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Transient receptor potential cation channels in normal and dystrophic mdx muscle

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Abstract

To investigate the defective calcium regulation of dystrophin-deficient muscle fibres we studied gene expression and localization of non-voltage gated cation channels in normal and mdx mouse skeletal muscle. We found TRPC3, TRPC6, TRPV4, TRPM4 and TRPM7 to be the most abundant isoforms. Immunofluorescent staining of muscle cross-sections with antibodies against TRP proteins showed sarcolemmal localization of TRPC6 and TRPM7, both, for mdx and control. TRPV4 was found only in a fraction of fibres at the sarcolemma and around myonuclei, while TRPC3 staining revealed intracellular patches, preferentially in mdx muscle. Transcripts of low abundance coding for TRPC5, TRPA1 and TRPM1 channels were increased in mdx skeletal muscle at certain stages. The increased Ca^{2+}-influx into dystrophin-deficient mdx fibres cannot be explained by increased gene expression of major TRP channels. However, a constant TRP channel expression in combination with the well described weaker Ca^{2+}-handling system of mdx fibres may indicate an imbalance between Ca^{2+}-influx and cellular Ca^{2+}-control.

Keywords: Duchenne muscular dystrophy; mdx mouse; Calcium influx; TRP channels; TRPC3; TRPC6; TRPV4; TRPM7; Sarcolemma

1. Introduction

Duchenne muscular dystrophy (DMD) and its animal model murine muscular dystrophy (mdx) are characterized by the lack of dystrophin, a submembraneous cytoskeletal protein [1,2]. Dystrophin deficiency leads to necrosis of muscle fibres, muscle damage, fibrosis and progressive muscle weakness [3]. Though mdx mice show a much milder phenotype than DMD patients, their fibres also undergo the typical cycles of necrosis and regeneration [4]. In contrast to muscles of DMD patients, mdx muscle is characterized by continuous regeneration and tissue remodelling, effects that seem to compensate effectively for the lack of dystrophin. Nevertheless, the mdx mouse is regarded as a suitable model to study the pathomechanism that connects dystrophin deficiency to fibre necrosis [5].

It is widely accepted that an abnormal calcium influx and abnormal intracellular calcium handling are key factors in the destructive process leading to muscle fibre degeneration in dystrophin-deficient muscle [6–10]. The total muscle calcium content is increased in DMD [11,12] and mdx muscle [13] while the free global cytoplasmic calcium level of mdx muscle fibres is unchanged or at least close to that of normal fibres [14–16]. It is assumed that effective compensatory mechanisms extrude excessive calcium from the cytoplasm and that fibres can adjust a nearly normal free cytoplasmic calcium level at least at rest. However, a more sophisticated approach revealed that mdx myotubes can develop much higher calcium transients in the sub-sarcolemmal area than in the bulk cytosol. Carbachol-induced Ca^{2+} transients below the plasma membrane were in average 4.5-fold higher in mdx myotubes compared with controls [17]. In adult, resting mdx fibres the sub-sarcolemmal calcium concentration was estimated to be three-

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fold higher than that of control fibres using Ca^{2+}-activated K^+ channels as indicators [18]. Increased Ca^{2+} influx and increased Ca^{2+} levels may stimulate proteolytic activity which is indeed higher in mdx compared to control muscle [19]. As a consequence, affected muscle fibres may become necrotic or apoptotic and release mitogenic chemoattractants which initiate inflammatory processes [20]. Finally, the continuous cycles of degeneration and partial regeneration lead to irreversible muscle wasting and replacement of muscle tissue by connective and adipose tissue.

While Ca^{2+} influx is increased in mdx muscle fibres, the concentration of certain calcium binding proteins is reduced. Calsequestrin-like proteins [21] and sarcalumenin [7], proteins that are involved in sarcoplasmic Ca^{2+} storage, are markedly decreased and show an abnormal staining pattern in mdx muscle. This observation may indicate that dystrophin-deficient fibres have a weaker system to keep their free intracellular Ca^{2+} within the physiological range.

