

Original Paper

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# Ion Transporters Involved in Dissipation of Transmembrane $\text{Na}^+$ and $\text{K}^+$ Gradients in C2C12 Myotubes Triggered by Electrical Pulse Stimulation

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## Key Words

C2C12 myotubes • Electrical pulse stimulation •  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio • Ouabain • Excitation–transcription coupling

## Abstract

**Background/Aims:** Cultured skeletal muscle cells subjected to electrical pulse stimulation (EPS) are widely employed as an *in vitro* model of exercising skeletal muscle. Numerous studies demonstrated that sustained excitation of skeletal muscle results in the dissipation of the transmembrane gradient of monovalent cations. During exercises the impending loss of excitability has to be counterbalanced by rapid restoration of the  $\text{Na}^+_i/\text{K}^+_i$  ratio. To understand mechanisms of the maintenance of muscle contractility, it is necessary to know which transporters are participated in the dissipation of  $\text{Na}^+_i$  and  $\text{K}^+_i$  gradients and how to activate Na,K-ATPase for its regeneration. This study was aimed at the identification of ion transporters involved in the dissipation of the transmembrane gradients of  $\text{Na}^+$  and  $\text{K}^+$  induced by EPS, and Na,K-ATPase isoforms involved in its restoration. **Methods:** The differentiated C2C12 myotubes were subjected to electrical pulse stimulation in the presence or absence of different ion transport systems inhibitors followed by measurement of intracellular monovalent cations by flame atomic absorption spectrometry. **Results:** Electrical pulse stimulation of C2C12 myotubes results in the dissipation of  $\text{Na}^+_i/\text{K}^+_i$  gradient, which is maintained by  $\alpha 2$ -Na,K-ATPase. Na-K-2Cl cotransporter (NKCC), voltage-gated  $\text{Na}^+$  ( $\text{Na}_v$ ), and large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\text{BK}_{\text{Ca}}$ ), and Na/H exchanger (NHE) are involved in the dissipation of this gradient. Suppression of calmodulin-dependent protein kinase II (CaMKII) increases  $\text{Na}^+$  efflux in EPS-treated C2C12 myotubes. **Conclusion:** NKCC,  $\text{Na}_v$ ,  $\text{BK}_{\text{Ca}}$ , and NHE are involved in the dissipation of  $\text{Na}^+_i/\text{K}^+_i$  gradient in EPS-treated C2C12 myotubes.

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## Foreword

Electrical pulse stimulation (EPS) of murine differentiated myotubes (C2C12 cells) results in the dissipation of transmembrane gradient of monovalent cations and elevation of the  $\text{Na}^+/\text{K}^+$  ratio. It resulted in the change of the genes transcription by  $\text{Ca}^{2+}$ -mediated and  $\text{Ca}^{2+}$ -independent manner. Inhibitor analysis was used to reveal a relative impact of  $\alpha 1$ - and  $\alpha 2$ -containing isoforms of Na,K-ATPase in the maintenance of  $\text{Na}^+/\text{K}^+$  gradient in EPS-treated myotubes, and ion transporters involved in their dissipation. Data obtained in this study demonstrate that: 1) EPS increased the relative impact of  $\alpha 2$ -Na,K-ATPase in the maintenance of transmembrane  $\text{Na}^+/\text{K}^+$  gradients in C2C12 myotubes; 2) EPS increased activity of Na-K-2Cl cotransporters measured as ouabain-resistant, bumetanide-sensitive component of  $\text{Rb}^+$  influx; 3) dissipation of transmembrane  $\text{Na}^+/\text{K}^+$  gradients observed in EPS-treated C2C12 myotubes is mainly due to the activation of tetrodotoxin-sensitive voltage-gated  $\text{Na}^+$  channels (Nav), Na/H exchanger (NHE) and charibdotoxin-sensitive large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels ( $\text{BK}_{\text{Ca}}$ ), respectively. Inhibitor of voltage-gated Kv1.2 and Kv1.3  $\text{K}^+$  channels noxiustoxin and inhibitor of ATP-sensitive  $\text{K}^+$  channels glibenclamide as well as the inhibitors of intermediate and small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (TRAM-34 and apamin, respectively) did not affect the loss of  $\text{K}^+$  induced by EPS. Thus, we can suggest that these channels do not participate in the dissipation of  $\text{K}^+$  gradient induced by EPS.

We also used inhibitor analysis to identify elements of the EPS-activated signaling system(s). The data obtained indicate that calmodulin-dependent protein kinase II suppresses  $\text{Na}^+$  efflux through Na,K-ATPase and/or other ion-transport system(s). Nifedipine (an inhibitor of L-type voltage-gated  $\text{Ca}^{2+}$  channels), cyclosporin A (an inhibitor of calcineurin), and PD08059 (an inhibitor of Erk 1/2) had no effect on  $\text{Na}^+$  and  $\text{K}^+$  content in EPS-treated C2C12 myotubes.

