Mechanisms underlying increases in rat soleus Na\(^+\)-K\(^+\)-ATPase activity by induced contractions

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Sandiford, S. D. E., H. J. Green, and J. Ouyang. Mechanisms underlying increases in rat soleus Na\(^+\)-K\(^+\)-ATPase activity by induced contractions. J Appl Physiol 99: 2222–2232, 2005. First published August 18, 2005; doi:10.1152/japplphysiol.00577.2005.—Acute regulation of the Na\(^+\)-K\(^+\)-ATPase activity in rat soleus muscle was investigated in response to 15 and 90 min of electrically induced contractile activity (500-ms trains at 30 Hz every 1.5 s). Kinetic measurements of Na\(^+\)-K\(^+\)-ATPase activity, assessed by the 3-O-methylfluorescein K\(^+\)-stimulated phosphatase assay (3-O-MFP), were performed on crude homogenates (Hom) and on tissue separated into two membrane fractions, the sarcolemmal/particulate (SLP) and endosomal (En), in both stimulated (Stim) and contralateral control (Con) muscles. Maximal 3-O-MFP activity (V\(_{\text{max}}\), nmol·mg protein\(^{-1}\)·h\(^{-1}\)) was elevated (P < 0.05) in Stim by 40% and by 53% in Hom and by 37 and 40% in SLP at 15 and 90 min, respectively. The 38% increase (P < 0.05) in the α\(_2\)-isoform subunit distribution in SLP at 15 min, as assessed by quantitative immunoblotting, persisted at 90 min, whereas for En a 42% decrease (P < 0.05) was observed only at 15 min. For the α\(_1\)-subunit at 15 min, a 27% decrease (P < 0.05) was observed in En, whereas the 13% increase observed in SLP was not significant (P = 0.08). At 90 min, α\(_1\) was increased (P < 0.05) by 14% in SLP and by 29% in En. No changes were observed in β\(_2\)-subunit distribution in En and SLP regardless of time of stimulation. Immunoprecipitation with antiphasagensine antibody and quantitative immunoblotting with α\(_1\)- and α\(_2\)-antibodies indicated changes in tyrosine phosphorylation of 51% in α\(_2\) at 15 min only. These results suggest that the increases in V\(_{\text{max}}\) during contractile activity are mediated both by increased phosphorylation and by translocation of the enzyme to the plasma membrane.

Skeletal muscle; Na\(^+\)-K\(^+\) pump; exercise; translocation; intrinsic activity

To sustain membrane excitability, it is necessary to translate increased neural motor command signals into repetitive action potentials, and ultimately mechanical behavior, active transport of Na\(^+\) and K\(^+\) across the plasmalemma must increase dramatically (7). Moreover, for the mechanical behavior to remain protected during prolonged stimulation, active transport of these cations must not be significantly compromised (7). The active transport of Na\(^+\) and K\(^+\) across the plasmalemma is regulated by the Na\(^+\)-K\(^+\)-ATPase, a cation pump (Na\(^+\)-K\(^+\) pump) that translocates 3 Na\(^+\) out of and 2 K\(^+\) into the cell at the expense of the energy provided by 1 ATP (33).

The Na\(^+\)-K\(^+\)-ATPase is a heterodimer consisting of α- and β-subunits. The α is the catalytic subunit containing the binding sites for the substrates of the enzyme (Na\(^+\), K\(^+\), Mg\(^2+\), ATP), whereas the β-subunit appears to regulate the assembly and expression of the Na\(^+\)-K\(^+\) pump (3). The β-subunit is essential for the catalytic activity of the enzyme (33). Isoforms of the α- and β-subunits also exist. In skeletal muscle, two isoforms of α (α\(_1\) and α\(_2\)) and two isoforms of β (β\(_1\) and β\(_2\)) predominate (3). There is also evidence suggesting the existence of α\(_3\) and β\(_3\) in skeletal muscle depending on the species (3). The isoforms also appear to be distributed in a fiberspecific manner. In rodent soleus (Sol) muscle, which contains an abundance of slow-twitch (type I) fibers of high oxidative potential, isoform distribution appears mainly restricted to α\(_1\), α\(_2\), and β\(_1\) (12). In rodent white gastrocnemius, which is high in oxidative potential, contains all four isoforms, namely α\(_1\) and α\(_2\) and β\(_1\) and β\(_2\) (12).

