Tissue Specific Role of the Na,K-ATPase α2 Isozyme in Skeletal Muscle *

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Background: The Na,K-ATPase α2 isozyme is the major Na,K-ATPase of skeletal muscles.

Results: Its targeted knockout in mouse skeletal muscle impairs contractility and fatigability without change in resting muscle function.

Conclusion: The α2 isozyme is regulated by muscle use and enables working skeletal muscles to maintain contraction and resist fatigue.

Significance: The Na,K-ATPase α2 isozyme is vital to muscle and movement.

SUMMARY

The Na,K-ATPase α2 isozyme is the major Na,K-ATPase of mammalian skeletal muscle. This distribution is unique compared to most other cells which express mainly the Na,K-ATPase α1 isoform, but its functional significance is not known. We developed a gene-targeted mouse (skα2−/−) in which the α2 gene (ATP1A2) is knocked out in the skeletal muscles, and examined the consequences on exercise performance, membrane potentials, contractility and muscle fatigue. Targeted knockout was confirmed by genotyping, Western Blot and immuno histochemistry. Skeletal muscle cells of skα2−/− mice completely lack α2 protein and have no α2 in the transverse tubules, where its expression is normally enhanced. The α1 isoform, which is normally enhanced on the outer sarcolemma, is upregulated 2.5-fold without change in subcellular targeting. Skα2−/− mice are apparently normal under basal conditions but show significantly reduced exercise capacity when challenged to run. Their skeletal muscles produce less force, are unable to increase force to match demand, and show significantly increased susceptibility to fatigue. The impairments affect both fast and slow muscle types. The subcellular targeting of α2 to the transverse tubules is important for this role. Increasing Na,K-ATPase α1 content cannot fully compensate for the loss of α2. The increased fatigability of skα2−/− muscles is reproduced in control EDL muscles by selectively inhibiting α2 enzyme activity with ouabain. These results demonstrate that the Na,K-ATPase α2 isoform performs an acute, isoform-specific role in skeletal muscle. Its activity is regulated by muscle use and enables working muscles to maintain contraction and resist fatigue.

The Na,K-ATPase is a vital enzyme in all living cells. It maintains the Na⁺ and K⁺ gradients and cell volume, provides the driving force for many other membrane transport processes, and serves as a membrane receptor in...
cell signaling and other non-transport roles (1). The functional enzyme is composed of a primary catalytic α subunit, a β subunit and, in some cells a FXYD subunit. Four α subunit isoforms (α1, α2, α3, and α4), three β subunit isoforms (β1, β2, and β3), and seven FXYD subunits have been identified (reviewed in: 2-4). The α1 isoform is ubiquitously expressed and is the predominant or sole α subunit in most cells, while the α2–α4 isoforms show more restricted cellular and subcellular distributions (4). The α1 isoform is widely considered to be the “housekeeping” isozyme which maintains basal ion homeostasis.

Adult skeletal muscles are distinct in that they express the Na,K-ATPase α2 subunit as the major isoform, at up to 87% of total Na,K-ATPase alpha subunit content (5-6); the α1 isoform is a minor Na,K-ATPase subunit. However, the physiological significance of this distribution is not known.

A striking finding in skeletal muscle is that the α2 isozyme, despite its significantly greater abundance, makes only a minor contribution to basal Na⁺ and K⁺ gradients and the resting potential. Removal of α2 in a global α2 knockout mouse (α2/-; 7) depolarizes the resting potential of neonatal diaphragm muscle by 2-4 mV (8-9). The diaphragm, in contrast to other skeletal muscles, expresses α2 from birth. Similarly, adult skeletal muscles lacking 50% of α2 content (α2-/- HET knockout) maintain normal Na⁺ and K⁺ concentrations and resting potentials (6,8). The low basal activity of α2 is not due to sequestration in intracellular pools because it readily binds extracellular ligands including labeled ouabain (10). Measurements in native, intact rat skeletal muscles also support this conclusion. Basal Na⁺/K⁺ transport by α2, measured as the electrogenic component of the resting potential, contributes 4-5 mV to the resting potential of rat diaphragm; while α1 activity generates 15-18 mV (11-12). Therefore, α2 contributes only 25% of the basal Na⁺/K⁺ transport required to set resting ion concentrations and the resting potential. Because skeletal muscles at rest utilize less than 5% of their total capacity for active Na⁺/K⁺ transport by both isoforms (13), the low basal activity of α2 represents less than 1% of the total capacity of skeletal muscles for Na⁺/K⁺ transport. These independent findings support the conclusion that the Na,K-ATPase α2 isozyme is not highly active in resting skeletal muscle and beg the question: What is its physiological role?

It is known that Na,K-ATPase activity is stimulated up to 20-fold within seconds of the onset of contraction (13) and that this stimulation is absolutely required to maintain excitation and contraction (14). However, the contributions of α1 and α2 to contraction-related Na,K-ATPase activity are not known.

To investigate the role of α2 during contraction, we generated a novel gene targeted mouse in which the Na,K-ATPase α2 subunit gene (ATP1A2) is knocked out in the skeletal muscles (skα2-/- mouse). This targeting strategy overcame problems with the global α2 knockout mouse which dies at birth (15). The α2 gene is developmentally regulated in mice and plays no role in early development (5). It is induced in voluntary skeletal muscles at 2-3 weeks postnatal (16), when the transverse tubules form and skeletal muscle cells undergo terminal differentiation (17). Therefore, this mouse model is a targeted, naturally conditional α2 knockout in adult voluntary skeletal muscles. We compared the following parameters in control and skα2-/- mice: cellular and subcellular content and localization of α1 and α2 protein, resting membrane potentials, exercise performance, contractility and fatigability of skeletal muscles in vivo, and contractility and fatigability of isolated skeletal muscles in vitro.

Our results show that Na,K-ATPase α2 isozyme activity is acutely regulated by muscle use and that its contraction-related transport enables working skeletal muscles to maintain contraction and resist fatigue. This role is isoform-specific and cannot be performed by the Na,K-ATPase α1 alone, despite its compensatory upregulation in the skeletal muscles of skα2-/- mice. These results are consistent with the proposal that the Na,K-ATPase α2 isoform may provide a reserve capacity which comes into play to meet the increased demand for Na⁺/K⁺ transport during contraction.
**EXPERIMENTAL PROCEDURES**

