Ionic mechanisms of excitation-induced regulation of Na\(^+\)-K\(^+\)-ATPase mRNA expression in isolated rat EDL muscle

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Murphy, K. T., W. A. Macdonald, M. J. McKenna, and T. Clausen. Ionic mechanisms of excitation-induced regulation of Na\(^+\)-K\(^+\)-ATPase mRNA expression in isolated rat EDL muscle. Am J Physiol Regul Integr Comp Physiol 290: R1397–R1406, 2006. First published December 15, 2005; doi:10.1152/ajpregu.00707.2005.—This study investigated the effects of electrical stimulation on Na\(^+\)-K\(^+\)-ATPase isoform mRNA, with the aim to identify factors modulating Na\(^+\)-K\(^+\)-ATPase mRNA in isolated rat extensor digitorum longus (EDL) muscle. Interventions designed to mimic exercise-induced increases in intracellular Na\(^+\) and Ca\(^2\+) content and membrane depolarization were examined. Muscles were mounted on force transducers and stimulated with 60-Hz 10-s pulse trains producing tetanic contractions three times at 10-min intervals. Ouabain (1.0 mM, 120 min), veratridine (0.1 mM, 30 min), and monensin (0.1 mM, 30 min) were used to increase intracellular Na\(^+\) content. High extracellular K\(^+\) (13 mM, 60 min) and the Ca\(^2\+) ionophore A-23187 (0.02 mM, 30 min) were used to induce membrane depolarization and increased intracellular Ca\(^2\+) content, respectively. Muscles were analyzed for Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)–\(\alpha_3\) and \(\beta_1\)–\(\beta_3\) mRNA (real-time RT-PCR). Electrical stimulation had no immediate effect on Na\(^+\)-K\(^+\)-ATPase mRNA; however at 3 h after stimulation, it increased \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) mRNA by 223, 621, and 892%, respectively (\(P = 0.010\)), without changing \(\beta\) mRNA. Ouabain, veratridine, and monensin increased intracellular Na\(^+\) content by 769, 724, and 598%, respectively (\(P = 0.001\)) but did not increase mRNA of any isoform. High intracellular K\(^+\) concentration elevated \(\alpha_1\)-mRNA by 160% (\(P = 0.021\)), whereas A-23187 elevated \(\alpha_2\)-mRNA by 123% (\(P = 0.035\)) but reduced \(\beta_1\) mRNA by 76% (\(P = 0.001\)). In conclusion, electrical stimulation induced subunit-specific increases in Na\(^+\)-K\(^+\)-ATPase mRNA in isolated rat EDL muscle. Furthermore, Na\(^+\)-K\(^+\)-ATPase mRNA appears to be regulated by different stimuli, including cellular changes associated with membrane depolarization and increased intracellular Ca\(^2\+) content but not increased intracellular Na\(^+\) content.

IN SKELETAL MUSCLE, Na\(^+\)-K\(^+\)-ATPase activity maintains the transmembrane Na\(^+\) and K\(^+\) concentration gradients necessary for repeated action potential generation. The Na\(^+\)-K\(^+\)-ATPase comprises a catalytic \(\alpha\)-subunit and a glycosylated \(\beta\)-subunit, with different genes encoding for four \(\alpha\)-isoforms (\(\alpha_1\)–\(\alpha_4\)) and three \(\beta\)-isoforms (\(\beta_1\)–\(\beta_3\)). Human skeletal muscle has recently been shown to express mRNA for each of the \(\alpha_1\) to \(\alpha_3\) and \(\beta_1\) to \(\beta_3\)-isoforms (24, 29) and also for the \(\alpha_4\)-isoform (29). Rat skeletal muscle has also been reported to express mRNA for each of the \(\alpha_1\) to \(\alpha_3\)- and \(\beta_1\)- and \(\beta_2\)-isoforms (31, 43), whereas the mRNA for the \(\alpha_4\)- and \(\beta_3\)-isoforms do not appear to have been probed.

Only ∼6 min of intense exercise elevated the mRNA expression of the \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms in human muscle (24), whereas more prolonged exercise elevated the mRNA expression of the \(\alpha_1\)-, \(\alpha_3\)-, and \(\beta_2\)-isoforms in human muscle (23) and the mRNA expression of the \(\alpha_1\)- and \(\beta_2\)-isoforms in rat muscle (43). Whether electrical stimulation exerts similar effects is unknown and was therefore investigated here. It was hypothesized that three bouts of high-frequency electrical stimulation of isolated rat extensor digitorum longus (EDL) muscle would increase the mRNA expression of one or several of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms.

The intracellular signals involved in the regulation in the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms in skeletal muscle have not been identified. Because acute exercise increases the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms in mammalian muscle (24, 43), it is likely that one or several of the transmembrane ionic fluxes and subsequent intracellular ionic concentration changes, which occur with exercise, may be involved in the signaling pathways inducing mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms.