At present it is unclear how the isoform combination of the heterodimer regulates the kinetic activity of the enzyme. This issue has been examined by Crambert et al. (9), who expressed nine different human Na\(^+\)-K\(^+\)-ATPase isoforms in Xenopus oocytes and then studied the functional behavior of the various α- and β-isofoms combinations. These investigators concluded that isoforms formed with α\(_1\)-isoforms play a housekeeping role and work at optimum rates under physiological conditions. It was suggested that, at least in vivo, isozymes with the α\(_2\)-subunit can vary the catalytic activity over a wide range to maintain membrane excitability in the face of increased demands, as during exercise (9). The importance of the α- and β-isoform combinations is unclear (3). Regulation of the acute increases in Na\(^+\)-K\(^+\)-ATPase activity during muscle stimulation has been attributed both to changes in intrinsic activity and to changes in the subcellular distribution of the enzyme. Increases in intrinsic activity are believed to occur via phosphorylation of the α-subunits secondary to protein kinase C (PKC) or protein kinase A (PKA) (33). Increases in α-subunit phosphorylation leading to increases in Na\(^+\)-K\(^+\)-ATPase activity have also been observed to occur with tyrosine kinase-dependent mechanisms (5). In skeletal muscle, increased Na\(^+\)-K\(^+\)-ATPase activity has been associated with PKA-mediated phosphorylation (7). Alternatively, translocation of the subunits to the plasmalemma from different intracellular sites may increase the number of functionally active heterodimers contributing to the increase in catalytic activity of the enzyme (23, 24, 36). However, the significance of translocation, at least during contractile activity, has recently been disputed (28). A major limitation in the studies used to investigate the translocation-mediated hypothesis is the possibility of a selective yield of the Na\(^+\)-K\(^+\)-
ATPase given the notoriously low recovery of the enzyme in the sarcolemma-enriched fractions (7). The relatively recent publication of a subcellular fractionation protocol for separation of tissue samples into sarcolemmal and endosomal components (16) has provided an opportunity to reexamine this issue.

The purpose of this study was to characterize the acute regulation of Na\(^{+}\)-K\(^{+}\)-ATPase activity in repetitively stimulated rat soleus muscle and to investigate the underlying mechanisms that are involved. We have hypothesized that repetitive activity would result in an increase in the maximal activity of the enzyme in the absence of changes in the substrate binding affinity for K\(^{+}\). Moreover, we have hypothesized that the increase in maximal activity would be mediated both by increased intrinsic activity and by translocation of functional heterodimers to the sarcolemma. The increases in intrinsic activity are postulated to occur by regulatory site phosphorylation, whereas increases in functional heterodimers are postulated to occur via translocation of both \(\alpha_1\beta_1\) and \(\alpha_2\beta_1\) heterodimers.

**METHODS**

*Animals.* Twelve-week-old, adult male Sprague-Dawley rats (total \(n = 55\)) weighing 368.0 ± 2.3 g (mean ± SE) were provided with food and water ad libitum and maintained on reverse light-dark cycles. In general, the animals were randomized into two groups. In one group, the plantar flexor muscles of one side were stimulated for 15 min (Stim-15) whereas in the other group the muscles were stimulated for 90 min (Stim-90). The different periods of stimulation were designed to provide insight into the time-dependent changes in the factors regulating the Na\(^{+}\)-K\(^{+}\)-ATPase behavior. Previous research has shown that translocation of the Na\(^{+}\)-K\(^{+}\)-ATPase to the plasma membrane occurs early in repetitive activity (23). The contralateral side served as the control (Con). Owing to tissue restrictions, separate subgroups of 15- and 90-min stimulated animals were used to determine the effects of electrical stimulation on K\(^{+}\)-stimulated activity and Western blotting on whole homogenates (Hom), K\(^{+}\)-stimulated activity and Western blotting on isolated sarcolemmal/particulate (SLP) and (En) endosomal fractions, tyrosine phosphorylation of \(\alpha\)-subunits, and \([\text{H]}\)ouabain binding. Animal care was in accordance with the guidelines of the Canadian Council on Animal Care and was approved by the Animal Care Committee of the University of Waterloo.