Creation of skeletal muscle targeted Na,K-
ATPase α2 knockout mice, α2<sup>Flox/Fl</sup>/MyoDiCre mice *(abbreviated ska2α*) – ska2α mice (Fig. 1) were produced by mating α2 LoxP mice (α2<sup>Flox/Fl</sup>) in which the ATP1A2 gene is flanked by LoxP sites (18) with heterozygous MyoDiCre knockin mice in which the iCre gene is knocked into the MyoD locus (19). Genotyping was determined through allele-specific PCR to detect the α2 5'loxP site using forward primer P1 (AGT CCT AAG GTG CCC GCT GCC) and reverse primer (GGG CAG GTA GGC GCT GAC CCT); recombination between the 5' and 3'loxP sites was detected with the forward primer P1 and recombination primer (CTT GTG GGC TAA GGA CC), and MyoDiCre was detected using forward primer (GGT GTC TCC AAA GGC ACT CC) and reverse primer (GGC GAT CCG AAT TCG AAG TTC C). The α2<sup>Flox/Fl</sup> mice, which possess the floxed α2 locus and endogenous MyoD promoter without the presence of iCre, served as our standard control. We confirmed that contractile parameters are not significantly different among the possible control mice – α2<sup>Flox/Fl</sup> mice, “true” wild type mice, or mice with iCre and no LoxP – validating the use of α2<sup>Flox/Fl</sup> mice as control for this study. Mice were produced and maintained by the Transgenic Mouse Service of the University of Cincinnati. Adult mice of 4 – 18 weeks of age were used for measurements. All procedures were performed in accordance with guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee. Tissue removal was performed using anesthesia (2.5% Avertin, 17 µl/kg) and the animals were euthanized after the experiment. To reduce possible differences in contractile history, mice of both genotypes were housed identically without exercise wheels or training.

**Microsomal membranes** – were prepared as described (20, 16). Protein concentration was determined using a BCA assay (Thermal Scientific, Rockford, IL).

**Primary antibodies** – a6F anti Na,K-
ATPase α1 monoclonal antibody (21); anti Na,K-ATPase α2 polyclonal antibody, affinity purified from antisera generated using the synthetic HERED peptide (22-23); anti-smooth muscle alpha actinin 1E12, anti-IIA MHC SC-71, anti-IIB MHC BF-F3, and anti-I MHC BA-F8 (University of Iowa Developmental Studies Hybridoma Bank).

**Western Blot** – Western Blot analysis was performed using specific antibodies and molecular weight standards, as described (16,24). Equal protein loading was confirmed by staining total protein with Ponceau S (Sigma-Aldrich) (25). Differences were confirmed in at least three independent samples from each genotype.

**Immunocytochemistry** – Muscles were dissected from anesthetized mice, coated with Tissue-Tek O.C.T. Compound and frozen for 1 min in isopentane cooled in liquid nitrogen. Serial cross-sections (8 - 10 µm) were cut and placed on glass slides, fixed in 100% acetone at -25°C for 5 min, transferred to PBS (pH 7.2, 22°C) and processed immediately. To unmask antigens, slides were treated with 10 mM Na citrate and 0.5% Tween-20 (pH 8) in a water bath at 100 C for 20 min, cooled for 10 min under running water, and washed twice for 1 min in room temperature PBS. Non-specific sites were blocked using 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc) in PBS for 1 hr at room temperature. Sections were incubated with primary antibody for 1.5 hrs at room temperature or overnight at 4°C, washed in PBS, and incubated with secondary antibody for 1 hr at room temperature, followed by washes in PBS, and mounting (Fluoromount-G, SouthernBiotech). Antibodies were diluted in PBS with 10% normal goat serum (a6F, 1:10; anti-Na,K-ATPase α2, 1:50; 1 E12 anti-smooth muscle alpha actinin, 1:20; myosin heavy chain (MHC) isoform antibodies, 1:20). Sections without primary antibody served as negative control. Secondary antibodies were Alexa Fluor 594 F(ab')<sub>2</sub> or Alexa Fluor 488 F(ab')<sub>2</sub> (1:200, Invitrogen). Some sections were dual labeled with Na,K-ATPase anti α2 antibody and α-bungarotoxin conjugated with Alexa Fluor 555 (Invitrogen) to confirm the presence of end plates, or anti α2 antibody with anti α-actinin antibody to identify vascular smooth muscle. Images were captured with a conventional fluorescence microscope or a confocal laser microscope (Carl Zeiss LSM510). Control and ska2α<sup>-/-</sup> samples were processed in parallel and imaged under identical conditions. Results were
confirmed with 3-4 independent control and ska2−/− muscles.

Bodipy-ouabain labeling of freshly isolated muscle – Freshly isolated EDL muscles of control and ska2−/− mice were incubated for 15 min. in physiological saline containing 500 nM bodipy-conjugated ouabain (Invitrogen), followed by washout in saline without ouabain. Superficial fibers were imaged in longitudinal view by confocal microscopy. We have previously shown, using a genetically modified mouse in which the Na,K-ATPase α2 isoform is rendered resistant to the binding of ouabain, that bodipy-ouabain at this concentration selectively binds the Na,K-ATPase α2 isoform in mouse skeletal muscle (10).

Treadmill exercise – Performed as described (24). Control and gene targeted mice were run in parallel.

Contractility in vivo – The extensor digitorum longus (EDL) muscle of anesthetized mice was surgically separated from surrounding tissues while preserving nerve and blood supply, and the distal tendon was connected to an isometric force transducer (Harvard Apparatus). The muscle was stimulated by platinum wire electrodes positioned under the sciatic nerve in the peroneal region.

Contractility of isolated skeletal muscles in vitro – Rest length was measured in vivo prior to removing the muscle. An isolated EDL or soleus muscle was mounted horizontally in a chamber between two platinum plate electrodes. One tendon was fixed and the other tendon was attached to an isometric force transducer (BG-50, Kulite Semiconductor Products, Inc.). The recording chamber was covered and contained 1 ml of physiological saline (mM): 118 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 0.026 EDTA, 11 glucose, equilibrated with 95% O2- 5% CO2 at room temperature (22°C), pH 7.4, perfused at 1.5 ml/min. Muscles were stimulated by 0.5 ms pulses. Supramaximal voltage was adjusted empirically and fixed at 1.5 x threshold. Optimal muscle length was adjusted to achieve maximal isometric twitch force. Resting and optimal lengths were 10-12% greater than muscle slack length determined with zero applied load and were not different between groups or for twitch and tetanic contraction (26). Approximately 5 mN load was required to reach optimal length for muscles of both genotypes. The optimal length for twitch and tetanic contractions was not significantly different, as expected (27). Force was recorded digitally (BioPac System, Goleta, CA). Specific force (mN/mm²) was calculated by normalizing force to muscle cross-sectional area estimated as muscle weight divided by muscle length (27).

Fatigability was assessed using a protocol which control and ska2−/− genotypes produced the same peak tetanic force in response to a single tetanic stimulation from the rested state. The protocol included, in order: 1) determine stimulus amplitude and optimal rest length (lₒ); 2) record reference tetanic force at 40 Hz, 400 ms; 3) apply single stimuli every 3 min to achieve constant twitch force; 4) apply tetanic stimuli of 40-125 Hz/400 ms every 3 min to determine peak tetanic force at each frequency and maximum tetanic force; 5) repeat reference tetanus (end); 6) apply fatiguing stimulation until force declines. 7) measure lₒ and muscle weight. This protocol provided multiple contractile measures from the same muscle with high reproducibility in a typical recording period of 30 min. (as shown in the inset to Fig. 8). In some experiments, control muscles were pre-incubated for 90 min. in physiological saline containing 5 µM ouabain (Sigma-Aldrich).