Repeated muscle contractions induce an elevation in intracellular Na\(^+\) content in human (40) and rat muscle (20). However, because this increases Na\(^+\)-K\(^+\)-ATPase activity (26), this elevation in intracellular Na\(^+\) content is transient. It is possible that this transient rise in intracellular Na\(^+\) content is involved in increasing the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms in skeletal muscle. In rat kidney cells, increasing the intracellular Na\(^+\) content and/or Na\(^+\) influx with ouabain, an inhibitor of Na\(^+\)-K\(^+\)-ATPase, induced an ∼100% increase in the mRNA expression of both the \(\alpha_1\)- and \(\beta_1\)-isoforms (36). Furthermore, in chicken skeletal muscle cells, veratridine, an activator of the voltage-gated Na\(^+\)-channels, induced a 70 and 150% increase in the mRNA expression of the \(\alpha\)- and \(\beta\)-subunit isoforms, respectively (42). Monensin, a specific Na\(^+\) ionophore, has also been used to elevate the intracellular Na\(^+\)-to-K\(^+\) ratio in rat skeletal muscle (8), but the effects of monensin on the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms are unknown. Interestingly, in rat hindlimb muscle, an increase in intracellular Na\(^+\) content induced by dietary K\(^+\) deficiency reduced the mRNA expression of the \(\alpha_2\)-isoform by 35%, with no significant effect on the mRNA expression of either of the \(\alpha_1\)- or \(\beta_1\)-isoforms (1). Despite the

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above-mentioned exception, it was therefore hypothesized that elevated intracellular Na\(^+\) content and/or Na\(^+\) influx, induced by either ouabain, veratridine, or monensin, would increase the mRNA expression of one or several of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms in rat skeletal muscle.

Repeated muscle contractions induce a reduction in intracellular K\(^+\) concentration and a subsequent increase in muscle extracellular K\(^+\) concentration ([K\(^+\)]\(_o\)), leading to membrane depolarization (38). During intense exercise in humans, muscle [K\(^+\)]\(_o\) can reach as high as \(\sim 13\) mM (41). In isolated rat EDL muscle, a [K\(^+\)]\(_o\) of 13 mM induced a membrane depolarization of 26 mV (13). No study has elevated muscle [K\(^+\)]\(_o\), to investigate the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms. However, in rat liver cells, low [K\(^+\)]\(_o\) (0.25–0.65 mM) induced a 250% increase in the mRNA expression of the \(\alpha\)-subunit isoform (35); in canine kidney cells, low [K\(^+\)]\(_o\) induced a 200% increase in the mRNA expression of both the \(\alpha\)- and \(\beta\)-subunit isoforms (3). Such low [K\(^+\)]\(_o\), would lead to membrane depolarization (10). It was therefore hypothesized that membrane depolarization induced by high [K\(^+\)]\(_o\), to replicate repeated muscle contractions, would increase the mRNA expression of one or several of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms in rat skeletal muscle.

Baseline cytosolic Ca\(^{2+}\) concentration increases during repeated muscle contractions in isolated mammalian muscle (44). In rat kidney cells, elevating the intracellular Ca\(^{2+}\) concentration from 0.1 to 4.0 \(\mu\)M induced a 300% increase in the mRNA expression of both the \(\alpha_1\)- and \(\beta_1\)-isoforms (37). Whether increased intracellular Ca\(^{2+}\) content exerts similar effects on the mRNA expression of the other Na\(^+\)-K\(^+\)-ATPase isoforms is unknown. The Ca\(^{2+}\) ionophore A-23187 has been shown to elevate both muscle Ca\(^{2+}\) content and \(45\)Ca uptake in rat EDL muscle (11), as well as the intracellular free Ca\(^{2+}\) content in cultured myotubes (16). On the basis of the findings by Rayson (37), it was hypothesized that elevated intracellular Ca\(^{2+}\) content and/or Ca\(^{2+}\) influx would increase the mRNA expression of one or several of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms in rat skeletal muscle.

The effects of electrical stimulation on the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms was studied here, with the aim to also identify the ionic factors modulating this expression in isolated rat EDL muscle. We show that electrical stimulation increased the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-isoforms but not of the \(\beta\)-isoforms in isolated rat EDL muscle. Furthermore, the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms appears to involve the cellular changes associated with membrane depolarization and increased intracellular Ca\(^{2+}\) content and/or Ca\(^{2+}\) influx but not of increased intracellular Na\(^+\) content.

**METHODS**

**Animals and preparation of muscles.** Experiments were carried out with 4-wk-old female and male Wistar rats weighing \(\sim 60–70\) g. Rats of this age were used because the relatively small size of their EDL muscles (~25 mg) minimizes the diffusional barriers to substrates, ions, and oxygen to the cell surface. The animals were fed ad libitum and were maintained in a temperature-controlled environment (21°C) with constant day length (12 h). The animals were killed by cervical dislocation, followed by decapitation, with intact EDL muscles, a predominantly fast-twitch fiber muscle (2), dissected out as previously described (27). All handling and use of animals complied with Danish animal welfare regulations.

Muscles were equilibrated for 30 min at 30°C in standard Krebs-Ringer bicarbonate buffer (KR) (pH 7.4) containing the following (in mM): 122.1 NaCl, 25.1 NaHCO\(_3\), 2.8 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.3 CaCl\(_2\), and 10.0 d-glucose; this was bubbled continuously with a mixture of 95% O\(_2\)-5% CO\(_2\). In buffer with 13.0 mM [K\(^+\)]\(_o\), an equivalent amount of Na\(^+\) was omitted to maintain isosmolarity.