## Introduction

Cultured skeletal muscle cells subjected to electrical pulse stimulation (EPS) are widely employed as an *in vitro* model of exercising skeletal muscle [1–3]. This approach is based on several observations. *First*, in differentiated myotubes obtained from murine [4, 5] and rat [6] skeletal muscles (C2C12 and L6 cell lines, respectively) as well as in primary cultures of rat [7] and human [1] skeletal muscles, EPS resulted in metabolic and adaptive changes detected in freshly isolated trained muscles. *Second*, C2C12 myotubes exhibit remarkable contractile responses [4, 8,9] and repetitive  $[\text{Ca}^{2+}]_i$  transients [8] triggered by EPS. *Third*, during the last two decades it was shown that skeletal muscle functions as an exercise-dependent endocrine organ secreting dozens of myokines with molecular weights of 15-30 kDa (for review, see [10–12]). Several research groups reported that in cultured myotubes EPS triggers secretion of several exercise-dependent myokines [4, 5,13–16].

Numerous studies demonstrated that sustained excitation of skeletal muscle results in the dissipation of the transmembrane gradient of monovalent cations. Thus, in humans, intensive dynamic and static exercises lead to up-to 2-fold elevation of venous  $[\text{K}^+]$  due to its release from skeletal muscle, i.e. a major source of intracellular  $\text{K}^+$  [17–20]). Calculation of femoral arterial-venous  $\text{Na}^+$  and  $\text{K}^+$  concentration differences based on the measurement of blood flow values,  $\text{Na}^+$  and  $\text{K}^+$  contents in muscle biopsies and changes of muscle water content demonstrated that in humans knee extensor intense exercises rapidly increase intracellular  $\text{Na}^+$  concentration ( $[\text{Na}^+]_i$ ) in skeletal muscle from 13 to 23 mM and reduce  $[\text{K}^+]_i$  from 162 to 129 mM [21]. Using flame photometry, Murphy and coworkers found that in rat soleus muscle 60 min of intermittent running resulted in ~2-fold elevation in  $[\text{Na}^+]_i$  and attenuation of  $[\text{K}^+]_i$  by 10-20% [22].

Data considered above allowed us to propose that along with the well-documented impact of excitation-transcription coupling mediated by elevation of  $[\text{Ca}^{2+}]_i$  and activation

of AMP-sensitive protein kinase (AMPK) and hypoxia-inducible factor HIF-1 $\alpha$  [23], myokine production in exercising skeletal muscle might be triggered by the dissipation of transmembrane gradient of monovalent cations [12]. To examine this hypothesis, we compared the action of EPS on the intracellular concentration of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, AMPK activity, and transcriptomic changes in C2C12 myotubes. We observed that similar to exercising skeletal muscle the transient EPS of C2C12 myotubes results in [Ca<sup>2+</sup>]<sub>i</sub> oscillations, dissipation of transmembrane gradients of monovalent cations and ~5-fold elevation in the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio [16, 24]. We also found that inhibition of L-type voltage-gated Ca<sup>2+</sup> channels by nifedipine abolished [Ca<sup>2+</sup>]<sub>i</sub> oscillations but did not affect the elevation of the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio observed in EPS-treated myotubes [16]. Two-hr EPS resulted in differential expression of more than 3000 genes. Importantly, the differential expression of hundreds of EPS-sensitive genes was preserved in the presence of nifedipine. Among the nifedipine-resistant genes, we noted 113 genes whose expression was also affected by partial Na,K-ATPase inhibition by 30  $\mu$ M ouabain providing the same elevation of the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio as in EPS-treated cells. Unlike the elevation of the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio and [Ca<sup>2+</sup>]<sub>i</sub> oscillation, EPS did not affect phosphorylation of acetyl-CoA carboxylase and Unc-51 like autophagy activating kinase-1, i.e. downstream markers of AMPK activation [16]. These results strongly suggested that elevation of the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio affects gene expression in EPS-treated myotubes *via* Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub>-sensitive, Ca<sup>2+</sup><sub>i</sub>- and AMPK-independent mechanism of excitation-transcription coupling.

During exercises, therefore, the impending loss of excitability has to be counterbalanced by rapid restoration of the Na<sup>+</sup><sub>i</sub>/K<sup>+</sup><sub>i</sub> ratio. To understand mechanisms of the maintenance of muscle contractility, it is necessary to know which transporters are participated in the dissipation of Na<sup>+</sup><sub>i</sub> and K<sup>+</sup><sub>i</sub> gradients and how to activate Na,K-ATPase for its regeneration.

This study was aimed at the identification of ion transporters involved in the dissipation of the transmembrane gradients of Na<sup>+</sup> and K<sup>+</sup> induced by EPS, and Na,K-ATPase isoforms involved in its restoration.

## Materials and Methods

### Cell culture

Murine C2C12 cells (RRID: CVCL\_0188) subjected to less than 10 passages were obtained from the Mammalian Cultures Collection of the Institute of Cytology of the Russian Academy of Sciences (Saint Petersburg, Russia). The cells were seeded at a density of 3 $\times$ 10<sup>4</sup> cells/well in 6-well plates containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin and kept at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Three-to-five days after plating, the cells reached 70-80% confluence and were subjected to differentiation in DMEM containing 25 mM glucose, antibiotics, 2% calf serum, and 1 nM insulin. The differentiation medium was changed daily. Cell morphology was evaluated by phase-contrast microscopy at  $\times$ 100 magnification without preliminary fixation. Consistent with previous publications [4, 5,8, 9,13], we found that 7-day exposure to medium depleted of serum-derived growth factors and supplied with insulin resulted in myogenic differentiation of C2C12 cells indicated by cell fusion, formation of prolonged multinucleated myotubes, and appearance of troponin T [24]. The differentiated C2C12 myotubes were washed with phosphate buffered saline (PBS) and supplied with 3 ml of differentiation medium with addition of compounds listed in tables and figures legends. In some experiments, cells were subjected to EPS at 37°C for 2 hours using a C-Pace pulse generator (C-Pace EP, IonOptix, USA) with voltage 40 V, stimuli duration 10 ms, and frequency 1 Hz. Cell viability was assessed using the AlamarBlue assay [25] as described in detail elsewhere [24].