Data analysis – Parameters are expressed as group means ± sem. Differences were analyzed by one-way analysis of variance (ANOVA followed by Tukey’s post-hoc test) or Student’s t-test, as appropriate, with statistical significance defined at P < 0.05.

RESULTS

The skeletal muscle targeted Na,K-ATPase α2 isoform knockout mouse, ska2−/− (Fig. 1), was produced by mating α2 LoxP mice (α2Flox/Flox) in which the ATP1A2 gene is flanked by LoxP sites (18) with MyoDiCre knockin mice in which the iCre gene is knocked into the MyoD locus (19). MyoDiCre mice faithfully express the recombinase in all skeletal muscle lineage cells (19).

The Na,K-ATPase α2 isoform is absent from muscle fibers of ska2−/− mice but retains its normal localization in non-muscle cells within the muscle tissue. Selective knockout of Na,K-ATPase α2 subunit protein was confirmed by
Western Blot (Fig. 2), and immuno histochemistry and labeled binding (Fig. 3). Na,K-ATPase α2 protein is not detected in the skeletal muscles of skα2−/− mice but remains at normal levels in other muscles (heart and aorta) and tissues. Na,K-ATPase α1 isoform protein is upregulated 2.5-fold in the skeletal muscles without change in its content in other tissues.

In control muscle, the Na,K-ATPase α2 isoform shows a highly specific cellular and subcellular localization. Within muscle cells (fibers), it is expressed on the transverse tubule membrane (3A, 3D), and discreet regions of the surface sarcolemma including the muscle end plate, caveolae and costameres (10,28,36), and on the sheath surrounding the muscle spindle (not shown). In non-muscle cells within the tissue (3B), α2 is expressed in some axons of the motor nerve, the nerve perinureum, and the vascular smooth muscle layer of arteries. The α2 isoform is absent in all skeletal muscle fibers of skα2−/− mice, but its distribution in non-muscle cells is not changed (3C). The transverse tubules are more easily visualized in longitudinal images of intact muscle labeled with a fluorescent ligand of the Na,K-ATPase. We have previously shown, using a genetically modified mouse in which the α2 isoform is rendered resistant to the binding of ouabain (10), that the Na,K-ATPase α2 isoform is present on the surface and transverse tubule membranes of mouse diaphragm muscle. Here, we confirm that α2 is present in these membranes of control EDL (3D), as expected, and is absent in skα2−/− EDL (3E). The transverse tubule labeling of Na,K-ATPase α2 is seen as double rows of bodipy-ouabain label having a periodicity of two rows per sarcomere, consistent with the location of transverse tubules at the A-I junctions of mammalian skeletal muscle.

Na,K-ATPase α1 isoform localization is not altered in skeletal muscles of skα2−/− mice. Because α1 protein is upregulated in the skeletal muscles of skα2−/− mice, we examined its subcellular localization (Fig. 4). Control muscle fibers express the α1 isofrom mainly on the surface sarcolemma, as reported (28). Skα2−/− muscle fibers show enhanced α1 expression on the surface sarcolemma, consistent with the increased α1 protein detected on Western Blot.

The Na,K-ATPase α1 isoform is reported to be absent from the transverse tubules (28). However, in some fixed longitudinal sections imaged with confocal optical sectioning and enhanced intensity, our α1 antibody detects it in the transverse tubules of both genotypes (4C-4F). Therefore, α1 may also be present in the transverse tubules, possibly at superficial regions and/or low abundance. The important finding is that the membrane targeting of α1 is not changed in skα2−/− muscle. Therefore, because α1 is enhanced on the surface sarcolemma and α2 is absent in skα2−/−, the skα2−/− muscle has little to no Na,K-ATPase of either isofrom in the transverse tubules.

Skα2−/− mice show significantly impaired exercise capacity. The skα2−/− mice are viable and apparently normal under basal laboratory conditions. They ambulate, obtain food, and perform other tasks of daily living. However, when challenged with graded treadmill running, they show significant impairment (Fig. 5). This test requires that the skeletal muscles increase and maintain force over the entire physiological range. Control mice easily run up to 26 m/min and beyond, and are able to maintain running for the duration of the 2 min. trial at each speed. In contrast, skα2−/− mice are unable to maintain running even at speeds as low as 4 meters/min. This speed is just a “fast walk” for normal mice. Skα2−/− mice fail significantly at higher speeds and their failure is evident immediately upon the onset of running, before other local and systemic factors that contribute to exercise performance in the animal have had time to fully develop.

The skeletal muscles of skα2−/− mice fatigue rapidly in vivo. The specific impairments in exercise performance, with rapid onset and dependence on exercise intensity, suggest that the skeletal muscles of skα2−/− mice are more susceptible to fatigue. Running requires that the skeletal muscles increase and maintain force in response to higher stimulation frequencies from the motor nerve. Therefore we compared the fatigability of skeletal muscles of control and skα2−/− mice in vivo in response to repetitive nerve stimulation, the physiological mode of muscle activation. In this condition, the muscle retains normal neuromuscular transmission, blood supply, and other systemic factors.
The only genetic difference is the presence or absence of the Na,K-ATPase α2 gene.

This analysis was performed using EDL muscles from the same cohort of mice used in the exercise trials. The mouse EDL has a fiber type composition representative of the hindlimb muscles used in running (29). It is a fast twitch, fatigue susceptible muscle which expresses the Na,K-ATPase α2 isoform at up to 87% of total α subunit content (6). Therefore, if the α2 isoform plays any role in fatigue resistance, its removal from this muscle is expected to cause an observable loss of function.

The EDL of skα2−/− mice is significantly more fatigue-susceptible than control (Fig. 6). It is completely unable to produce force after only 27 sec of continuous stimulation at 20 Hz (6A). This is a very mild stimulation protocol for a control EDL which still produces 70% of peak force at 27 seconds. A control EDL muscle can respond to continuous nerve stimulation at frequencies up to 125 Hz. When stimulated with a moderate fatigue producing protocol (intermittent 40 Hz tetani, 6B), force in skα2−/− EDL decays to 15% of peak in only 3 min (6B), while control EDL muscles continue to maintain plateau force. The mean onset of fatigue is significantly different between genotypes (6C). Table 1 gives the average contractile parameters from all mice used to assess exercise and contractility in the animal.

These results exclude the possibility that the increased fatigability results from a failure in neuromuscular transmission. It was important to verify this point in vivo because the Na,K-ATPase α2 isoform is expressed at the neuromuscular junction (10). The ability of the skα2−/− EDL to generate force in response to nerve stimulation also indicates that the molecular mechanisms of muscle activation - from neuromuscular transmission and excitation of the plasma membrane through Ca2+ release from the sarcoplasmic reticulum- are functional in skα2−/− muscle.