**Effect of electrical stimulation on intracellular Na\(^+\) and K\(^+\) contents.** Intact muscles were mounted at resting length on electrodes for isometric contractions and equilibrated for 30 min in KR at 30°C. Muscles were then either rested or exposed to field stimulation across the central region through platinum electrodes, using either one, two, or three stimulation bouts, each comprising 10 s of continuous 60-Hz stimulation (0.2 ms, 12 V), given at 10-min intervals. We measured force (mN) using force displacement transducers, which were recorded with a chart recorder and/or digitally on a computer. Muscles exposed to two stimulation bouts were allowed to rest for 10 min after the second stimulation bout to represent the intracellular Na\(^+\) and K\(^+\) contents immediately before the third stimulation bout. Muscles were then washed for 4×15 min in ice-cold Na\(^+\)-free Tris succrose buffer (pH 7.45, containing the following in mM: 263.5 sucrose, 4.7 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.3 CaCl\(_2\)) to remove all extracellular Na\(^+\) (8). Muscles were blotted, tendons were removed, muscle wet weight was determined, and the muscles were soaked overnight in 0.3 M trichloroacetic acid (TCA) to give complete extraction of ions from the tissue (5). The Na\(^+\) content in the TCA extract was measured by flame photometry (FLM3; Radiometer, Copenhagen, Denmark) with lithium as internal standard. Values for Na\(^+\) content were then multiplied by 1.59 to correct for the loss of intracellular Na\(^+\) during the ice-cold washout (see Fig. 1, as described below). In contrast, the loss of K\(^+\) during the washout was minimal (8).

**Effect of washout in ice-cold Na\(^+\)-free Tris-sucrose buffer on intracellular Na\(^+\) content.** Control experiments were performed to determine the loss of intracellular Na\(^+\) during the 4×15 min (60
min) washout in ice-cold Na\(^+\)-free Tris sucrose buffer in the EDL muscle, as this has previously only been determined in the soleus muscle (8). Intact muscles were mounted at resting length on electrodes for isometric contractions and equilibrated for 30 min in KR, after which muscles were transferred to ice-cold Na\(^+\)-free Tris-sucrose buffer for washout for 30, 60, 90, 120, 150, or 180 min. Every 15 min during washout, muscles were transferred to new tubes and were kept agitated by continuous bubbling with air. After washout, muscles were blotted, tendons were cut off, muscle wet weight was determined, and the muscles were soaked overnight in 0.3 M TCA. The Na\(^+\) content in the TCA extract was measured by flame photometry, as described above. Figure 1 demonstrates that, after an initial 30 min of washout, which allows for removal of extracellular Na\(^+\) from the tissue, the muscle Na\(^+\) content showed an exponential decline with washout duration. This relationship can be tightly fitted by a linear regression line in the semi-logarithmic plot (r\(^2\) > 0.99) and is assumed to represent the washout of intracellular Na\(^+\) (33). To determine the intracellular Na\(^+\) content of the muscles at time 0 (t = 0; prewashout), the regression line was used to calculate a correction factor. The correction factor was calculated by dividing the intercept value of the regression line (t = 0) with the regression line value at 60 min of washout. From this, all values for intracellular Na\(^+\) contents determined after a 60-min (4 x 15 min) washout were corrected by multiplying by a factor of 1.59.

Effects of electrical stimulation on the mRNA expression of the Na\(^-\)-K\(^+\)-ATPase isoforms. Muscles were mounted for isometric contractions in thermostated chambers containing standard KR and were allowed to recover for a further 3 h in standard KR, before being incubated in the appropriate buffer for the indicated duration. All muscles were then allowed to recover for a further 3 h in standard KR (4 mM K\(^+\)), after which they were blotted, tendons were removed, and the muscle was frozen in liquid N\(_2\) for measurement of the mRNA expression of the Na\(^-\)-K\(^+\)-ATPase isoforms. Control muscles were incubated for durations matching their respective experimental muscles in standard KR.

Increased intracellular Na\(^+\) content and/or Na\(^+\) influx induced by ouabain, veratridine, and monensin. Matching groups of muscles were used to determine the mRNA expression of the Na\(^-\)-K\(^+\)-ATPase isoforms after ouabain (120 min, 1.0 mM), veratridine (30 min, 0.1 mM), or monensin (30 min, 0.1 mM) exposure and compared to those used to determine the changes in intracellular Na\(^+\) content arising from these interventions.

Membrane depolarization induced by high [K\(^+\)]. Membrane depolarization was induced by incubating muscles for 60 min in KR containing 13 mM [K\(^+\)]\(_o\) (13).

Increased intracellular Ca\(^{2+}\) content and/or Ca\(^{2+}\) influx induced by A-23187. Increased intracellular Ca\(^{2+}\) content and/or Ca\(^{2+}\) influx was induced by incubating muscles for 30 min in KR containing the Ca\(^{2+}\) ionophore A-23187 (0.02 mM) (11, 16).

Measurement of the mRNA expression of the Na\(^-\)-K\(^+\)-ATPase isoforms. Total RNA was extracted from ~10 mg of muscle using the FastRNA reagents (BIO 101, Vista, CA), with methods previously described (25). The resulting RNA pellet was dissolved in EDTA-treated water, and total RNA concentration was determined spectrophotometrically at 260 nm. The ratio of absorbance at 260 and 280 nm (260/280) was 1.95 (SD 0.33), and the concentration of yielded RNA was not significantly different between control and test muscles (P = 0.152). RNA (1 \(\mu\)g) was transcribed into cDNA using the Promega AMV reverse transcription kit (Promega, Madison, WI), with oligo(dT) primers, with the resulting cDNA stored at −20°C for further analysis.