### Rubidium influx

To examine the action of EPS on transmembrane monovalent cation fluxes, we employed Rb<sup>+</sup> as analogue of K<sup>+</sup> [26]. To study Rb<sup>+</sup> influx, control and EPS-treated C2C12 myotubes were washed with PBS and incubated in shaking water-bath at 37°C in medium A containing 135 mM NaCl, 5 mM RbCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM glucose and 20 mM HEPES-tris (pH 7.4). In part of experiments, medium

A contained inhibitors of ion transporters listed in figures legends. Fig. 1 displays kinetics of Rb<sup>+</sup> accumulation in C2C12 myotubes. To study the rate of Rb<sup>+</sup> influx, we limited incubation time to 10 min.

#### Measurement of intracellular Na<sup>+</sup>, K<sup>+</sup>, and Rb<sup>+</sup> content

Immediately after electrical pulse stimulation, six-well plates were transferred onto ice, the experimental medium was quickly removed, and the cells were washed three times with 3 ml of ice-cold 0.1 M MgCl<sub>2</sub> solution in doubly deionized (DDI) water. Then, 1.5 ml of 5% trichloroacetic acid (TCA) in DDI water was added to each well, followed by incubation at 4°C overnight for complete extraction of ions from myotubes. Cell precipitates were suspended and centrifuged for 5 min at 18, 000 relative centrifugal force. Supernatants were transferred to test tubes and stored at -20°C. Cell precipitates were resuspended in 0.75 ml of 0.1 M NaOH and incubated at 65°C for 1 h for complete protein dissolution. The Na<sup>+</sup>, K<sup>+</sup>, and Rb<sup>+</sup> contents in TCA extracts were measured by flame atomic absorption spectrometry using a Kvant-2m1 spectrometer (Cortec, Russia) with propane-air mixture in accordance with the manual at slit of 0.25 mm and wavelengths of 766.5 nm (K<sup>+</sup>), 589 nm (Na<sup>+</sup>), 780 nm (Rb<sup>+</sup>). Solutions of KCl (0.5-4 mg/liter K<sup>+</sup>), NaCl (0.05-2 mg/liter Na<sup>+</sup>), and RbCl (0.2-4 mg/liter Rb<sup>+</sup>) in 5% TCA in DDI water were used for calibration. The ion contents in each well were normalized to protein amount in the same well, which was determined by Lowry protein assay [27].

#### Materials

DMEM was purchased from both PanEco (Russia) and Gibco (USA). Trypsin-EDTA was obtained from PanEco (Russia), and penicillin-streptomycin, calf serum, and heat-inactivated fetal bovine serum were purchased from Gibco (USA). Cell culture equipment was from Corning (USA). AlamarBlue reagent was obtained from Invitrogen (USA). Insulin and ouabain were from Sigma-Aldrich (USA); MgCl<sub>2</sub> from Fluka Analytical (Switzerland); trichloroacetic acid (TCA) from Fisher BioReagents (USA). Bumetanide, amiloride, 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), furosemide, cyclosporin A was purchased from Sigma-Aldrich (USA); apamin from GenScript Biotech (USA); charybdotoxin from BaChem (Switzerland); glibenclamide from TCI (Japan); noxiustoxin from Alomone Labs (Israel); KN-62, nicardipine hydrochloride from ABCR (Germany); KN-93 from TargetMol Chemicals Inc. (USA); PD98059 from Calbiochem (USA). Unless otherwise noted, all chemicals were of the purest grade available from Sigma-Aldrich (USA), Fischer BioReagents (USA), Fluka Analytical (Switzerland), Amresco (USA) or ThermoFisher (USA).

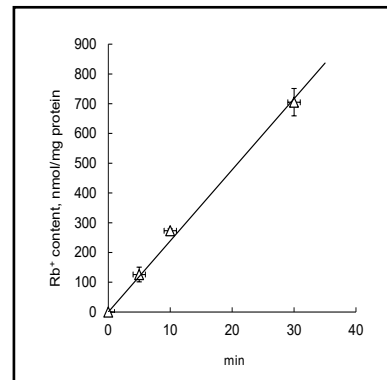
#### Statistical analysis

All data are expressed as means ± standard error and analyzed by Dunnett's multiple comparison OneWay ANOVA test. The threshold for significance was p < 0.05.