Isolated skeletal muscles of skα2−/− mice show similar contractile impairments and increased susceptibility to fatigue. The contractile impairment in vivo suggests that the Na,K-ATPase α2 isoform is essential for working muscles to resist fatigue and maintain contraction. To further examine this hypothesis, we measured membrane potentials and contractility in isolated intact skeletal muscles in vitro. The in vitro condition removes all systemic inputs and allows a more quantitative comparison of resting and contractile function under controlled conditions. It minimizes the possibility that knock-out of the α2 gene in the skeletal muscles may have introduced secondary changes in other systems which influence muscle performance in the animal.

The basal activity of the Na,K-ATPase performs an essential role in maintaining ion gradients and resting potential. However, reported measurements of resting potentials in skeletal muscles suggested that the Na,K-ATPase α2 does not perform this canonical role to any great extent in skeletal muscle (8-9, 11-12). Therefore we measured the resting potentials of adult EDL muscles of control and skα2−/− mice (Table 2). Resting membrane potentials in skα2−/− and control are not significantly different (P > 0.1), indicating that the Na,K-ATPase α2 isoform is not required to set the resting potential. This conclusion was further confirmed in control EDL muscles by selectively inhibiting Na,K-ATPase α2 activity with a low concentration of ouabain (5 μM). In rodent skeletal muscle, this concentration inhibits essentially all active transport by the α2 isoform while inhibiting ~ 20% of α1 transport (12). Control EDL muscles depolarized by -3.6 mV after 5 μM ouabain (P < .001). This small depolarization is comparable to that of adult rat diaphragm treated with 5 μM ouabain (12).

In contrast to its minor effect on resting muscle function, removal of α2 has a pronounced effect on contracting muscle. Peak twitch force and maximum tetanic force are 24% and 40% lower, respectively (Fig. 7A, 7B), indicating some muscle weakness in the skα2−/− mice. The increased ratio of twitch to tetanic force indicates that the contractile impairment is greater during repetitive stimulation. There were no significant differences among the three control genotypes, confirming that the presence of LoxP sites or the MyoDCre knockin does not change maximum twitch or tetanic force. The skα2−/− EDL is unable to increase force with increasing frequency of stimulation (Fig. 7C) and produces less tetanic force at all frequencies. Maximum tetanic force occurs at 80 Hz in the
skα2−/− EDL, and declines at higher frequencies; while control EDL muscles continue to increase force up to 125 Hz. The force-frequency responses of all control genotypes (α2Flox/Flox, wt cre−, and wt cre+) were not significantly different. Contractile parameters from all measurements in vitro are given in Table 3. Control EDL muscles are able to maintain force for the duration of a 400 ms tetanus at 125 Hz, while force in the skα2−/− EDL declines by 44% in this condition. There were small changes in the kinetics of twitch but not tetanic force.

Notably, the skα2−/− EDL is significantly more susceptible to fatigue (Fig. 8). In response to mild fatiguing stimulation, force declines to 50% in less than 3.5 min, compared to 4.5 min in control. There was no difference in the onset of fatigue in any control genotype (Fig. 8A). The skα2−/− EDL also shows a small initial increase in force preceding the decline (Fig. 8B). Table 4 summarizes the fatigue parameters measured from all mice in vitro.

These independent measures indicate that skeletal muscles of skα2−/− mice have a reduced ability to resist fatigue and are unable to increase and maintain force to match demand. 40 Hz is a mild stimulation frequency for the EDL, which can normally respond up to 120 Hz. The failure of skα2−/− EDL at this mild frequency underscores its significantly increased susceptibility to fatigue. The impaired contractility of skα2−/− muscles per se, both in vivo and in vitro, predicts the impaired exercise performance of the animal. A frequency-dependent impairment in contractility and decreased resistance to fatigue in the locomotor muscles is expected to impair exercise capacity in the animal.

Inhibition of the Na,K-ATPase α2 isoform in control EDL recapitulates the increased fatigue susceptibility of skα2−/− EDL. The Na,K-ATPase is capable of performing both transport and non-transport roles (1). The acute contribution of α2 to fatigue resistance suggests that enzyme transport is essential for this function because signaling actions require longer times. To examine this question, we measured the fatigability of control muscles after 90 min. incubation in 5 µM ouabain (Fig. 8). The treated muscles fatigue significantly faster than untreated controls (Fig. 8B). The development of fatigue in treated muscles follows a time course nearly identical to that of the skα2−/− EDL. It is possible that the membrane depolarization due to loss of α2 electrogenic activity (12) may impair contraction. However, the resting potential of control muscles depolarized by only -3.6 mV after 90 min. in 5 µM ouabain (Table 2), consistent with previous measurements of the small contribution of α2 to the resting potential (8,11,12). This depolarization is not expected to prevent action potential excitation under our conditions because the muscle is activated directly by a suprathreshold extracellular stimulus. This expectation was confirmed empirically. Single twitch amplitude and stimulus threshold in control muscles did not change during ouabain treatment (inset, 8B). Twitch force, maximum tetanic force, and the force frequency relationship of ouabain-treated control EDL were not obviously different from untreated controls. No significant changes in gene expression are expected over this time period and the only known receptor for ouabain is the Na,K-ATPase. Our finding that acute removal of α2 function in a control animal produces the same effect on fatigue as genetically removing the gene argues strongly that the functional phenotype in skα2−/− mice is due to lack of enzyme transport by the α2 Na,K-ATPase. This finding demonstrates that the contribution of the Na,K-ATPase α2 isoform to fatigue resistance requires enzyme activity.

The soleus muscle of skα2−/− mice shows weakness and greater fatigability. The majority of our analysis focused on the fast twitch EDL muscle because its fiber type composition closely matches the overall mix of fiber types used in running. Fig. 9 and Table 5 show that the slow-twitch soleus muscle of skα2−/− mice also produces less peak twitch and maximum tetanic force and is unable to maintain force for the duration of a 10 sec tetanus at frequencies ≥ 40 Hz. These impairments are remarkable for the soleus, a normally highly fatigue resistant muscle. They indicate that the Na,K-ATPase α2 isoform is required to support contraction and resist fatigue in both slow- and fast twitch muscles.
DISCUSSION

This study reports the following new findings. We have developed and characterized a novel gene targeted mouse in which the Na,K-ATPase α2 isoform, the major Na,K-ATPase isoform of adult skeletal muscle, is knocked-out in the skeletal muscles (skα2<sup>−/−</sup>) without change in its content or localization in other tissues. The successful use of MyoD<sup>cre</sup> mouse (19) to generate a skeletal muscle targeted knockout mouse and the efficiency of α2 knockout suggest that this strategy may be useful for targeting other skeletal muscle genes.

The Na,K-ATPase α1 subunit, the minor Na,K-ATPase isoform of adult skeletal muscle, is upregulated approximately 2.5-fold in the skeletal muscles of skα2<sup>−/−</sup> mice, without change in its expression in other tissues. In control muscles, the α1 isoform is localized largely to the surface sarcolemma (28) and its localization does not change in skα2<sup>−/−</sup> muscle. This result was expected since the Na,K-ATPase α1 subunit gene, including any targeting sequences, was not altered. Therefore, this genetic change the skα2<sup>−/−</sup> muscles with little or no Na,K-ATPase of either isoform in the transverse tubules.