Real-time PCR (GeneAmp 5700 sequence detection system) was run for 1 cycle (50°C for 2 min, 95°C for 10 min) and 40 cycles (95°C for 15 s, 60°C for 60 s). Primer sequences were designed for the rat Na\(^+\)-K\(^+\)-ATPase \(\alpha_1-\alpha_4\) and \(\beta_1-\beta_3\) genes from published sequences (Table 1). However, mRNA expression of the Na\(^-\)-K\(^+\)-ATPase

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Table 1. Rat Na\(^+\)-K\(^+\)-ATPase genes \(\alpha_1-\alpha_4\) and \(\beta_1-\beta_3\) and Cyc primer sequences used for mRNA analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sense Primer (5'(^-)3')</th>
<th>Antisense Primer (5'(^-)3')</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1)</td>
<td>NM_012504</td>
<td>CAGTGTTTCTCAAGCTACCCAAAGA</td>
<td>GCCGACTCCGAAACCAT</td>
<td>174</td>
</tr>
<tr>
<td>(\alpha_2)</td>
<td>NM_012505</td>
<td>GCTAGAGCAGACAGATCGATTTTCA</td>
<td>ATTACCTTTACAGACCTCTTCMTC</td>
<td>127</td>
</tr>
<tr>
<td>(\alpha_3)</td>
<td>M90659</td>
<td>GGCTTCTGCCAGGGCTCTG</td>
<td>GCCATTCTCAAAACGACAC</td>
<td>268</td>
</tr>
<tr>
<td>(\alpha_4)</td>
<td>NM_022848</td>
<td>TTTTGGTCCAGGGCTCTTGG</td>
<td>CGGAGTTCTTGATGCGTGA</td>
<td>204</td>
</tr>
<tr>
<td>(\beta_1)</td>
<td>NM_031113</td>
<td>TCCAAAGCTTGCAGGGCTC</td>
<td>ATCGTTATGCTCAGGTC</td>
<td>229</td>
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<td>(\beta_2)</td>
<td>D90048</td>
<td>AGGGCGCTGTCAGGGCTG</td>
<td>CGGCTTCTCCAGGCTGAC</td>
<td>230</td>
</tr>
<tr>
<td>(\beta_3)</td>
<td>XM_213132</td>
<td>AATCGGTGGCTGCTGCTG</td>
<td>TTTGCGATGCTGCTGCTG</td>
<td>126</td>
</tr>
<tr>
<td>Cyc</td>
<td>M19533</td>
<td>CAGTGTTTCTCAAGCTACCCAAAGA</td>
<td>GCCGACTCCGAAACCAT</td>
<td>174</td>
</tr>
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Primer sequences were designed with the use of Primer Express software (Applied Biosystems) from gene sequences obtained from GenBank. Primer specificity was determined by use of a BLAST search. Cyc, cyclophilin.
α4-isoform could not be detected in all muscle samples by RT-PCR. The sizes of the PCR fragments amplified with each primer (126–268 bp) are included in Table 1 and are within the size range for close to 100% PCR efficiency, thereby validating this method. All samples were run in triplicate, and measurements included a no-template control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the commonly used housekeeping control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the commonly used housekeeping control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the commonly used housekeeping control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the commonly used housekeeping control (no cDNA), as well as a rat skeletal muscle sample endogenous control. Primer sequences for the commonly used housekeeping control (no cDNA), as well as a rat skeletal muscle sample endogenous control.

Effect of electrical stimulation on intracellular Na+ and K+ contents and on the mRNA expression of the Na+-K+-ATPase isoforms. A representative trace of the force responses to the electrical stimulation paradigm is shown in Fig. 2. During the three stimulation bouts, each at 10 s of stimulation at 60 Hz, force decreased by 27.0% (SD 8.1), 17.3% (SD 7.0), and 15.1% (SD 9.4), respectively (n = 6). At 3 h after the final 10-s stimulation bout, peak tetanic force was 49.2% (SD 7.8) of initial peak tetanic force (n = 6).

The first 10-s stimulation bout immediately increased intracellular Na+ content by 32.2% (P = 0.003) and reduced intracellular K+ content by 7.9% (P = 0.049; Fig. 3). Intracellular Na+ content was 28.9% (P = 0.016) lower in muscles exposed to two stimulation bouts followed by a 10-min recovery than in resting muscles (Fig. 3). The third 10-s stimulation bout, given at 20 min after the first 10-s stimulation bout, increased intracellular Na+ content by 50.7% (P = 0.001) and reduced intracellular K+ content by 4.4% (P = 0.030), compared with before the third stimulation bout (Fig. 3).

Immediately after the third stimulation bout, there was no significant increase in the mRNA expression of any of the α1-, α2-, or α3-isoforms (Fig. 4). At 3 h after stimulation, these were, however, increased by 223% (P = 0.011), 621% (P = 0.001), and 892% (P = 0.001), respectively (Fig. 4). The mRNA expression of the α2- and α3-isoforms at 3 h poststimu-

