mRNA is unknown and can reflect disturbances in the translocation between intracellular and membrane pools of Na,K-ATPase. The decrease in electrogenic activity of Na,K-ATPase was shown previously in skeletal muscle of mdx mice under conditions of unchanged expression and phosphorylation of α1 isoform of Na,K-ATPase [11]; functional status of α2 isoform was not estimated [11]. We found no published data on the function of the Na,K-ATPase in dysferlin-deficient mice. Thus, we for the first time demonstrated disturbances in the membrane localization of α2 isoform of Na,K-ATPase that can be a result of adaptation to chronic motor dysfunction in this mouse model of dysferlinopathy.

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