## Results and Discussion

### *α2-Na,K-ATPase provides the maintenance of Na<sup>+</sup>/K<sup>+</sup> gradient in EPS-treated C2C12 myotubes*

Na,K-ATPase comprises α- and β-subunits, which together form a functional αβ-heterodimer [28]. Major Na,K-ATPase α-subunit isoforms expressed in skeletal muscle [29] and C2C12 cells [30] are α1- and α2-subunits. In contrast to number of other cell types studied, both C2C12 cells [30] and skeletal muscle (for review, see [31, 32]) are abundant with α2-isoform of Na,K-ATPase whose content is 3-5-fold higher compared to housekeeping α1-isoform. Importantly, in mice and several other rodents the affinity of α1-isoform for inhibitory action of ouabain is 3 order of magnitude less compared to α2-Na,K-ATPase



**Fig. 1.** Kinetics of Rb<sup>+</sup> influx by C2C12 myotubes. Rb<sup>+</sup> uptake was measured in medium A containing 5 mM RbCl, respectively. Means ± S.E. obtained in 3 experiments are shown.

[33]. Thus, using NIH 3T3 fibroblasts transfected with cDNAs encoding distinct  $\alpha$ -subunits O'Brien and co-workers demonstrated that the apparent affinity of rat  $\alpha 2$ - and  $\alpha 1$ -Na,K-ATPase for ouabain calculated from [ $^3$ H]-ouabain binding and ATPase assay yielded values of  $\sim 115$  and  $48,000$  nM, respectively [34]. In mouse and rat skeletal muscle,  $10 \mu\text{M}$  ouabain completely inhibits  $\alpha 2$ -Na,K-ATPase without significant effect on  $\alpha 1$ -Na,K-ATPase whose full-scale inhibition was observed in the presence of  $1000$ - $3000 \mu\text{M}$  ouabain [35]. We used this feature of mice-derived C2C12 cell line to examine the relative impact of  $\alpha 1$ - and  $\alpha 2$ -isozymes in the regulation of intracellular  $\text{Na}^+_i$  and  $\text{K}^+_i$  content in control and EPS-treated myotubes.

Consistently with previous reports [16, 24], we observed that 2-hr EPS resulted in the 2-fold elevation of  $\text{Na}^+_i$  and attenuation of  $\text{K}^+_i$  intracellular content (Table 1). In accordance with results

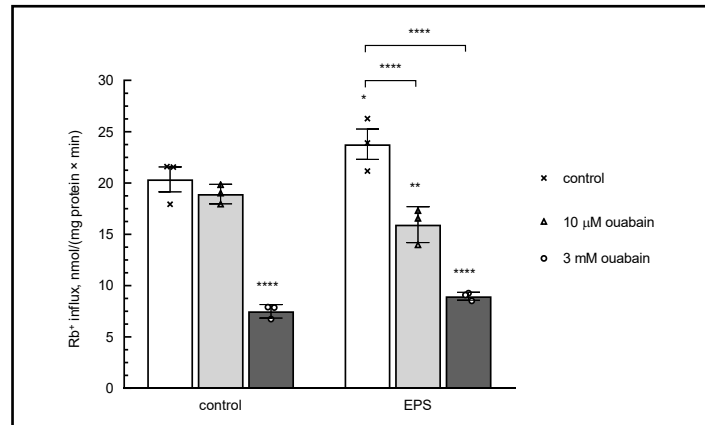
obtained in rat skeletal muscle strips [36], the complete inhibition of Na,K-ATPase by  $3000 \mu\text{M}$  ouabain led to the sharp elevation of the  $\text{Na}^+_i/\text{K}^+_i$  ratio and abolished the action of EPS. At the concentration of  $10 \mu\text{M}$ , ouabain did not affect the baseline content of monovalent cations but increased the increment of the  $\text{Na}^+_i/\text{K}^+_i$  ratio evoked by EPS. These results suggest that EPS increases the relative impact of  $\alpha 2$ -containing Na,K-ATPase in the maintenance of transmembrane  $\text{Na}^+$  and  $\text{K}^+$  gradients.

Indeed, Fig. 2 shows that the activity of  $\alpha 2$ -containing Na,K-ATPase measured as a component of the rate of  $\text{Rb}^+$  influx inhibited by  $10 \mu\text{M}$  ouabain was increased in EPS-treated myotubes from  $1.9 \pm 2.1$  up to  $7.3 \pm 2.4$  nmol/mg protein  $\times$  min. In contrast to low doses of ouabain, the full-scale inhibition of Na,K-ATPase by  $3000 \mu\text{M}$  ouabain resulted in  $\sim 3$ -fold attenuation of  $\text{Rb}^+$  influx in both control and EPS-treated myotubes (Fig. 2).

We suggest that the stronger effect of the Na,K-ATPase containing  $\alpha 2$ -subunit on the maintenance of  $\text{Na}^+_i$  and  $\text{K}^+_i$  gradient in EPS-treated C2C12 myotubes is associated not only with the high abundance of this isoform but also with its activation. It was demonstrated that in intact mouse fibers the apparent affinities of  $\alpha 1$ - and  $\alpha 2$ -Na,K-ATPase for extracellular  $\text{K}^+$  are  $1.3$  and  $4$  mM, respectively [37]. It is known also that  $\text{Na}^+$ -sensitivity of  $\alpha 1$ - and  $\alpha 2$ -Na,K-ATPase is the same [38]. These data and the measurement of  $\text{K}^+_o$ -dependent regulation of the electrical membrane potential in isolated muscle fibers allowed to propose that in exercising skeletal muscle  $\alpha 2$ -Na,K-ATPase activity is stimulated by an increment of  $\text{K}^+_o$  concentration in the T-tubule limited space [39].

