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Electrical pulse stimulation decreases electrochemical Na^+ and K^+ gradients in C2C12 myotubes

Kirill Danilov [a, b, **], Svetlana Sidorenko [a, c], Kseniya Milovanova [c], Elizaveta Klimanova [a], Leonid V. Kapilevich [c], Sergei N. Orlov [a, c, *]

[a] M.V. Lomonosov Moscow State University, Moscow, 1-12 Leninskie Gory, 119234, Russia
[b] Skolkovo Institute of Science and Technology, Moscow, 4 Nobel Street, 143026, Russia
[c] National Research Tomsk State University, Tomsk, 36 Lenin Ave, 634050, Russia

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ABSTRACT

Electrical pulse stimulation (EPS)-treated cultured myotubes are widely employed as an *in vitro* model of muscle contraction. Here we examined time-dependent EPS action and dose-dependent ouabain action on $[Na^+]_i$ and $[K^+]_i$ in C2C12 myotubes. After 2 h of EPS (40 V, 1 Hz, 10 ms) $[Na^+]_i$ increased by ~150% whereas $[K^+]_i$ declined by ~20%. 3 μM ouabain had a negligible impact on $[Na^+]_i$ and $[K^+]_i$ in control cells but increased the $[Na^+]_i/[K^+]_i$ ratio in EPS-treated myotubes by 85%. Thus, our results show for the first time that EPS results in dissipation of Na^+ and K^+ gradients in cultured myotubes and suggest that the augmented production of endogenous cardiotonic steroids may contribute to elevation of the $[Na^+]_i/[K^+]_i$ ratio in exercising muscle.

1. Introduction

Numerous laboratories reported that vigorous exercises had lead to rapid elevation of plasma K^+ concentration up to 8–10 mM (for review, see Refs. [1–4]). Keeping in mind that skeletal muscle in mammals is a major pool of potassium [2] it might be assumed that the increment of plasma $[K^+]$ triggered by exercise is caused by dissipation of transmembrane gradients of monovalent cations in myotubes. Indeed, it was shown by calculation of femoral artery-venous Na^+ and K^+ concentration differences that 6 min intense knee extensor exercise in human skeletal muscle increased $[Na^+]_i$ from 13 to 23 mM and reduced $[K^+]_i$ from 162 to 129 mM [5]. More recently, Murphy and co-workers using flame photometry found that in rat soleus muscle 60 min of intermittent running resulted in ~2-fold elevation of $[Na^+]_i$ and attenuation of $[K^+]_i$ by 10–20% [6].

Cultured skeletal muscle cells subjected to EPS are widely employed as an *in vitro* model of exercising skeletal muscle. This approach is based on several observations. *First*, in differentiated myotubes obtained from murine C2C12 [7,8], rat L6 [9] and primary rat [10] and human [11] skeletal muscle cells EPS resulted in metabolic and adaption changes detected in freshly isolated trained muscles [12]. *Second*, C2C12 myotubes exhibit remarkable contractile responses [7,13,14] and repetitive $[Ca^{2+}]_i$ transients [13] triggered by EPS. *Third*, in C2C12 myotubes [7,15,16] and primary cultured human myotubes [17] EPS triggers secretion of interleukin-6 and several other myokines whose plasma content levels were sharply increased by exercising human and animals (for review, see Refs. [18–20]).

Electrochemical gradients of monovalent cations in animal cells are under control of Na^+,K^+-ATPase consisting of catalytic α1-α4 and regulatory β1-β3 subunits. In contrast to other cell types, skeletal muscle is abundant with α2 isozyme whose content is 3–5-fold higher compared to housekeeping α1 subunit (for review see Refs. [21,22]). Another striking finding is that α2 isozyme, despite its significantly greater abundance, makes negligible contribution to baseline Na^+ and K^+ gradient but plays a key role in regulation of the $[Na^+]_i/[K^+]_i$ ratio in contracting skeletal muscle treated with electrical and pharmacological stimuli [23,24]. Importantly, the affinity of α1 isoform for inhibitory action of ouabain is 3 order of

Abbreviations: EPS, electrical pulse stimulation; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PBST, PBS supplemented with Tween 20; DDI, doubly deionized; TCA, trichloroacetic acid; RCF, relative centrifugal force.

* Corresponding author. Department of Biomembranes, Faculty of Biology, Lomonosov Moscow State University, 1-12 Leninskie Gory, 119234, Moscow, Russia.
** Corresponding author. Department of Biochemistry, Faculty of Biology, Lomonosov Moscow State University, 1-12 Leninskie Gory, 119234, Moscow, Russia.
E-mail addresses: danilovkiri@yandex.ru (K. Danilov), sergeinorlov@yandex.ru (S.N. Orlov).

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magnitude less compared to α2-Na^+,K^+-ATPase in mice [25] as well as in several other rodents. This study examined the action of EPS and ouabain on $[Na^+]_i$ and $[K^+]_i$ content in mouse C2C12 myotubes.

2. Materials and methods

2.1. Cell culture

Murine C2C12 cells subjected to less than 10 passages were obtained from 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 3×10^4 cells per well in 6-well plates containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 100 units·ml^{-1} penicillin and 100 μg·ml^{-1} streptomycin and kept at 37°C in humidified 5% CO_2 atmosphere. Three-five days after plating, the cells had reached 70–80% confluence and were subjected to differentiation in DMEM containing 5 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 × 100 magnification without preliminary fixation.