*Experimental procedure.* Animals were anesthetized with a dose of pentobarbital sodium (−0.1 ml/100 g body wt) (Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). Animals stimulated for 15 min received only one dose. Ninety-minute animals were given an additional dose of 0.1 ml Somnotol at 60 min of stimulation to ensure adequate anesthesia for the duration of the protocol. The hindlimbs were prepared as previously described (6). Briefly, both limbs were prepared for stimulation, with Con or Stim limbs randomly assigned. Preparation of electrical stimulation involved removal of external skin and fascia followed by separation of the gastrocnemius-plantaris-soleus muscle group from the fibula. The tendon of this muscle complex was attached to a force transducer by a stainless steel S-hook that was sutured in place. Stainless-steel 18-gauge needles were guided through the joint capsule of the knee to secure the knee joint to the stimulation apparatus. A small incision was made along the gluteal muscle to expose the sciatic nerve of the Stim limb and a small hook electrode guided around the nerve. A similar incision was made on the Con limb. The nerve was located and exposed on this limb, but no electrode was applied. During surgical preparation, muscle and nerve tissues were kept moist with saline warmed to 37°C. The total time for surgical preparation did not exceed 40 min. Animals were kept warm throughout the procedure by placing them prone on a covered heating pad. Skinned muscles were periodically heated by gauze, soaked in saline (pH 7.4), and warmed to 37°C. The supramaximal stimulation protocol involved intermittent 30-Hz, square-wave pulses using a Grass S48 stimulator (Grass Medical Instruments, Quincy, MA). The stimulation protocol was modified from Chin et al. (6) and Taketa and Ikata (32) and involved 500-ms trains delivered every 1.5 s. After stimulation, Sol muscles both Con and Stim were quickly extracted and frozen in liquid nitrogen. Samples were stored at −80°C until further analysis.

Force measurements were recorded from the Con and Stim gastrocnemius-plantaris-soleus muscle groups to confirm the stability of our preparation and to assess the collective effects of stimulation. For the Stim muscles, force declined to 28 ± 4% at 90 min. In Con muscles, force was not altered over the period of stimulation.

*Whole homogenate preparation.* Whole Hom were prepared by homogenizing small portions of previously frozen Sol (−30 mg) in 20 vol of ice-cold buffer containing (in mM) 10 trometamol, 2-amino-2-(hydroxymethyl)-1-propanol (Tris base), 2 EDTA, and 250 sucrose (pH 7.4), and a commercially prepared combination of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Samples were homogenized for 2 × 20 s at 25,000 rpm with a PT 3100 Polytron homogenizer (Kinematica, Littau, Switzerland), aliquoted, and quickly frozen in liquid nitrogen until further analysis. These samples were used to assess K\(^{+}\)-stimulated kinetic behavior. The kinetic properties assessed included the maximal activity (V\(_{\text{max}}\)), the free K\(^{+}\) required for half-maximal activity (K\(_{\text{m}}\)), and the Hill coefficient (m\(_{\text{H}}\)), which is a measure of the cooperative K\(^{+}\)-binding of the Na\(^{+}\)-K\(^{+}\)-ATPase enzyme. As well, Western blotting analysis was employed to assess whether electrical stimulation over 15 or 90 min resulted in alterations in the protein expression of the enzyme subunit isoforms.