**Table 1.** Effect of ouabain on intracellular sodium and potassium content in control and EPS-treated C2C12 myotubes. C2C12 myotubes were supplied with 3 ml of differentiation medium containing compounds listed in the left column. In some experiments, cells were subjected to EPS for 2 hours with voltage  $40 \text{ V}/10 \text{ ms}/1 \text{ Hz}$ . Means  $\pm$  S.E. obtained in  $n$  experiments are shown.  $^*^*$   $p < 0.01$  and  $0.002$  compared to control (EPS-untreated) cells

Tested compounds	$\text{Na}^+_i$ , nmol/mg protein		$\text{K}^+_i$ , nmol/mg protein	
	control	EPS	control	EPS
1. None (control) (n=4)	$112 \pm 11$	$229 \pm 19^{**}$	$972 \pm 42$	$750 \pm 29^*$
2. Ouabain, $10 \mu\text{M}$ (n=4)	$132 \pm 19$	$334 \pm 31^*$	$934 \pm 50$	$606 \pm 33^*$
3. Ouabain, $3000 \mu\text{M}$ (n=3)	$917 \pm 90$	$989 \pm 109$	$142 \pm 22$	$150 \pm 24$
$p_{1,2}$	NS	$< 0.05$	NS	$< 0.02$
$p_{1,3}$	$< 0.001$	$< 0.001$	$< 0.001$	$< 0.001$



**Fig. 2.** Effect of ouabain on the rate of  $\text{Rb}^+$  influx in C2C12 myotubes. In part of experiments, cells were subjected to EPS as indicated in Table 1 legend. Then, control and EPS-treated myotubes were supplied with medium A containing  $\text{RbCl}$  and with or without  $10$  or  $3000 \mu\text{M}$  ouabain and incubated during  $10$  min in the presence or absence of EPS. Means  $\pm$  S.E. obtained in  $3$  experiments are shown.

We may also suggest that the increase of  $[Na^+]_i/[K^+]_i$  ratio during EPS may result in the elevation of the  $\alpha 2$ -subunit expression. It was shown that physical exercises induce an increase in  $\alpha 2$ -subunit Na,K-ATPase expression in skeletal muscle [40], and the modulation of  $Na^+$  transport into cells also triggers  $\alpha 2$ -isoform-specific expression in C2C12 [30]. The physiological significance of  $\alpha 2$ -subunit of Na,K-ATPase in contracting muscle is confirmed by the data of Radzyukevich and coworkers who developed a gene-targeted *ska2*<sup>-/-</sup> mouse in which Na,K-ATPase containing  $\alpha 2$ -subunit is completely absent in the transverse tubules of skeletal muscle cells, where its expression in wild-type animals is normally enhanced compared to housekeeping  $\alpha 1$ -isoform [41]. They reported that *ska2*<sup>-/-</sup> mice are normal under baseline conditions but exhibit reduced exercise capacity when forced to run.

*Voltage-gated  $Na^+$  ( $Na_v$ ), and large conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ), and Na/H exchanger (NHE) are responsible for  $Na^+ / K^+$  gradient dissipation in EPS-treated C2C12 myotubes*

It is known that sarcolemma and T-tubule of skeletal muscle are abundant with  $Na_v 1.4$ ,  $Na_v 1.5$ ,  $Na_v 1.6$  voltage-gated  $Na^+$  channels,  $K_v 1.4$ ,  $K_v 3.4$ ,  $K_v 7.5$  voltage-gated  $K^+$  channels as well as with large conductance ( $K_{Ca} 1.1$ ) and small conductance ( $K_{Ca} 2.2$  and  $K_{Ca} 2.3$ )  $Ca^{2+}$ -activated  $K^+$  channels [42, 43]. Simon et al. demonstrated the presence of  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits of epithelial sodium channels (ENaC) in rat muscle spindles [44]. Their expression and activity in resting and EPS-treated C2C12 myotubes have not yet been investigated.

Table 2 shows that the gain of  $Na^+_i$  seen in EPS-treated myotubes was completely abolished by 0.5  $\mu M$  tetrodotoxin, an inhibitor of voltage-gated  $Na^+$  channel, and by 10  $\mu M$  EIPA, an inhibitor of Na/H exchanger, whereas the loss of  $K^+_i$  was suppressed by 0.1  $\mu M$  charybdotoxin, an inhibitor of large conductance  $Ca^{2+}$ -activated  $K^+$  channels. Neither the inhibitor of voltage-gated  $Kv 1.2$  and  $Kv 1.3$   $K^+$  channels noxiustoxin, nor the inhibitor of ATP-sensitive  $K^+$  channels glibenclamide affected the loss of  $K^+_i$  triggered by EPS. We also did not detect any significant impact of inhibitors of intermediate and small conductance  $Ca^{2+}$ -activated  $K^+$  channels (TRAM-34 and apamin, respectively) on this parameter. None of the tested compounds affected the intracellular content of  $Na^+_i$  and  $K^+_i$  in control EPS-untreated myotubes. The data clearly demonstrate that the dissipation of transmembrane  $Na^+_i$  and  $K^+_i$  gradient observed in EPS-treated C2C12 myotubes is mainly associated with the activation of tetrodotoxin-sensitive voltage-gated  $Na^+$  channels ( $Na_v$ ), Na/H exchanger (NHE) and charybdotoxin-sensitive large conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ). Our results do not contradict the previously published data. Thus, voltage-gated  $Na^+$  channels and large conductance  $Ca^{2+}$ -activated  $K^+$  channels are necessary to maintain the excitability of skeletal muscle cells [45, 46]. At the same time, changes in skeletal muscle pH during exercise are observed, and Na/H exchanger is participated in intracellular pH recovery following it [47].