### Table 2. Intra-assay variability of 2-ΔCT values

<table>
<thead>
<tr>
<th>Gene</th>
<th>2-ΔCT CV</th>
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<tr>
<td>α1</td>
<td>11.2</td>
</tr>
<tr>
<td>α2</td>
<td>11.0</td>
</tr>
<tr>
<td>α3</td>
<td>9.8</td>
</tr>
<tr>
<td>β1</td>
<td>12.2</td>
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<tr>
<td>β2</td>
<td>11.6</td>
</tr>
<tr>
<td>β3</td>
<td>11.1</td>
</tr>
<tr>
<td>Cyc</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Values are percent. Each sample was run in triplicate wells in the same real-time PCR run; n = 241. CV, coefficient of variation; CT, cycle threshold.
Ouabain exposure followed by a 3-h recovery had no significant effect on the mRNA expression of any of the \(\alpha_1\)-, \(\alpha_2\)-, \(\alpha_3\)-, or \(\beta_1\)-isoforms but reduced the mRNA expression of the \(\beta_2\)- and \(\beta_3\)-isoforms, by 76% (\(P = 0.044\)) and 92% (\(P = 0.009\)), respectively (Fig. 6). Neither veratridine nor monensin exposure, followed by a 3-h recovery, had any significant effect on the mRNA expression of any of the \(\alpha_1\)-, \(\alpha_3\)-, \(\beta_1\)-, or \(\beta_2\)-isoforms, but both reduced the mRNA expression of the \(\beta_3\)-isofrm, by 87% (\(P = 0.013\)) and 90% (\(P = 0.011\)), respectively, whereas veratridine also reduced the mRNA expression of the \(\alpha_2\)-isofrm by 69% (\(P = 0.001\); Fig. 6).

**Effect of 13 mM \([K^+]_o\) on the mRNA expression of the \(Na^+-K^+\)-ATPase isoforms.** Exposure to 13 mM \([K^+]_o\), followed by a 3-h recovery increased the mRNA expression of the \(\alpha_1\)-isofrm by 160% (\(P = 0.021\)) and tended to increase the mRNA expression of the \(\beta_1\)-isofrm (\(P = 0.055\); Fig. 7). There was no significant effect of 13 mM \([K^+]_o\), on the mRNA expression of any of the \(Na^+-K^+\)-ATPase \(\alpha_2\)-, \(\alpha_3\)-, \(\beta_1\)-, or \(\beta_2\)-isoforms (Fig. 7).

# Fig. 3
Effects of 3 bouts of high-frequency electrical stimulation (stim) on intracellular Na\(^+\) (A) and K\(^+\) contents (B) in rat EDL muscle. Muscles were mounted for isometric contractions, equilibrated for 30 min in standard KR, and either allowed to rest for 10 s or stimulated for 10 s at 60 Hz, at 10-min intervals, for either 1 (1 × 10 s), 2 (2 × 10 s), or 3 stimulation bouts (3 × 10 s). Muscles stimulated for 2 stimulation bouts were then allowed to rest for a further 10 min to represent the intracellular Na\(^+\) and K\(^+\) contents immediately after the third stimulation bout. All muscles were then washed for 4 × 15 min in ice-cold Na\(^+\)-free Tris-sucrose buffer and blotted; tendons were then removed, weighed, and taken for flame photometric analysis of Na\(^+\) and K\(^+\) contents. Values for Na\(^+\) content were multiplied by 1.59 to correct for the loss of intracellular Na\(^+\) during the washout. Bars denote 10-s stimulation bouts.

Data are means (●) and SD; \(n = 4\)–10. *\(P < 0.05\) vs. rest, †\(P < 0.05\) vs. 1 × 10 s, ‡\(P < 0.05\) vs. 2 × 10 s plus 10-min recovery.

**Fig. 4.** Effects of 3 bouts of high-frequency electrical stimulation and subsequent 3-h recovery on mRNA expression of the \(Na^+-K^+\)-ATPase \(\alpha_1\)- to \(\alpha_3\)- and \(\beta_1\)- to \(\beta_3\)-isoforms in rat EDL muscle. Isoform mRNA expression is expressed relative to control (Con) (1.0). Muscles were mounted for isometric contractions, equilibrated for 30 min in standard KR, and stimulated electrically for 10 s at 60 Hz at 10-min intervals. Muscles were then either immediately removed (Stim) or allowed to recover in standard KR for a further 3 h (Stim+3h). Data are means and SD; \(n = 8\) except Stim where \(n = 6\). *\(P < 0.02\), greater than Con; †\(P < 0.02\), greater than Stim.
Fig. 5. Effects of ouabain, veratridine, and monensin on intracellular Na\(^+\) content in rat EDL muscle. Muscles were placed in polyethylene baskets, equilibrated for 30 min in standard KR, and then incubated without (solid bars) or with ouabain (120 min, 1.0 mM), veratridine (30 min, 0.1 mM), or monensin (30 min, 0.1 mM). They were then allowed to recover in standard KR for a further 3 h (hatched bars). Muscles were then washed for 4 \times 15 min in ice-cold Na\(^+\)-free Tris-sucrose buffer and blotted; tendons were then removed, weighed, and taken for flame photometric analysis of Na\(^+\) content. Values for Na\(^+\) content were multiplied by 1.59 to correct for the loss of intracellular Na\(^+\) during the washout. Data are means and SD; \(n = 13\) for Con, \(n = 4\)–7 for ouabain, and \(n = 4\)–6 for veratridine and monensin. *\(P < 0.001\), greater than Con.

Effect of A-23187 on the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms. A-23187 exposure followed by a 3-h recovery increased the mRNA expression of the \(\alpha_2\)-isoform by 123% (\(P = 0.035\)) and, in contrast, reduced the mRNA expression of the \(\beta_2\)-isoform by 76% (\(P = 0.001\); Fig. 8). There was no significant effect of A-23187 on the mRNA expression of any of the \(\alpha_1\)-, \(\alpha_2\)-, \(\beta_2\)-, or \(\beta_3\)-isoforms (Fig. 8).