*Enriched sarcolemmal fraction preparation.* Soleus muscle samples were separated into two membrane fractions, namely SLP and En, as described by Fuller et al. (16). All steps were carried out at 4°C. Briefly, Sol muscles (−140 mg) were minced and incubated for 30 min at 4°C in a salt solution containing 0–5 M NaCl and 20 mM HEPES, pH 7.4. The incubated tissue was then homogenized in 10 times the volume of tissue buffer containing (in mM) 250 sucrose, 1 Tris (pH 7.4), and a commercially prepared combination of protease inhibitors (Roche Diagnostics). Homogenates were centrifuged at 1,000 rpm for 5 min. The pellets were resuspended and centrifuged at 1,000 g for another 5 min. The combined supernatants were centrifuged at 100 g for 10 min. The supernatant from this centrifugation was then pelleted at 5,000 g for 10 min. The pellets from this spin were stored on ice, and the resulting supernatant was centrifuged at 20,000 g for 30 min. Pellets from the 5,000 g and 20,000 g spins were combined, resuspended in ~400-µl buffer, frozen in liquid nitrogen, and stored at −80°C until use. This represents the SLP fraction. The supernatant from the 20,000 g spin represented the En fraction. The En fraction (500–600 µl) was suspended in the same buffer as described for SLP, frozen, and stored under similar conditions. The two resulting fractions (SLP and En) were assayed to determine the K\(^{+}\)-stimulated kinetic behavior of the Na\(^{+}\)-K\(^{+}\)-ATPase (V\(_{\text{max}}\), K\(_{\text{m}}\), and m\(_{\text{H}}\)) as well as to perform Western blotting using antibodies against α (α\(_1\) and α\(_2\)) and β (β\(_1\)) Na\(^{+}\)-K\(^{+}\)-ATPase subunits. These measurements enabled the assessment of the fractions for the possibility of subunit redistribution with electrical stimulation.

This isolation protocol was favored over other methods because only two fractions are generated from the tissue samples. Only a small amount of the membranes are discarded. The SLP fraction, at least in cardiac tissue, contains in excess of 85% of the ouabain-sensitive ATPase activity (16). Fuller et al. have demonstrated that the high-salt solution detaches the membranes from the myofilaments. The myofilaments are then pelleted at low speed and discarded.

3-O-methylfluorescein phosphatase activity. The K\(^{+}\)-stimulated 3-O-methylfluorescein phosphatase activity (3-O-MFase) was assessed fluorometrically by using a modified procedure of Huang and
Askari (21) and Horgan and Kuyper’s (20), by using a higher substrate concentration (15). The use of 1.25 mM EGTA was confirmed in a separate set of experiments to decrease nonspecific activity in the assay and optimize assessment of K⁺-stimulated activity in rat muscle (1). Samples (~6 μg protein for Hom and 2 μg for SLP and En) were diluted 5× in cold homogenate buffer containing 0.1% deoxycholate before being incubated for 4 min in medium containing (in mM) 5 MgCl₂, 1.25 EDTA, 1.25 EGTA, 100 Tris base, and varying concentrations of KCl (pH 7.40) at 37°C. A SpectraMax GeminiXS microplate fluorometer (Molecular Devices, Sunnyvale, CA) was used to analyze the samples. The 96-well fluorescence plate allowed the successive assay of samples incubated in concentrations of KCl (in mM): 0, 0.2, 0.5, 0.75, 1, 1.5, 3, and 5. The reaction was started with the addition of 160 μM 3-O-methylfluorescein phosphate (3-O-MFP). The metabolism of this substrate, and subsequent appearance of the fluorescing compound 3-O-MFP, was measured over 3 min. The resulting slope was corrected against a known standard of 3-O-MFP (a standard curve was performed each day), and the K⁺-stimulated 3-O-MFPase activity was determined as the difference between slopes generated from samples incubated in medium containing the different concentrations of KCl (see above) and “blank” samples incubated in medium without KCl. The specificity of the assay was confirmed by the use of ouabain to inhibit K⁺-stimulated activity in assayed samples. Incubation with 2–5 mM ouabain inhibited >90% of the K⁺-stimulated 3-O-MFPase activity and confirmed the specificity of the assay.

Activity was based on the average of two separate trials. For each animal, samples of Con and Stim limbs were analyzed on the same day. The order of assay between Con and Stim limbs was randomized. Measurements on two to four animals were generally completed in one analytical session. The intra- and interday coefficients of variation were 5 and 12%, respectively, for Hom samples. Respective values of 5 and 22% were obtained for SLP. Activities achieved by using the K⁺ concentrations listed above (0 to 5 mM) were entered into a GraphPad program where data were fit to a modified hyperbolic (Michaelis-Menten) model. Kinetic parameters (Vmax, Km, and nH) were determined on the basis of the relationship between free K⁺ concentration and Na⁺–K⁺-ATPase activity by using the following equation:

\[ Y = \frac{V_{max}}{X} / \left[ \frac{K_m + X}{H} \right] \]

where \( Y \) is the activity of the sample at a specific substrate concentration (X), \( V_{max} = V_{max} \cdot H \) is the Hill slope, and \( K_m \) is Michaelis-Menten constant.