Though many abnormalities in calcium handling have been described in dystrophin-deficient fibres, the mechanisms of Ca^{2+} entry are still a matter of debate. Voltage-gated L-type Ca^{2+} channels do not seem to account for the increased Ca^{2+} influx, since they have a low open probability at resting potential. It was shown that L-type Ca^{2+} current densities increased with age in mdx fibres, but were always significantly smaller than those of age-matched control fibres [22]. Stretch-activated channels [23], calcium leak channels [6] mechanosensitive channels [24], insulin-like growth factor (IGF-1) activated channels [25] and store-operated channels [26] are regarded as candidates for influx pathways of Ca^{2+} into muscle fibres. Recently, physiological and pharmacological data indicated that stretch-activated channels and store-operated channels belong to the same channel population in skeletal muscle or share common constituents [27].

We have also described an influx pathway for divalent cations into skeletal muscle fibres. It is active at rest and the rate of influx in mdx fibres was found to be twofold higher as in normal fibres [28]. Based on physiological data on divalent cation influx and sarcolemmal channel activity we hypothesized that candidates for influx channels could be members of the degenerin/epithelial Na^+-channel (DEG/ENaC) family of cation channels or members of the transient receptor potential (TRP) ion channel superfamily. Vandebrouck and coworkers showed that TRPC1, 4 and 6 are localized at the sarcolemma of mdx muscle fibres [29] and suggested that these proteins are the molecular counterparts of the above mentioned Ca^{2+}-leak channels. However, in the latter study only one subfamily, TRPC, of the large TRP channel superfamily [30] has been investigated. The superfamily of TRP channels has been divided into six subfamilies in mammals, TRPC, TRPV, TRPM, TRPA, TRPP and TRPML according to structural similarities and modes of activation [30]. The members of subfamilies TRPC, TRPV, TRPM and TRPA comprise the Group 1 TRPs, while the TRPP and TRPML members are classified as the Group 2 TRPs [30]. The overexpression of TRPV2 can lead to pathological changes due to Ca^{2+} overload in cardiac muscle [31]. Thus, TRPV channel dysfunction may contribute to the pathogenesis of degenerative diseases, such as muscular dystrophy.

The present study was designed to identify candidates of cation channels responsible for the abnormal Ca^{2+} influx in dystrophin-deficient muscle. To obtain an overview about the expression of cation channel genes in skeletal muscle, we studied the expression of all DEG/ENaC channels and all group1 TRP channels on the RNA level in control and mdx mice. For the first time we established a complete set of gene expression data for all relevant non-voltage gated cation channels. In addition, the presence of four major Ca^{2+} conducting TRP channels was studied on the protein level and their cellular localization was investigated by immunochemistry.

2. Materials and methods

2.1. Animals

Mice of the inbred strains C57Bl/10Sc/J (control) and C57Bl/100ScSn-*Dmd*^{mdx}/J (mdx) were originally obtained from Charles River (Sulzfeld, Germany). Animals of either sex were used for the study at ages 30, 100 and 365 d. Both strains were bred in the Department of Laboratory Animal Science of the Medical Faculty at the University of Greifswald. All animals were killed using ether inhalation according to the regulations of the University of Greifswald. Tissue samples were removed quickly and frozen in liquid nitrogen for real-time RT-PCR and situated in formalin-buffer for *in situ* hybridisation and immunohistochemistry. Muscle tissue from TRPC3 knockout mice was kindly provided by Dr. L. Birnbaumer, National Institute of Environmental Health Service, NC, USA.

2.2. TaqMan RT-PCR

TaqMan RT-PCR assays of mouse TRP channel genes were performed as described previously [32]. To quantify gene expression of mouse DEG/ENaC channels, voltage gated Na^+ channels SCN4A and SCN5A and the skeletal muscle ryanodine receptor (RyR1) we purchased gene-specific TaqMan PCR primers and probes from PE Applied Biosystems (Weiterstadt, Germany, Table 1) or TIBMOLBIOL (Berlin, Germany, Table 2) with each probe having

Table 1
Primers and probes for real-time RT-PCR of ion channel transcripts

Gene	Accession Nos.	Assay-on-demand
ACCN1	NM_007384	Mm00475691_m1
SCN4A	NM_133199	Mm00500103_m1
SCN5A	NM_021544	Mm00451971_m1
RYR1	NM_009109	Mm01175172_g1
α1-Actin	NM_009109	Mm01175172_g1

The sets of probes and primers for detection by TaqMan RT-PCR were obtained from PE Applied Biosystems.