DISCUSSION

This study investigated the effects of high-frequency electrical stimulation on the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms in isolated rat EDL muscle. Factors modulating this expression were also explored, using interventions designed to induce either increased intracellular Na\(^+\) or Ca\(^{2+}\) content or membrane depolarization. The first main finding was that three bouts of high-frequency electrical stimulation followed by 3 h of recovery induced subunit-specific Na\(^+\)-K\(^+\)-ATPase mRNA expression, with the mRNA expression of each of the catalytic \(\alpha_1\)-, \(\alpha_2\)-, and \(\alpha_3\)-isoforms increased with stimulation. The second main finding was that ouabain, veratridine, and monensin each markedly increased intracellular Na\(^+\) content but, surprisingly, did not increase the mRNA expression of any isoform. In fact, each of these interventions reduced the mRNA expression of the \(\beta_2\)-isoform and ouabain and veratridine also reduced the mRNA expression of the \(\alpha_2\)- and \(\alpha_2\)-isoforms, respectively. In contrast, 13 mM [K\(^+\)]\(_{\text{o}}\), which induces a 26-mV membrane depolarization (13), increased the mRNA expression of the \(\alpha_1\)-isoform, whereas A-23187, which elevates intracellular Ca\(^{2+}\) content (17) and Ca\(^{2+}\) influx (11), increased the mRNA expression of the \(\alpha_3\)-isoform but reduced the mRNA expression of the \(\beta_1\)-isoform. Thus the mRNA expression of the six Na\(^+\)-K\(^+\)-ATPase isoforms expressed in isolated rat EDL muscle appears to be regulated by different intracellular stimuli.

Three bouts of high-frequency electrical stimulation specifically increased the mRNA expression of the \(\alpha\)-isoforms. The effects of three bouts of high-frequency electrical stimulation on the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms clearly differs between the \(\alpha\)- and \(\beta\)-subunits. These findings are in contrast to the previously reported increase in the mRNA expression of the \(\beta_2\)-isoform but not of the \(\alpha_1\)-isoform in fast-twitch muscles after 1 h of treadmill running in rats (43). Furthermore, in human vastus lateralis muscle, –6 min of intense exercise elevated the mRNA expression of all six Na\(^+\)-K\(^+\)-ATPase isoforms (24) whereas ~55 min of submaximal exercise elevated the mRNA expression of the \(\alpha_1\)-, \(\alpha_3\)-, and \(\beta_2\)-isoforms (23). Thus the changes in the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms induced with whole body exercise are not necessarily matched with those induced with in vitro electrical stimulation in isolated rat skeletal muscle. This difference may suggest that the mRNA expression of the Na\(^+\)-K\(^+\)-ATPase isoforms in skeletal muscle may involve both systemic (e.g., hormonal) and local factors. However, a recent study in humans demonstrated that, despite the concentrations of epinephrine and norepinephrine being significantly higher after exercise involving both arms and legs compared with that involving only legs, there was no difference in the mRNA expression of any of the \(\alpha_1\)-, \(\alpha_2\)-, \(\beta_1\)-, \(\beta_2\)-, \(\beta_3\)-, and \(\alpha_3\)-subunits.
and \( \beta_3 \)-isoforms between the two exercise regimens (29). Thus it appears likely that local factors rather than systemic effects are involved in the mRNA expression of the \( \text{Na}^+ \cdot \text{K}^- \)-ATPase isoforms in skeletal muscle.

The upregulatory effect of electrical stimulation on the mRNA expression of the \( \alpha \)-isoforms was not evident until 3 h after stimulation. This is consistent with the higher mRNA expression of genes regulating energy metabolism in the 2- to 4-h period after exercise (34), indicating that the mechanisms involved in increasing mRNA expression (i.e., accelerated transcription, attenuated mRNA degradation, or a combination of both) require several hours to induce a detectable increase in mRNA expression of the \( \text{Na}^+ \cdot \text{K}^- \)-ATPase isoforms. Despite an apparent increase, the lack of significant change in the mRNA expression of any of the \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \)-isoforms immediately after the final stimulation bout may reflect a type II error (14); however, the statistical power values of 0.72, 0.67, and 0.75, respectively, suggest that this is unlikely. Importantly, we also found that the elevations in mRNA expression of the \( \alpha \)-isoforms with electrical stimulation were not due to an artifact of the experimental design, specifically due to the stretching of muscles to optimal force-generating length.

A significant increase in the mRNA expression of the \( \alpha \)-isoforms, but not of the \( \beta \)-isoforms, with three bouts of 10 s of electrical stimulation at 60 Hz was also observed in an experiment involving 90 s of 60-Hz electrical stimulation followed by 3 h of recovery in isolated rat EDL muscle (Murphy et al., unpublished observation). Thus \( \alpha \)-subunit-specific \( \text{Na}^+ \cdot \text{K}^- \)-ATPase mRNA expression may be an obligatory response to high-frequency electrical stimulation in isolated rat EDL muscle. This response may reflect the overabundance (5.5-fold for mRNA; 1.4- to 3.3-fold for protein) of the \( \beta \)-subunit in mammalian skeletal muscle (18, 29). Thus only an increased expression of the \( \alpha \)-subunit may be required for the formation of additional \( \alpha \beta \)-heterodimers. This response may also reflect the catalytic nature of the \( \alpha \)-isoforms, predisposing these isoforms to tight regulation imposed by changes associated with altered ion fluxes. As previously discussed, such changes were thought to include elevations in intracellular \( \text{Na}^+ \) content (36, 42) and intracellular \( \text{Ca}^{2+} \) concentration (37) and also membrane depolarization (3, 35). Indeed, as evidenced with the first and