The nonspecific enzyme activity was not different between the Con and Stim muscles at either 15 or 90 min for all of the preparations examined, namely Hom, SLP, and En (data not reported).

**Western blotting.** Soleus samples containing either 20 μg (SLP) or 30 μg (Hom, En) of protein were electrophoresed on 7.5% sodium dodecyl polyacrylamide gels (Bio-Rad Mini-PROTEAN II) according to the general procedures previously published from our laboratory (12). A biotinylated ladder was used as a molecular weight standard (Cell Signalling Technology, Beverly, MA). Proteins were transferred to polyvinylidene difluoride membranes (PVDF membrane, Bio-Rad) and blocked for 1 h or overnight in 5% nonfat milk or 7.5% BSA in Tris-buffered saline (TBS, pH 7.5) for α₁ monoclonal and α₂ and β₁ polyclonal antibodies, respectively (Upstate Biotechnology, Lake Placid, NY). Membranes were incubated with primary monoclonal antibodies against α₁ (1:5,000) overnight and polyclonal α₂ (1:500) and β₁ (1:1,000) for 1 h. After being washed (1 × 15 min followed by 2 × 5 min) in 0.1% TBS Tween-20 (TBS-T), membranes were incubated for 60 min in secondary antibodies for α₁ (goat anti-mouse) and α₂ and β₁ (goat anti-rabbit) (Chemicon International, Temecula, CA) diluted to 1:2,000, 1:3,000, and 1:3,000 in TBS-T, respectively. An enhanced chemiluminescence procedure was used to identify antibody content (Amersham, Buckinghamshire, UK). Blots were analyzed by use of a Chemi Genius² model bio imaging system (SynGene, Frederick, MD) with SynGene software version 1.0. In PM and En fractions, protein expression of all isoforms in the Stim limbs was expressed relative to a brain standard and then to their corresponding Con values. Alternatively, instead of correcting all Hom samples to a brain standard, samples analyzed for the presence of α₁ and α₂ were first corrected by using an antibody against actinin (5CS) (Sigma Chemical, St. Louis, MO), and then Stim samples were expressed relative to their respective Con values. Actinin is abundant in skeletal muscle, and its levels are not believed to be altered by acute repetitive contraction (37). In the case of the β₁-isoforms, samples were deglycosylated before Western blot analysis with N-glycosan (Roche Diagnostics) following the manufacturer’s instructions. The linearity between blot signal and the amount of protein applied to the gel was determined in pilot work.

In the event of subunit translocation between fractions, controls are required to confirm the suggestion of redistribution. To provide support for the isolation procedure, polyclonal antibodies against the glucose transporter (GLUT-4, 1:1,000) (Chemicon International, Temecula, CA) and monocarboxylate transporter (MCT1), as well as a monoclonal antibody against the dihydropyrodine receptor (Affinity Bioreagents and Golden, CO) (DHPR, 1:500) were used in addition to those listed above. The premise behind the choice of these particular antibodies is that GLUT-4 will undergo redistribution upon electrical stimulation whereas MCT1 (35) and DHPR (4) will not.