In epithelial cells, 1  $\mu M$  amiloride almost completely suppresses ENaC activity (for review, see [48]). Unlike epithelial cells, in skeletal muscle  $Na^+$  channels triggered by stretch were slightly inhibited by 1  $\mu M$  amiloride but completely blocked after the addition of 1000  $\mu M$  amiloride [44]. Table 2 shows that both the elevation of  $[Na^+]_i$  and the attenuation of  $[K^+]_i$  triggered by EPS were resistant to 1  $\mu M$  amiloride but sharply diminished in the presence of 1000  $\mu M$  amiloride indicating the presence of channels with low affinity for this compound. A direct effect of 1000  $\mu M$  amiloride on NHE is also possible, since such an effect has been observed previously in chick muscle cells [49]. However, it should be emphasized that high amiloride concentrations can also inhibit the activity of diverse protein kinase involved in intracellular signaling cascades, side-by-side with ENaC and NHE [50]. It is worth noting that 1000  $\mu M$  amiloride also eliminates EPS-mediated  $K^+$  efflux (Table 2). However, there is only one mention in the literature of amiloride-sensitive  $K^+$  channels identified in rabbit renal brush border membrane [51]. It has also been shown that *c* wave produced by  $K^+$  currents in rabbit electroretinogram reduced by 100  $\mu M$  amiloride [52].

**Table 2.** Effects of ion transport inhibitors on intracellular sodium and potassium content in control and EPS-treated C2C12 myotubes. C2C12 myotubes were supplied with 3 ml of differentiation medium containing compounds listed in the left column. In some experiments, cells were subjected to EPS with voltage 40 V/10 ms/1 Hz. Means ± S.E. obtained in n experiments are shown. \*, \*\*, \*\*\* p<0.05, <0.01 and <0.001 compared to control (EPS-untreated) cells

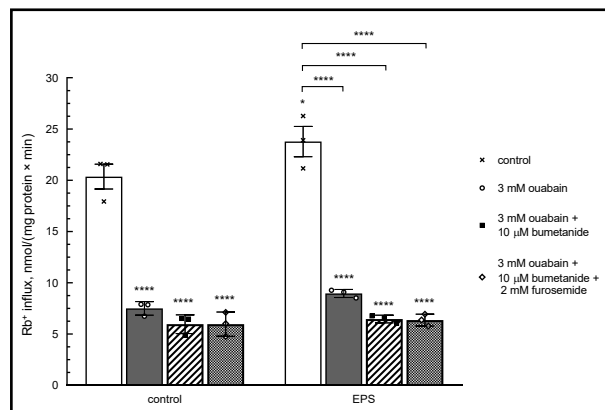
Tested compounds	Na <sup>+</sup> <sub>i</sub> , nmol/mg protein		K <sup>+</sup> <sub>i</sub> , nmol/mg protein	
	control	EPS	control	EPS
1. None (control) (n=12)	120±12	223±18***	999±55	753±47**
2. Tetrodotoxin, 0.5 μM (n=6)	137±18	156±21	981±81	834±42
3. Amiloride, 1 μM (n=4)	120±21	239±20**	1018±69	790±50*
4. Amiloride, 1000 μM (n=3)	109±28	168±41	1001±62	937±43
5. Charybdotoxin, 0.1 μM (n=6)	109±14	198±18**	1089±87	922±73
6. TRAM-34, 0.1 μM (n=4)	110±21	216±30*	1007±52	708±33*
7. Apamin, 2 μM (n=4)	116±25	238±34*	1023±87	703±54*
8. Noxiustoxin, 0,1 μM (n=5)	99±31	205±30*	987±69	742±56*
9. Glibenclamide, 50 μM (n=4)	122±18	241±29**	924±41	721±38**
10. EIPA, 10 μM (n=3)	139±15	115±38	1050±9	887±10*
11. Furosemide, 2000 μM (n=3)	96±11	166±11*	1122±21	965±44

*EPS increases Na,K,2Cl cotransporter activity in C2C12 myotubes*

Na-Cl cotransporter (NCC), Na-K-2Cl cotransporters (NKCC), and K-Cl cotransporters (KCC) belong to the family of solute carriers involved in the transport of ions across biological membranes along or against their electrochemical gradient. It is known that NCC and NKCC are inhibited by low doses of bumetanide and thiazide derivatives, respectively. There are two potent specific inhibitors of KCC (VU0255011 and VU0463271), and their activity is known to be partially attenuated in the presence of high doses of furosemide [53].

Immunoreactive NKCC1 was detected in both red and white skeletal muscle as well as in rat skeletal muscle-derived immortalized L6 myoblasts [54]. Two isoforms of KCCs (KCC1 and KCC3) have been identified in skeletal muscles (for review, see [55, 56]). In L6 myoblasts, bumetanide inhibited Rb<sup>+</sup> uptake by 50-60% [54, 57] thus indicating the highly active NKCC. Unlike L6 myoblasts, bumetanide did not affect Rb<sup>+</sup> uptake in freshly isolated rat soleus and plantaris skeletal muscles [58]. CCC activity in C2C12 myotubes has not been examined yet. To understand which of these transporters are involved in the dissipation of Na<sup>+</sup><sub>i</sub> and K<sup>+</sup><sub>i</sub> gradient, we performed an inhibitor analysis.