Skα2<sup>−/−</sup> mice exhibit a phenotype characterized by significantly reduced exercise capacity with rapid onset. The impairment is seen at low exercise intensities and increases with increasing exercise intensity. The exercise impairment in the animal is due, in part, to a specific loss of function in the locomotor skeletal muscles. The skeletal muscles produce less twitch and tetanic force, are unable to increase force to match demand, and are significantly more susceptible to fatigue. The impairment is seen early into muscle contraction and occurs both in vivo and in isolated skeletal muscles in vitro, confirming that the targeted gene change has produced a specific functional deficit in the skeletal muscles per se. The measurements in vitro also confirm that the molecular mechanisms of muscle activation - from neuromuscular transmission and excitation of the plasma membrane through Ca<sup>2+</sup> release from the sarcoplasmic reticulum- are preserved in skα2<sup>−/−</sup> mice. The contractile deficits occur in both slow and fast muscle types and predict the exercise impairment in the animal.

Skeletal muscle fibers of skα2<sup>−/−</sup> mice maintain normal resting potentials, despite the removal of 60-90% of total Na,K-ATPase content. This confirms that Na,K-ATPase α2 activity is not essential for setting the resting potential of skeletal muscles.

Treatment of control EDL muscles with a low concentration of ouabain to selectively inhibit the α2 isoform reproduces the increased fatigability of skα2<sup>−/−</sup> EDL. The increased fatigability of ouabain-treated control muscles is not explained by impaired membrane excitation resulting from the loss of α2 electrogenic activity. This finding demonstrates that the acute contribution of the Na,K-ATPase α2 isoform to muscle function requires enzyme activity.

Collectively, these findings identify a fundamental, tissue- and isoform-specific role of the Na,K-ATPase α2 in working skeletal muscles. The Na,K-ATPase α2 isoform supports exercise capacity in the animal by enabling working skeletal muscles to resist fatigue and maintain contraction.

The Na,K-ATPase α2 isoform is regulated by muscle use and its activity becomes significant during muscle contraction. The finding that skα2<sup>−/−</sup> muscles maintain near normal resting potentials in the complete absence of Na,K-ATPase α2 indicates that the α2 isoform is not highly active in resting skeletal muscles. This same conclusion was made from previous measurements using the neonatal diaphragm of global α2<sup>−/−</sup> knockout mice (8-9), adult EDL of α2<sup>−/−</sup> HET mice (6, 8), and adult diaphragm of control rats in which α2 isoform activity was inhibited with low concentrations of ouabain (11-12).

Voluntary skeletal muscles function in a bipolar manner, alternating between long periods of rest and brief periods of intense activity. The needs of resting and active muscle for Na<sup>+</sup>/K<sup>+</sup> transport vary accordingly. Our findings suggest that the Na,K-ATPase α2 isoform provides a reserve Na<sup>+</sup>/K<sup>+</sup> transport capacity that is largely inactive in resting muscle but is rapidly recruited upon the onset of muscle use, to support the increased demands of contraction for Na<sup>+</sup>/K<sup>+</sup> transport.

This finding is consistent with previous studies showing that the Na,K-ATPase is stimulated within seconds of the onset of
contraction and that its transport activity is absolutely required to maintain excitation and contraction (13-14,30-34). Our present findings identify the Na,K-ATPase α2 isozyme as a key player in contraction-related Na+/K+ transport.

Na,K-ATPase α2 isoform activity plays an essential, acute role in resistance to muscle fatigue. Removal of Na,K-ATPase α2 in skeletal muscles renders them significantly more susceptible to fatigue. Fatigue occurs when a muscle cannot continue to generate force during sustained use. The development of fatigue in skα2−/− muscle can be reproduced in control muscles by inhibiting α2 activity with a low concentration of ouabain. Therefore, active Na+/K+ transport by the Na,K-ATPase α2 isozone enables working skeletal muscles to maintain contraction during sustained use.

Upregulation of Na,K-ATPase α1 content cannot fully compensate for the lack of α2. The 2.5-fold upregulation of α1 in skeletal muscles is likely a compensatory response to the loss of α2. The Na,K-ATPase α1 isoform is also upregulated in the neonatal diaphragm of α2−/− mice (1.9-fold; 24) and in adult EDL muscles of α2−/− mice (1.4 - 1.5 fold; 6,8). These increases are at the upper range of reported changes in α1 content in skeletal muscle under various physiological conditions (28). Therefore, it is remarkable that α1, even when upregulated to its physiological limit, is unable to substitute for the loss of α2. Its inability to functionally compensate may be because it is not targeted to where it is needed during contraction.

The ability of Na,K-ATPase α2 to resist fatigue in working muscle is due in part to its ability to transport K+ in the transverse tubules—Working skeletal muscles lose K+ and gain Na+ as a consequence of repetitive action potential activity. This K+ load increases with action potential frequency and accumulates in the muscle extracellular spaces, especially in the diffusion-restricted transverse tubules. If not cleared, it causes depolarization, excitation failure and loss of contraction (35).

Our results are consistent with the proposal that skα2−/− muscles are more susceptible to fatigue because they are unable to clear K+ in the transverse tubules during muscle activity. The transverse tubules of skα2−/− muscle have little to no Na,K-ATPase of either isoform with which to clear dynamic K+ changes in the transverse tubules. The similar frequency dependence of fatigue in skα2−/− muscle and control muscles with α2 inhibited, and the requirement for active transport by α2 support this interpretation. The localization of α2 in the transverse tubules positions it to oppose the use-dependent K+ load where it is greatest. While, the enhanced expression of α1 on the surface sarcolemma positions it better to clear extracellular K+ in the muscle interstitial space. The contraction-related transport by α2 is expected to maintain membrane excitability in the face of elevated extracellular K+, particularly in the transverse tubules, by transporting K+ back into the muscle and also by directly hyperpolarizing the membrane potential due to the electrogenic nature of Na,K-ATPase transport.

Our results confirm and extend previous studies of the subcellular localization of Na,K-ATPase α isoforms in skeletal muscle. In studies using immuno histochemistry, membrane fractionation, and co-immunoprecipitation, the α2 isoform has been found in the transverse tubule membranes and at discreet regions of surface sarcolemma including the muscle end plate, caveolae and costameres (10,28,36). Our results confirm this and, further, show that α2 is localized in a distinct pattern within the muscle tissue where it is expressed in some axons or cells of the motor nerve, the nerve perinureum, and arterial vascular smooth muscle. The α1 isoform has been detected on the surface sarcolemma at the end plate, caveolae and costameres (10, 28,36), but not in the transverse tubules (28). Our present results suggest that α1 may also be present in the transverse tubules but at low abundance. Therefore, α1 is expressed largely on the surface sarcolemma while α2 content is enhanced in the transverse tubules.

In astrocytes and arterial myocytes, a 90 amino acid sequence in the cytoplasmic N-terminus of α2 targets it to regions of the plasma membrane which overlay the endoplasmic/sarcoplasmic reticulum (37). This sequence may participate in targeting α2 to the transverse tubules in skeletal muscle. The transverse tubules invaginate from caveolae and form junctions with the sarcoplasmic reticulum along 80% of their length. Alternatively, the
Na,K-ATPase α2 isoform associates with multiple partners to form macromolecular complexes capable of complex interactions (1,10), and its targeting to the transverse tubules may utilize such interactions.