**Immoprecipitation of tyrosine phosphorylated Na⁺–K⁺-ATPase.** The primary isoform in rat skeletal muscle is α₁ (17). Owing to the inability to find a commercially available α₂ antibody suitable for immunoprecipitation, we decided to immunoprecipitate all tyrosine-phosphorylated proteins and probe for the presence of α₁ and α₂ as previously described (5, 34). Preliminary trials were run to establish that enough antibody and agarose were added to immunoprecipitate all tyrosine-phosphorylated residues. For these initial trials, samples were prepared as described below, and immunoblot analysis was performed using phospho-tyrosine antibody PY20 (BD Biosciences Canada, Mississauga, ON). For experimental samples, whole Sol homogenates (750 μg of protein at ~8 μg/μl) were prepared as detailed above, resuspended, and incubated for 1 h at 4°C in ice-cold lysis buffer, at an approximate 1:3 (vol/vol) ratio, containing (in mM) 20 Tris (pH 8.0), 135 NaCl, 10 Na₂PO₄, 10 NaF, 1 NaVO₃, 10% glycerol, 0.1% Tween-20, 1% Triton-X. The samples were centrifuged at 300 g for 10 min, and the supernatant was pretreated with 60 μl of a 50% protein A-agarose bead slurry (KPL, Gaithersburg, MD) for 1 h at 4°C. Samples were centrifuged at 14,000 g for 20 s, and the supernatant was incubated with 50 μl of anti-phosphotyrosine (PY69) antibody (BD Biosciences Canada) along with 100 μl of the protein A-agarose slurry and incubated for 4 h at 4°C. Samples were centrifuged at 14,000 g for 20 s, and the supernatant was saved for contamination profiling and later analysis. The agarose was washed four times with lysis buffer, followed by one wash in 0.1 M Tris (pH 8.0) and 0.5 M LiCl; one wash in 10 mM Tris (pH 7.6) 150 mM NaCl, and 1 mM EDTA; and one final wash in 20 mM HEPES, 5 mM MgCl₂, and 1 mM dithiothreitol as performed by Chibalin and coworkers (5). Samples were suspended in 20 μl each of homogenization medium and 2× SDS-PAGE sample buffer and heated at 95°C to elute the antigen from the antibody-agarose complex. Tyrosine-phosphorylated samples were then probed for the presence of α₁ and α₂ by using Western blotting methods described above.

For all assays, protein content was determined in duplicate according to Lowry as modified by Schacterle and Pollack (31) using bovine serum albumin as the standard.

**Maximal [³H]ouabain binding.** [³H]Ouabain binding was used to determine maximal binding (Bmax) as described previously (30) and as employed in our laboratory (12). Two muscle samples weighing between 2 and 8 mg were prewashed twice for 10-min periods in a Tris-sucrose buffer containing (in mM) 10 Tris·HCl, 3 MgSO₄, 1 Tris-vanadate, 250 sucrose, and NaVO₃ at 0°C. Samples were incubated in the Tris-sucrose buffer with a saturating concentration of [³H]Ouabain (1 μM, at a specific activity of 0.9 Ci/ml) for 180 min at
37°C. After the unbound ouabain was removed by washing three times for 15 min in ice-cold buffer, the samples were blotted, weighed, placed in 1.5-ml Eppendorf tubes, and soaked in 1 ml 5% TCA for 16 h at room temperature. A 0.5-ml of the above sample was then counted for [3H]ouabain binding capacity, corrected for the loss of specifically bound [3H]ouabain binding sites, and expressed as picomoles per gram wet weight as described previously (30). The [3H]ouabain binding protocol has been shown to detect the majority of functional Na⁺-K⁺ pump in skeletal muscle (7).

Data analysis. Statistical analysis was performed using Statistica for Windows R.4.5 software (Statsoft, Tulsa, OK, 1993) with significance set at \( P < 0.05 \). For all comparisons, one-way ANOVA with repeated measures was used to analyze Western blotting data (including those results used to determined whether there was evidence of redistribution and the determination of tyrosine phosphorylation). [3H]ouabain binding was also analyzed by a one-way ANOVA. Two-way ANOVA with repeated measures were used to identify differences between K⁺-stimulated activity dependencies between control and stimulated limbs. Post hoc analysis of mean values was performed using a Newman-Keuls test.