Fig. 3 shows that EPS increased NKCC activity measured as ouabain-resistant, bumetanide-sensitive component of the rate of Rb<sup>+</sup> influx (1.7±0.4 and 2.8±0.5 nmol/mg protein×min in control and EPS-treated myotubes, respectively). In the presence of bumetanide, addition of 2000 μM furosemide did not affect the rate of Rb<sup>+</sup> uptake thus



**Fig. 3.** Effect of bumetanide and furosemide on the rate of Rb<sup>+</sup> influx in C2C12 myotubes. In part of experiments, cells were subjected to EPS as indicated in Table 3 legend. Then, control and EPS-treated myotubes were supplied with medium A containing RbCl, 3000 μM ouabain and with or without 20 μM bumetanide and 2000 μM furosemide and incubated during 10 min in the presence or absence of EPS. Means ± S.E. obtained in 3 experiments are shown.

indicating a negligible activity of inwardly directed KCC. Neither bumetanide nor furosemide affected the intracellular  $\text{Na}^+_i$  and  $\text{K}^+_i$  content under baseline conditions, as well as the increment of the  $\text{Na}^+_i/\text{K}^+_i$  ratio triggered by EPS (Table 3). All data together demonstrate that in C2C12 myotubes EPS appears to increase the activity of Na-K-2Cl cotransporter providing  $\text{K}^+$  influx. On the other hand, when interpreting these data, it should be kept in mind that the analysis of ion fluxes was performed after the end of electrical pulse stimulation and not during it. This fact does not exclude that NKCC may be activated to restore gradients of monovalent cations.

*Suppression of calmodulin-dependent protein kinase II (CaMKII) by KN-62 and KN-93 increases  $\text{Na}^+$  efflux in EPS-treated C2C12 myotubes*

In order to identify elements of EPS-activated signaling system(s), we also used an inhibitor assay. Table 4 shows that inhibition of calmodulin-dependent protein kinase II (CaMKII) activity by 10  $\mu\text{M}$  KN-62 or 10  $\mu\text{M}$  KN-93 results in reduced  $\text{Na}^+$  content in EPS-treated myotubes compared with control EPS-treated samples. At the same time, 10  $\mu\text{M}$  KN-93 also decreased  $\text{Na}^+_i$  content in EPS-untreated C2C12 myotubes. Previously, 5  $\mu\text{M}$  KN-93 was shown to reduce  $\text{Na}^+$  currents in rat ventricular myocytes [59]. It is possible that this effect of KN-93 is nonspecific, unrelated to CaMKII activity [60]. The effect of these inhibitors was diminished but did not disappear completely after the inhibition of Na,K-ATPase by ouabain. Apparently, CaMKII suppresses Na,K-ATPase activity and/or affects some other  $\text{Na}^+$  transporters which leads to the activation of  $\text{Na}^+$  influx or to the inhibition of  $\text{Na}^+$  efflux. It is most likely that CaMKII inhibition decreases the activity of the Na/H exchanger, which contributes to the gain of  $\text{Na}^+_i$  in EPS-treated C2C12 myotubes (Table 3). This observation is supported by data obtained using rat cardiomyocytes that CaMKII activates the Na/H exchanger in these cells in order to recover intracellular pH [61].

**Table 3.** Effect of bumetanide and furosemide on intracellular sodium and potassium content in control and EPS-treated C2C12 myotubes. C2C12 myotubes were supplied with 3 ml of differentiation medium containing compounds listed in the left column. In some experiments, cells were subjected to EPS with voltage 40 V/10 ms/1 Hz. Means  $\pm$  S.E. obtained in n experiments are shown. \*, \*\*  $p < 0.05$  and  $< 0.01$  compared to control (EPS-untreated) cells

Tested compounds	$\text{Na}^+_i$ , nmol/mg protein		$\text{K}^+_i$ , nmol/mg protein	
	control	EPS	control	EPS
1. None (control) (n=4)	119 $\pm$ 16	220 $\pm$ 21**	1073 $\pm$ 87	769 $\pm$ 53*
2. Bumetanide, 10 $\mu\text{M}$ (n=4)	108 $\pm$ 19	200 $\pm$ 26*	901 $\pm$ 69	750 $\pm$ 50*
3. Bumetanide + Furosemide, 2000 $\mu\text{M}$ (n=3)	107 $\pm$ 15	195 $\pm$ 18*	931 $\pm$ 41	769 $\pm$ 40*

**Table 4.** Effect of  $\text{Ca}^{2+}$ -activated signaling systems inhibitors on intracellular sodium and potassium content in control, 3000  $\mu\text{M}$  ouabain and EPS-treated C2C12 myotubes. C2C12 myotubes were supplied with 3 ml of differentiation medium containing compounds listed in the left column. In some experiments, cells were subjected to EPS with voltage 40 V/10 ms/1 Hz. Means  $\pm$  S.E. obtained in n experiments are shown. \*, \*\*, \*\*\*, \*\*\*\* 0.0189, 0.0038, 0.0009 and  $< 0.0001$  compared to control (ouabain and EPS-untreated) cells