**Reduced force in skα2-/- muscles.** Peak twitch and maximum tetanic force are reduced in skα2-/- muscles, suggesting that they also have some weakness. The reduction in force was significant only in vitro, although there was a tendency for skα2-/- muscles to produce less force in vivo (Table 1). Measurement of absolute force is considered more reliable in vitro. Therefore, the reduction in force from the rested state is likely a real impairment of skα2-/- muscle that is distinct from its increased fatigability.

Muscle weakness was not seen in an earlier global α2 knockout model (6). In fact, the EDL of a heterozygous global α2 knockout mice shows greater peak twitch and tetanic force, without change in the onset of fatigue (6). The two models are not directly comparable because they involve different genetic manipulations. Targeted removal of 100% of α2 in adult skeletal muscles is not necessarily expected to double the effect of removing 50% of α2 in all tissues from conception. Nevertheless, they suggest that the Na,K-ATPase α2 isoform plays some role in determining muscle force, in addition to its role in countering fatigue. Force in skeletal muscles is directly proportional to the intracellular Ca2+ change. Therefore, the Na,K-ATPase α2 in skeletal muscle may interact in as yet unknown ways with proteins of the excitation-contraction coupling complex or other Ca2+ handling proteins.

**Potential contribution of other factors to the phenotype.** Isoform-specific differences in the regulation of α1 and α2 enzyme activity may also contribute to differences in function. While many studies have examined Na,K-ATPase regulation in the kidney and heart, much less is known about the mechanisms which regulate its activity in resting and contracting skeletal muscle (reviewed in: 34). In kidney and heart, the Na,K-ATPase can be regulated by phosphorylation of FXYD proteins, a regulatory Na,K-ATPase subunit with tissue-specificity (2), and this mechanism may operate in skeletal muscle as well (12). The Na,K-ATPase in skeletal muscle is also regulated by electrical activity, adrenergic receptor activation, insulin, calcitonin gene related peptide, ACh (34; 10), purines, the cardiotonic receptor site (24), and other small molecules. However, these regulatory mechanisms typically have a modest fold-change and/or their kinetics are not sufficiently fast to explain the rapid, up to 20-fold stimulation that occurs within seconds of the onset of muscle contraction. Therefore, other mechanisms must exist (34).

Changes in gene expression are not expected in the short time of this experiment and the only known receptor for ouabain is the Na,K-ATPase. Our finding that the acute removal of α2 function in a control animal produces the same effect on fatigue as genetically removing the gene argues strongly that the functional phenotype in the gene-altered animal is due to lack of an essential function of the α2 Na,K-ATPase.

The chronic absence of α2 and/or an altered pattern of use secondary to the removal of α2 may cause long-term muscle fiber remodeling. We examined this possibility by immuno histochemistry of cross-sections of soleus, vastus and tibialis muscle dual-labeled with antibodies to myosin heavy chain (MHC) isoforms I, IIA and IIB, and the Na,K-ATPase (Radzykevich and Heiny, unpublished). The locomotor muscles of adult skα2-/- mice are differentiated and express all of the same fiber types as control. The slow soleus retains its predominance of type I fibers and fast muscles retain their predominance of type II fibers. There is a trend towards fewer type II fibers and more or larger type I or type X in some but not all muscles. These changes, when present, are in a direction to reduce fatigue and therefore may be compensatory for the loss of α2. Importantly, they cannot explain the increased fatigability seen in all muscle types.

Exercise training can also remodel skeletal muscles. We controlled for a training effect by housing mice in identical conditions without wheels or training.

Additional factors may impair exercise capacity in the animal. For example, removal of Na,K-ATPase α2 in the skeletal muscles may affect the involuntary respiratory muscles. However, diaphragm or respiratory muscle
fatigue is not expected early into exercise at low exercise intensities.

In summary, this study reveals a fundamental, tissue- and isoform-specific role of the Na,K-ATPase α2 enzyme in skeletal muscle. In contrast to the canonical role of the Na,K-ATPase α1 isoform in setting basal ion gradients and the resting potential of most cells, the Na,K-ATPase α2 isoform in skeletal muscle is not required for this function. Its contribution becomes significant during muscle use. Na,K-ATPase α2 activity is regulated by muscle use and its contraction-related activity enables working skeletal muscles to maintain contraction, match force to demand, and resist fatigue. This role cannot be performed by the Na,K-ATPase α1 isoform alone, despite its compensatory upregulation. The inability of α1 to fully substitute for α2, the frequency dependent impairment in the absence of α2, and the requirement for active transport by α2 all suggest that the localization of α2 in the transverse tubules is important for its role in fatigue resistance. These results establish a foundation for future studies to address important questions regarding the structural bases for differences in Na,K-ATPase α2 isoform targeting and function, and the molecular mechanisms that acutely regulate its activity.
References

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FIGURE LEGENDS

FIGURE 1. Generation of ska22/− mice. Homozygous α2 LoxP mice (α2Flox/Flox; 18) having a targeted allele of the Na,K-ATPase α2 gene (ATP1A2) were mated with heterozygous MyoDiCre knockin mice (19) in which the iCre gene is knocked into the MyoD locus. MyoDiCre-mediated recombination and deletion of α2 in skeletal muscle cells results in offspring which are homozygous for the α2 null allele in all skeletal muscle lineage cells.

FIGURE 2. Na,K-ATPase α2 (A) and α1 (B) protein content in different tissues of control (-) and ska22−/+ (mouse. A) Representative Western Blot and average expression measured in independent blots using microsomal membrane samples from 3 control and 3 ska22−/− mice, except for aorta samples which were pooled and run on one blot to obtain a detectable signal. *P < 0.05. The α2 isoform is not detected in kidney and liver, as expected. LC, loading control was Ponceau staining (25), shown at ~37 kDa band. Protein expression was determined by densitometry and normalized to LC. B) same for α1 expression. Protein loaded for α1 and α2 (µg, µg): hindlimb skeletal muscle (20, 20), liver (20, 20), brain (10, 10), kidney (2.5, 20), aorta (50, 150), heart (5, 80).