![Fig. 7. Effects of 13 mM extracellular \( \text{K}^+ \) concentration ([\( \text{K}^+ \)]) on mRNA expression of the \( \text{Na}^+ \cdot \text{K}^- \)-ATPase \( \alpha_1 \)-to \( \alpha_3 \)- and \( \beta_1 \)- to \( \beta_3 \)-isoforms in rat EDL muscle. Isoform mRNA expression is expressed relative to control (1). Muscles were placed in polyethylene baskets, equilibrated for 30 min in standard KR, and then incubated for 60 min at 13 mM [\( \text{K}^+ \)]o, before being allowed to recover in normal KR (4 mM \( \text{K}^+ \)) for a further 3 h. Data are means and SD; \( n = 12 \). *\( P < 0.03 \), greater than Con; \( \# P < 0.06 \), greater than Con.](http://ajpregu.physiology.org/)

![Fig. 8. Effects of A-23187 on mRNA expression of the \( \text{Na}^+ \cdot \text{K}^- \)-ATPase \( \alpha_1 \)- to \( \alpha_3 \)- and \( \beta_1 \)- to \( \beta_3 \)-isoforms in rat EDL muscle. Isoform mRNA expression is expressed relative to control (1). Muscles were placed in polyethylene baskets, equilibrated for 30 min in standard KR, and then incubated with A-23187 (30 min, 0.02 mM), before being allowed to recover in normal KR for a further 3 h. Data are means and SD; \( n = 6 \). *\( P < 0.04 \) vs. Con.](http://ajpregu.physiology.org/)
third stimulation bouts, the electrical stimulation protocol used in the present study significantly elevated intracellular Na⁺ content and reduced intracellular K⁺ content, which would lead to membrane depolarization (21). The observed under-shoot in intracellular Na⁺ content in muscles exposed to two stimulation bouts followed by a 10-min recovery, compared with that in resting muscles, reflects excitation-induced activation of the Na⁺–K⁺-ATPase (26). This finding suggests that the first 10-s stimulation bout was sufficient to increase Na⁺–K⁺-ATPase activity for at least 20 min. If the excitation-induced increase in intracellular Na⁺ content is involved in triggering the increase in Na⁺–K⁺-ATPase mRNA expression, it is surprising that such a short-lasting event is a sufficient signal. Although not measured in the present study, it is well-documented that there is an elevation in baseline cytosolic Ca²⁺ concentration with repeated muscle contractions in isolated mammalian muscle (44).

Ouabain, veratridine, and monensin increased intracellular Na⁺ content but did not increase the mRNA expression of any of the Na⁺–K⁺-ATPase isoforms. Despite a clear increase in intracellular Na⁺ content with each of ouabain (120 min, 1.0 mM), veratridine (30 min, 0.1 mM), and monensin (30 min, 0.1 mM), there was no significant increase in the mRNA expression of any of the Na⁺–K⁺-ATPase isoforms. These findings are in contrast to those with cultured rat kidney cells, where in both cultured cells and isolated muscles.

13 mM [K⁺]o increased mRNA expression of the α₁-isoform. The elevation of [K⁺]o to levels mimicking those occurring in contracting muscle during exercise (41) induced an increase in the mRNA expression of the α₁-isoform and a tendency (P = 0.055) toward an increased mRNA expression of the β₂-isoform but had no significant effect on the mRNA expression of any of the α₂-, α₃-, β₁-, or β₂-isoforms. In rat liver cells, low [K⁺]o (0.65 mM) was shown to increase the mRNA expression of the α₂-subunit isoform by 250% (35), whereas in canine kidney cells, an even lower [K⁺]o (0.25 mM) induced a 200% increase in the mRNA expression of both the α₁- and β₂-subunit isoforms (3). Because such low [K⁺]o would have actually depolarized the muscle (10) and the 13 mM [K⁺]o used in the present study would have also depolarized the muscle (13), these results suggest that membrane depolarization may increase the mRNA expression of the Na⁺–K⁺-ATPase isoforms in both cultured cells and isolated muscles.

The effects of membrane depolarization on the mRNA expression of the Na⁺–K⁺-ATPase isoforms is unlikely to be due to any increase in intracellular Ca²⁺ content. In skinned fibers from rat EDL muscle, membrane depolarization induced via reduction of intracellular K⁺ concentration decreased sarcoplasmic reticulum Ca²⁺ release, as indicated by a reduction in twitch force (28), whereas in isolated rat EDL muscles, 30-min incubation in 20 mM [K⁺]o had no significant effect on [Ca²⁺]i (9). It therefore appears that intracellular Ca²⁺ content was more likely to have been reduced, rather than elevated, with the membrane depolarization induced here with 13 mM [K⁺]o.