Tested compounds	$\text{Na}^+_i$ , nmol/mg protein			$\text{K}^+_i$ , nmol/mg protein		
	control	ouabain	EPS	control	ouabain	EPS
1. None (control) (n=6)	140 $\pm$ 13	1643 $\pm$ 55****	700 $\pm$ 29****	1101 $\pm$ 12	180 $\pm$ 4****	1029 $\pm$ 26*
2. 10 $\mu\text{M}$ KN-62 (n=3)	166 $\pm$ 10	1507 $\pm$ 22****	517 $\pm$ 3**	1101 $\pm$ 34	193 $\pm$ 13****	992 $\pm$ 27*
3. 10 $\mu\text{M}$ KN-93 (n=3)	24 $\pm$ 1	1463 $\pm$ 27****	451 $\pm$ 23***	1099 $\pm$ 16	190 $\pm$ 6****	941 $\pm$ 4***
4. 10 $\mu\text{M}$ nicardipine (n=3)	97 $\pm$ 14	1553 $\pm$ 56****	573 $\pm$ 42**	1121 $\pm$ 30	230 $\pm$ 5****	983 $\pm$ 3**
5. 10 $\mu\text{M}$ CsA (n=3)	64 $\pm$ 22	1484 $\pm$ 89****	587 $\pm$ 105**	1184 $\pm$ 37	151 $\pm$ 1****	889 $\pm$ 78**
6. 10 $\mu\text{M}$ PD98059 (n=3)	180 $\pm$ 30	1625 $\pm$ 69****	752 $\pm$ 50****	1167 $\pm$ 15	175 $\pm$ 5****	951 $\pm$ 38****
7. 40 $\mu\text{M}$ PD98059 (n=3)	157 $\pm$ 24	1422 $\pm$ 16****	811 $\pm$ 57****	1165 $\pm$ 20	174 $\pm$ 8****	949 $\pm$ 14****
p1,2	NS	NS	NS	NS	NS	NS
p1,3	$p < 0.002$	NS	0.0088	NS	NS	NS
p1,4	NS	NS	NS	NS	0.0007	NS
p1,5	NS	NS	NS	NS	NS	NS
p1,6	NS	NS	NS	NS	NS	NS
p1,7	NS	NS	NS	NS	NS	NS



We did not detect any significant action of 10  $\mu\text{M}$  nifedipine (an inhibitor of L-type voltage-gated  $\text{Ca}^{2+}$  channels), 10  $\mu\text{M}$  cyclosporin A (an inhibitor of calcineurin), 10 and 40  $\mu\text{M}$  PD98059 (an inhibitor of Erk 1/2) on intracellular content of monovalent cations in control C2C12 myotubes as well as in EPS-treated samples.

## Conclusion

The data obtained in the present studies led us to five major conclusions.

*First*, the dissipation of transmembrane  $\text{Na}^+_i$  and  $\text{K}^+_i$  gradients observed in EPS-treated C2C12 myotubes is mainly caused by the activation of tetrodotoxin-sensitive voltage-gated  $\text{Na}^+$  channels ( $\text{Na}_v$ ),  $\text{Na}/\text{H}$  exchanger (NHE) and charybdotoxin-sensitive  $\text{Ca}^{2+}$ -activated large-conductance  $\text{K}^+$  channels ( $\text{BK}_{\text{Ca}}$ ).

*Second*, EPS is accompanied by the activation of  $\alpha 2$ - $\text{Na},\text{K}$ -ATPase that partially suppresses the dissipation of transmembrane  $\text{Na}^+_i$  and  $\text{K}^+_i$  gradients.

*Third*, in C2C12 myotubes, EPS appears to increase the activity of  $\text{Na}-\text{K}-2\text{Cl}$  cotransporters measured as ouabain-resistant bumetanide-sensitive component of  $\text{Rb}^+$  influx.

*Fourth*, we did not find any evidence that voltage-gated  $\text{Kv}1.2$  and  $\text{Kv}1.3$   $\text{K}^+$  channels, as well as ATP-sensitive  $\text{K}^+$  channels and small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels affect EPS-induced  $\text{K}^+_i$  loss in C2C12 cells.

*Fifth*, calmodulin-dependent protein kinase II appears to stimulate  $\text{Na}^+$  influx in EPS-treated C2C12 myotubes.

The impact of  $\text{Na}^+$  and  $\text{K}^+$  fluxes through different transporters in the dissipation of  $\text{Na}^+_i$  and  $\text{K}^+_i$  gradients is the subject of further studies, as well as the role of various protein kinase that are inhibited by high amiloride concentrations.

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### Author contributions

**Svetlana V. Sidorenko:** Investigation, Software, Visualization; **Elizaveta A. Klimanova:** Data curation, Writing - Original draft preparation, Reviewing and Editing; **Olga D. Lopina:** Writing - Original draft preparation, Reviewing and Editing; **Eugene G. Maksimov:** Writing - Reviewing and Editing; **Sergei N. Orlov:** Conceptualization, Methodology, Supervision, Writing - Original draft preparation.

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The authors have no ethical conflicts to disclose.

## Disclosure Statement

The authors have no conflicts of interest to declare.

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