FIGURE 3. The Na,K-ATPase α2 isoform is absent from all skeletal muscle fibers of ska22−/− mice, but retains its normal expression pattern in other cell types within the tissue. (A) Fluorescence micrograph of control skeletal muscle labeled with an Na,K-ATPase α2-antibody. The α2 isoform is found in the surface sarcolemma (arrows), the muscle end plate (●), and diffusely in the fiber interior reflecting the transverse tubules, as reported (20,13). (B) Same section in a region with a motor nerve (N) and artery (A). The α2 isoform is present in vascular smooth muscle, the nerve perineurium, and in some axons or cells within the nerve bundle. (C) Skeletal muscle of a ska22−/− mouse. The α2 isoform is absent from muscle fibers but retains its expression in vascular smooth muscle, the nerve and nerve perineurium. Identification of neuromuscular junctions and vascular smooth muscle was confirmed as described in Methods. 8 µm cross sections of tibialis anterior muscles from control (A & B) and ska22−/− (C) littermates, processed in parallel and imaged under identical conditions. (D) Confocal image of an isolated EDL muscle labeled with 500 nM bodipy-conjugated ouabain (Invitrogen). Ouabain at this concentration selectively binds the α2 isoform in mice (8). The α2 isoform is expressed on the surface sarcolemma and in the transverse tubules of control muscle, evident as double rows of label per sarcomere as expected from the location of transverse tubules at the A-I junctions in mammalian skeletal muscle. (E) α2 label isoform is absent in the EDL of ska22−/− mice.

FIGURE 4. The Na,K-ATPase α1 isoform is upregulated in skeletal muscle fibers of ska22−/− mice, without change in its subcellular localization. (A) Transverse section of control muscle labeled with Na,K-ATPase α1-isoform specific antibody (yellow, Alexa fluor 594 conjugated secondary antibody) and imaged with confocal microscopy. (B) ska22−/− muscle labeled and imaged under identical conditions. 8 µm sections of tibialis anterior muscle, x 40 magnification, from the same mice used for Fig 3. (C - F) Longitudinal fixed sections (8 µm) of EDL from control (C, E) and ska22−/− (D,F) mice labeled with the same primary antibody (green, Alexa fluor 488 conjugated secondary antibody) and imaged with confocal optical sections. x80 magnification. Transverse tubule staining is evident in some optical sections of both genotypes as double rows of label per sarcomere.
FIGURE 5. Skα2−/− mice show a striking inability to perform physical exercise. Untrained α2Flox/Flox (control) and skα2−/− mice were run on a treadmill as running speed (meters/min) was increased every 2 minutes at a fixed incline of 6°. Failures were defined as 1 sec intervals spent on the shock bar. *, ** and *** indicate significantly different from control at P < .05, P < .01, P < .001. 7 control and 5 skα2−/− mice.

FIGURE 6. The EDL muscle of skα2−/− mice fatigues rapidly in vivo compared to control (α2Flox/Flox, grey lines and open circles). (A) Representative force recordings elicited by continuous stimulation of the motor nerve (20 Hz for 27 sec) or (B) intermittent tetanic stimulation (40 Hz, 750 ms train applied every 4 sec) in anesthetized mice. (C) Average onset of fatigue in vivo. ** significant difference at P < .01

FIGURE 7. Isolated EDL muscles of skα2−/− mice show significantly impaired contractility in vitro. (A) maximum twitch force. (B) maximum tetanic force (80 Hz tetanus). (C) Force-frequency response elicited by a 400 ms train at the indicated frequency. The skα2−/− EDL (n = 11) was compared with three possible control genotypes: our standard control (■, α2Flox/Flox, n=6-8), true WT (wt cre−, n=3-4), and MyoD iCre (wt cre+, n=7-8). None of the control groups were significantly different from each other at P > .67 (twitch) and P > .4 (tetanus). *** significant difference at P < .0001 (ANOVA). Inset. Representative recordings of tetanic force (40 Hz) produced by a skα2−/− EDL at the start and end of recording.

FIGURE 8. Isolated EDL muscles of skα2−/− mice develop significantly greater fatigue compared to control. (A) Control genotypes. Fatigue was compared in vitro using a stimulation protocol which elicited the same peak tetanic force in all genotypes (40 Hz, 750 ms tetani every 7.7 sec). Same control genotypes defined in Fig. 7. (B) Fatigability of skα2−/− EDL (●), control EDL treated with 5 µM ouabain (△), and untreated control (○). Inset: Reproducibility of twitch force produced by a control EDL from the rested state before and after 90 min. in 5 µM ouabain. *** significant difference at P < .0001

FIGURE 9. The soleus muscle of skα2−/− mice (black lines) shows greater fatigability compared to control (grey lines, α2Flox/Flox) and cannot maintain peak tetanic force above 40 Hz. Representative force recordings in vitro in response to continuous stimulation at the indicated frequency. The skα2−/− soleus is unable to maintain force for the duration of a 10 sec tetanus at frequencies above 40 Hz.
TABLE 1. Contractility and fatigability of the EDL muscle of control and \( sk\alpha^{2-} \) mice *in vivo*. Data were obtained in the animal from the same cohort of mice used for the treadmill exercise trials. \( Lo \), optimal rest length. Intermittent fatiguing stimulation, 40 Hz 750 ms trains repeated every 4 sec. * \( P < .05 \), ** \( P < .001 \)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (weeks)</th>
<th>Body wt (g)</th>
<th>EDL wt (mg)</th>
<th>EDL ( Lo ) (mm)</th>
<th>Twitch force ( mN/mm^2 )</th>
<th>Peak Tetanic Force at 40Hz ( mN/mm^2 )</th>
<th>Peak Tetanic Force at 70Hz ( mN/mm^2 )</th>
<th>Fatigability after 27 s of continuous stimulation at 20 Hz ( F/F_o )</th>
<th>Fatigability after 1.5 min of intermittent tetanic stimulation at 40 Hz ( F/F_o )</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>15.6±0.6 (11)</td>
<td>25.7±1.1 (10)</td>
<td>9.8±0.8 (9)</td>
<td>11.6±0.5 (9)</td>
<td>40.6±3.8 (8)</td>
<td>80.7±7.4 (8)</td>
<td>110.5±12.6 (8)</td>
<td>0.9±0.1 (6)</td>
<td>0.76±0.06 (6)</td>
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<tr>
<td>( \alpha^{2Flox/Flox} )</td>
<td>16.3±0.4 (10)</td>
<td>25.8±1.5 (10)</td>
<td>11.6±0.5 (10)</td>
<td>12.2±0.1 (10)</td>
<td>31.2±3.7 (10)</td>
<td>62.5±9.6 (9)</td>
<td>100.5±24.9 (9)</td>
<td>0.4±0.2 (6) *</td>
<td>0.33±0.1 (5) **</td>
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</tbody>
</table>
TABLE 2. Resting membrane potentials of the EDL muscle of control and \( sk\alpha 2^- \) mice. Mean ± sem of (n) measurements from 5 control and 5 \( sk\alpha 2^- \) muscles. Values in 5 \( \mu \)M ouabain were obtained after 90 min. incubation. ** P < .001