A-23187 increased mRNA expression of the α₂-isoform but reduced mRNA expression of the β₁-isoform. The Ca²⁺ ionophore A-23187 (0.02 mM) was used to induce an elevation in intracellular Ca²⁺ content and/or Ca²⁺ influx. Indeed, in iso-
lated rat EDL muscle, only 15 min of incubation with 0.02 mM A-23187 significantly increased $^{45}$Ca uptake by 323% (11). Additionally, in cultured rabbit myocytes, only 10 min of incubation with 0.4 μM A-23187 was sufficient to increase intracellular free Ca$^{2+}$ content by 200–900% (16). In the present study, A-23187 induced complex changes in the mRNA expression of the Na$^+$-K$^+$-ATPase isoforms, by increasing the mRNA expression of the α$_1$-isoform, reducing the mRNA expression of the β$_1$-isoform, and having no significant effect on the mRNA expression of any of α$_2$-, α$_3$-, β$_2$-, or β$_3$-isoforms. These findings are in contrast to the 300% elevations in the mRNA expression of both the α$_1$- and β$_1$-isoforms found in rat kidney cells after 1-h incubation in solution containing 1.0 μM Ca$^{2+}$ compared with that containing 0.1 μM Ca$^{2+}$ (37). In that study, the author confirmed that the increase in extracellular Ca$^{2+}$ (0.1–1.0 μM) also induced an increase in intracellular Ca$^{2+}$ (0.1–4.0 μM). However, the validity of those results is uncertain because the extracellular Ca$^{2+}$ concentrations used in that study are nonphysiologically low, with the normal resting extracellular Ca$^{2+}$ concentration being 1.0–1.5 mM, as utilized here.

The physiological significance of an elevation in the mRNA expression of the α$_3$-isoform and a reduction in the mRNA expression of the β$_1$-isoform in response to increased intracellular Ca$^{2+}$ content and/or Ca$^{2+}$ influx is unclear. Nonetheless, because the relative mRNA expression of the α$_3$-isoform in skeletal muscle is likely to be low (29), the quantitative importance of an increase in the mRNA expression of the α$_3$-isoform is uncertain.

Importantly, the effects of A-23187 on the mRNA expression of the Na$^+$-K$^+$-ATPase isoforms were unlikely to be due to any A-23187-induced contracture because no increase in baseline tension was previously found with application of A-23187 (11). Although the mechanisms responsible for the elevation in mRNA expression of the α$_3$-isoform and the reduction in mRNA expression of the β$_1$-isoform with A-23187 are unknown, they may involve altered rates of transcription and degradation, respectively. In rat kidney cells, the elevation in mRNA expression of the α$_1$-isoform with an increase in extracellular Ca$^{2+}$ concentration could almost completely be accounted for by an acceleration in the transcription rate of the α$_1$-isoform (37). On the other hand, the effect of an increase in extracellular Ca$^{2+}$ concentration in the mRNA expression of the β$_1$-isoform was thought to be mediated by an altered degradation rate of the β$_1$-isoform. However, in that study, an increase in extracellular Ca$^{2+}$ concentration was thought to attenuate the degradation rate of the β$_1$-isoform.

**Perspectives**

The functional significance of an increase in mRNA expression of the Na$^+$-K$^+$-ATPase isoforms with three bouts of 10-s electrical stimulation is uncertain. In isolated rat soleus and EDL muscles, protein expression of the Na$^+$-K$^+$-ATPase α$_2$-isoform was unchanged with up to 240 min of electrical stimulation (22). In human vastus lateralis muscle, $[^3]$Houabain binding was not changed after 72 min of exercise (19) but was increased by 13% with ~10 h of running (32). It therefore appears that a time course discrepancy exists between the mRNA and protein expression of the Na$^+$-K$^+$-ATPase. Thus mRNA expression of the Na$^+$-K$^+$-ATPase may be elevated with only 30 s of repeated muscle contractions, whereas the protein expression of the functional Na$^+$-K$^+$-ATPase may only be elevated after several hours of repeated muscle contractions.

The mRNA expression of the Na$^+$-K$^+$-ATPase isoforms appears to be regulated by different stimuli in rat skeletal muscle. In the EDL, a muscle comprising predominantly fast-twitch fibers, the present study suggests that membrane depolarization and elevated intracellular Ca$^{2+}$ content and/or Ca$^{2+}$ influx may induce increased mRNA expression of the α$_1$- and α$_3$-isoforms, respectively. Thus other factors not explored in this study may be responsible for inducing the increased mRNA expression of the other Na$^+$-K$^+$-ATPase isoforms observed after electrical stimulation and exercise. This may include increased reactive oxygen species (ROS) since repeated muscle contractions increase ROS in both rat and human muscle (15, 39). Furthermore, there is accumulating evidence that increased ROS may act as a second messenger in signaling pathways involved in the mRNA expression of the cardiac Na$^+$-K$^+$-ATPase (45). Further work is required to investigate the role of increased ROS in the mRNA expression of the Na$^+$-K$^+$-ATPase isoforms in skeletal muscle.

In conclusion, the effects of three bouts of high-frequency electrical stimulation on the mRNA expression of the Na$^+$-K$^+$-ATPase isoforms in rat EDL muscle were subunit specific, with increases in mRNA expression of the α$_1$-isoforms but not of the β$_1$-isoforms. Furthermore, mRNA expression of the Na$^+$-K$^+$-ATPase isoforms appears to be regulated by different stimuli, including the cellular changes associated with high [K$^+$], such as membrane depolarization, as well as with A-23187 such as elevated intracellular Ca$^{2+}$ content and/or Ca$^{2+}$ influx. Surprisingly, there was no increase in the mRNA expression of any of the Na$^+$-K$^+$-ATPase isoforms with interventions used to elevate intracellular Na$^+$ content but rather a decreased mRNA expression of several isoforms. Thus a surprising diversity of signals appears to be involved in upregulating the mRNA expression of the different Na$^+$-K$^+$-ATPase isoforms. However, this is consistent with the expression of multiple isoforms, with presumably a corresponding diversity in function.

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