2.2. Electric pulse stimulation

The differentiated C2C12 myotubes in 6-well plates were washed with phosphate buffered saline (PBS), supplied with 3 ml DMEM containing 25 mM glucose and subjected to EPS for up to 4 h using a C-Pace pulse generator (C-Pace EP, IonOptix, USA) with voltage 40 V, stimuli duration 10 ms and frequency 1 Hz. Previously, it was shown that EPS in C2C12 triggers repetitive $[Ca^{2+}]_i$ transients [13] as well as release of IL-6 and several other myokines [7,16] at these voltage and frequency. Ouabain was added at final concentration of 1–3000 μM in a part of experiments.

2.3. Cell viability measurement

The AlamarBlue assay [26] was carried out according to manufacturer's instructions. Briefly, experimental medium was removed and wells were filled with 1.5 ml of fresh experimental medium. 45 μl of AlamarBlue reagent were added to 1.5 ml of medium in each well and mixed gently. Following 2 h of incubation fluorescence of medium was measured at the excitation and emission wavelength of 570 and 585 nm, respectively, using a Synergy H4 Multi-Mode Hybrid Microplate Reader (BioTek, USA). For each experiment, wells containing only medium with AlamarBlue reagent without cells were also prepared and incubated during 2 h for quantification of background fluorescence. Fluorescence signals from each well were normalized on protein amount in the same well, which was determined by Lowry protein assay [27]. Viability of control wells was referred to as 100%.

2.4. Western blot analysis

The harvested cell lysates before and after differentiation were prepared by solubilisation of cells precipitates in PBS 1× (pH 7.4) and repeated freezing and melting. Protein concentrations were determined using Lowry protein assay [27]. SDS-polyacrylamide gel electrophoresis was performed in accordance with Laemmli method [28] with 5% stacking gel and 12% running gel. Proteins were transferred from gel to a nitrocellulose membrane (BioRad, USA) followed by blocking with 5% milk powder (Valio, Finland) in PBST 1× (PBS supplemented with Tween 20 0.1%) for 1 h at room temperature. Detecting of skeletal troponin-T was achieved employing an overnight incubation with 10 μg ml^{-1} anti-troponin-T antibodies each at 4°C in 5% milk powder in PBST. Incubation with HRP-conjugated secondary antibodies was carried out for 1 h at room temperature in 5% milk powder in PBST. Visualization of antigen-antibody complexes was performed using the ECL kit and ChemiDoc XRS + Molecular Imager (BioRad, USA).

2.5. Measurement of intracellular Na^+ and K^+

6-well plates were transferred onto ice, experimental medium was quickly removed and cells were washed three times with 3 ml of an ice-cold 0.1 M $MgCl_2$ solution in doubly deionized (DDI) water. Then, 1.5 ml of 5% trichloroacetic acid (TCA) in DDI water were added to each well, followed by incubation at 4°C overnight for complete extracting of ions from myotubes. Cell precipitates were suspended and centrifuged during 5 min at 18000 RCF. Supernatants were transferred into 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 during 1 h for complete protein dissolving. The protein solutions gained were used for protein amount quantification by Lowry protein assay [27]. The Na^+ and K^+ contents in TCA extracts were measured by flame atomic absorption spectrometry using the Kvant-2m1 spectrometer (Cortec, Russia) with propane-air mixture in accordance with the manual. KCl (0.5–4 mg L^{-1} K^+) and NaCl solutions (0.05–2 mg·L^{-1} Na^+) in 5% TCA in DDI water were used for calibration. The Na^+ and K^+ contents in each well were normalized on protein amount in the same well.

2.6. Materials

DMEM was purchased from both PanEco (Russia) and Gibco (USA). Trypsin-EDTA was obtained from PanEco (Russia), 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). Murine monoclonal anti-troponin-T antibodies (clones 1A11, 2F3 and 1C11, HyTest Ltd., Finland) were a gift from A.V. Kharitonov (Lomonosov Moscow State University, Moscow, Russia). Secondary anti-mouse HRP-conjugated antibodies were obtained from Biosource (USA, cat.# AMI3704, 1:5000). The ECL kit (SuperSignal West Femto Maximum Sensitivity Substrate) was obtained from Thermo Scientific (USA). Unless otherwise noted, all chemicals were of the purest grade available from Sigma-Aldrich (USA), Fischer BioReagents (Germany), Fluka Analytical (USA) or Amresco (USA).

2.7. Statistical analysis

All data are expressed as means ± SEM with number of trials provided. Pairwise differences between two means were tested using two-tailed *t*-test. P values are denoted as follows: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$.

3. Results and discussion

Consistently with previous publications [7,8,13,14,16], 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 and formation of prolonged multinucleated myotubes (Fig. 1A). This conclusion is also supported by appearance of troponin T, i.e. a well documented skeletal muscle marker (Fig. 1B).

Fig. 2A shows that 2 h EPS did not affect viability of C2C12 myotubes estimated by the AlamarBlue assay whereas after 4 h this parameter decreased by 20%. We did not find any cytotoxic action of 6 h incubation in the presence of ouabain in the range from 1 to

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