<table>
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<tr>
<th>Genotype</th>
<th>Resting Membrane Potential</th>
</tr>
</thead>
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<tr>
<td></td>
<td>mV</td>
</tr>
<tr>
<td>Control (( \alpha 2^{Flox/Flox} ))</td>
<td>-79.5 ± 0.6 (128)</td>
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<tr>
<td>( Sk\alpha 2^- )</td>
<td>-78.3 ± 0.5 (149)</td>
</tr>
<tr>
<td>Control + 5 ( \mu )M ouabain</td>
<td>-75.9 ± 0.6 (98) **</td>
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TABLE 3. Twitch and tetanus parameters measured in vitro from EDL of control and $\alpha_2^{\alpha_{2-/-}}$ mice. Three control genotypes were: $\alpha_2^{\text{Flox/Flox}}$, wt cre- (true wild type), and wt cre+ (mice with iCre under control of MyoD). Tetanus parameters were measured at 80 Hz, a frequency at which the $\alpha_2^{\alpha_{2-/-}}$ EDL generates maximum force. * P < .05, ** P < .001, *** P< .0001. None of the control genotypes were significantly different for any parameter.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age weeks</th>
<th>Body wt g</th>
<th>EDL wt mg</th>
<th>EDL Lo mm</th>
<th>Twitch force $\text{nN/mm}^2$</th>
<th>Twitch rise time ms</th>
<th>Twitch half relaxation time ms</th>
<th>Peak tetanic Force $\text{nN/mm}^2$</th>
<th>Tetanus rise time (10-75%) ms</th>
<th>Tetanus half relaxation time ms</th>
<th>Twitch/tetanus ratio</th>
<th>Force decline during 400 ms 125 Hz tetanus %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_2^{\text{Flox/Flox}}$</td>
<td>17.6 ±0.5 (9)</td>
<td>27.2 ±1.5 (9)</td>
<td>9.6 ±0.3 (9)</td>
<td>11.5 ±0.4 (9)</td>
<td>50.1 ±3.1 (9)</td>
<td>21.8 ±0.6 (9)</td>
<td>23.3 ±1.5 (9)</td>
<td>167.2 ±11.7 (7)</td>
<td>1781 ±194 (7)</td>
<td>41 ±0.5 (6)</td>
<td>0.30 ±0.01 (6)</td>
<td>3.8 ±1.0 (8)</td>
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<tr>
<td>wt cre-</td>
<td>16.0 ±0.4 (5)</td>
<td>26.1 ±1.1 (5)</td>
<td>9.0 ±0.1 (5)</td>
<td>11.8 ±0.3 (5)</td>
<td>51.2 ±1.8 (5)</td>
<td>21.7 ±0.6 (5)</td>
<td>23.7 ±0.6 (5)</td>
<td>168.4 ±6.1 (2)</td>
<td>1737 ±154 (4)</td>
<td>42.0 ±1.2 (3)</td>
<td>0.31 ±0.02 (4)</td>
<td>6.0 ±2.1 (3)</td>
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<tr>
<td>wt cre+</td>
<td>16.9 ±0.4 (8)</td>
<td>28.2 ±2.0 (8)</td>
<td>10.8 ±0.6 (8)</td>
<td>11.8 ±0.2 (8)</td>
<td>49.3 ±2.0 (8)</td>
<td>22.8 ±0.4 (8)</td>
<td>24.8 ±0.5 (8)</td>
<td>156.6 ±80 (8)</td>
<td>2021 ±274 (5)</td>
<td>43.3 ±2.4 (6)</td>
<td>0.32 ±0.02 (7)</td>
<td>2.7 ±0.5 (7)</td>
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<tr>
<td>$sk\alpha_2^{\alpha_{2-/-}}$</td>
<td>17.4 ±0.5 (11)</td>
<td>26.1 ±0.7 (11)</td>
<td>11.6 ±0.5 (11) *</td>
<td>12.5 ±0.2 (11) *</td>
<td>38.1±1.1 (11) ***</td>
<td>25.5 ±1.0 (11) *</td>
<td>30.2 ±2.4 (11) *</td>
<td>99.9±4.7 (11) ***</td>
<td>1771±140 (11)</td>
<td>49.1±3.2 (11) *</td>
<td>0.39±0.0 (11) *</td>
<td>43.6±8.1 (11) ***</td>
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</table>
**TABLE 4. Fatigability of the EDL *in vitro*.** Same control genotypes used for Table 2. Fatigue was produced by repeated tetani of 40 Hz, 750 ms duration applied every 7.7 sec. * P < .05, ** P < .001, *** P < .0001. None of the control genotypes were significantly different for any parameter.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Initial Force</th>
<th>Initial time-to-peak</th>
<th>Time to 50% force</th>
<th>Time-to 50% peak</th>
<th>Time to half decline</th>
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<tr>
<td></td>
<td>mN/mm²</td>
<td>ms</td>
<td>min</td>
<td>ms</td>
<td>ms</td>
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<tr>
<td>α² Flox/Flox</td>
<td>122.7 ±11.6 (7)</td>
<td>272.0 ±30.1 (6)</td>
<td>4.2 ±0.2 (7)</td>
<td>2.39 ±0.29 (6)</td>
<td>2.05 ±0.1 (7)</td>
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<tr>
<td>wt cre-</td>
<td>136.9 ±9.0 (5)</td>
<td>256.0 ±27.2 (4)</td>
<td>4.2 ±0.2 (5)</td>
<td>2.61 ±0.58 (5)</td>
<td>2.12 ±0.1 (5)</td>
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<tr>
<td>wt cre+</td>
<td>121.7 ±7.6 (7)</td>
<td>280.9 ±11.6 (7)</td>
<td>3.9 ±0.2 (7)</td>
<td>2.02 ±0.32 (7)</td>
<td>2.31 ±0.1 (7)</td>
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<tr>
<td>sk α²+</td>
<td>87.8 ±3.8 (11) ***</td>
<td>259.2 ±18.6 (10)</td>
<td>3.0 ±0.1 (11) ***</td>
<td>0.55 ±0.02 (10) ***</td>
<td>2.02 ±0.12 (11) ***</td>
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</table>
TABLE 5. Contractility of the isolated soleus (SOL) muscle.
Maximum tetanic force at 60 Hz. * P < .05, *** P < .0001

<table>
<thead>
<tr>
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<th>SOL wt</th>
<th>SOL Lo</th>
<th>Twitch force</th>
<th>Peak tetanic force</th>
<th>Twitch/tetanus ratio</th>
<th>Force decline after 60 Hz, 10s tetanus</th>
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<tr>
<td></td>
<td>mg</td>
<td>mm</td>
<td>mN/mm²</td>
<td>mN/mm²</td>
<td>%</td>
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<td>control</td>
<td>8.7 ± 0.7 (6)</td>
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<td>31.2 ± 1.8 (4)</td>
<td>132.3 ± 2.0 (4)</td>
<td>0.24 ± 0.01 (4)</td>
<td>10.7 ± 1.1 (5)</td>
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<tr>
<td>skα2−</td>
<td>8.8 ± 0.4 (8)</td>
<td>11.2 ± 0.4 (7)</td>
<td>19.2 ± 2.6 (7) *</td>
<td>61.2 ± 6.5 (6) ***</td>
<td>0.35 ± .04 (6) ***</td>
<td>37.3 ± 3.9 (6) ***</td>
</tr>
</tbody>
</table>
FIGURE 7

A

Max. twitch force, mN/cm²

B

Max. tetanic force, mN/cm²

C

Force, mN/cm²

Stimulation frequency, Hz

control
ska2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-~-