RESULTS

K⁺-stimulated 3-O-MFPase activity. The kinetic properties of the enzyme for Con and Stim limbs are summarized in Table 1. For Hom, stimulation resulted in an ~40 and 53% increase in \( V_{\text{max}} \) at 15 and 90 min of stimulation, respectively (Fig. 1). The increase in \( V_{\text{max}} \) was unaffected by the duration of stimulation. For the SLP, the increases in \( V_{\text{max}} \) with stimulation showed the same approximate percentage increase, namely 37 and 40% at 15 and 90 min, respectively (Fig. 2). In contrast, no differences were observed in \( V_{\text{max}} \) between Con and Stim muscles in the En fraction, regardless of the duration of contractile activity (Table 1). The other kinetic properties examined, namely the \( K_n \) and \( n_H \), were not affected by the stimulation protocol, regardless of the preparation.

Na⁺-K⁺-ATPase isoform distribution. Western blotting for detection of the \( \alpha_1 \), \( \alpha_2 \), and \( \beta_1 \)-isoforms was performed on the Hom, SLP, and En fractions for both Con and Stim muscles. For the Hom, the \( \alpha_1 \)-isofrom was observed to increase (15%) but only at 90 min of stimulation (Fig. 3). However, only the increase in content at 90 min was significant \( P < 0.05 \). For \( \alpha_2 \), the content in the SLP fraction was between 38 and 40% higher \( P < 0.05 \) compared with Con at both 15 and 90 min of stimulation.

### Table 1. Effects of repetitive stimulation on kinetic properties of the Na⁺-K⁺-ATPase in soleus muscle

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<th>15 min</th>
<th>90 min</th>
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<tr>
<td></td>
<td>Con</td>
<td>Stim</td>
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<tr>
<td>Hom</td>
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<tr>
<td>( V_{\text{max}} )</td>
<td>277±26</td>
<td>387±21*</td>
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<tr>
<td>( K_m )</td>
<td>0.70±0.17</td>
<td>0.81±0.10</td>
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<tr>
<td>( n_H )</td>
<td>1.10±0.30</td>
<td>1.17±0.15</td>
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<tr>
<td>SLP</td>
<td></td>
<td></td>
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<tr>
<td>( V_{\text{max}} )</td>
<td>2,224±211</td>
<td>3,040±271*</td>
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<tr>
<td>( K_m )</td>
<td>1.32±0.13</td>
<td>1.12±0.18</td>
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<tr>
<td>( n_H )</td>
<td>1.19±0.20</td>
<td>1.12±0.10</td>
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<tr>
<td>En</td>
<td></td>
<td></td>
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<tr>
<td>( V_{\text{max}} )</td>
<td>213±27</td>
<td>271±33</td>
</tr>
<tr>
<td>( K_m )</td>
<td>0.13±0.10</td>
<td>0.21±0.12</td>
</tr>
<tr>
<td>( n_H )</td>
<td>1.2±0.13</td>
<td>1.1±0.12</td>
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Values are means ± SE. For whole muscle homogenates (Hom), \( n = 8 \) and 10 at 15 and 90 min, respectively. For the sarcolemmal/particulate (SLP) and endosomal (En), fractions \( n = 7 \) at 15 and 90 min. Kinetic properties were determined from plots between 3-O-methylfluorescein phosphatase (3-O-MFPase) activity and \( K_m \), \( V_{\text{max}} \), maximal activity (mmol-mg protein⁻¹-h⁻¹). \( K_m \), \( V_{\text{max}} \), Hill slope. Time (min), duration of stimulation. Con, control; Stim, stimulated. *Significantly different from Con \( P < 0.05 \).
contractile activity (Fig. 4). The changes in the α-subunit composition in the En fraction depended on both the isoform type and the time of stimulation (Figs. 3 and 4). In this fraction, the α₂ content declined by 42% at 15 min but was unchanged at 90 min of stimulation relative to the Con. Decreases in α₁ content of 27% at 15 min were also observed in this fraction in Stim compared with Con, whereas at 90 min α₁ content was ~29% higher in Stim compared with Con. The content of the β₁-isofom was not affected by stimulation, regardless of duration, in any of the preparations examined (Fig. 5).

To determine whether electrical stimulation of the Sol muscle also induces translocation of other proteins between the SLP and En fractions, we have measured the content of GLUT-4, MCT1 and DHPR (Fig. 6). For GLUT-4, 15 min of stimulation resulted in a decrease in content of 16% in the En fraction in Stim relative to Con. The 11% increase in GLUT4 observed in the SLP fraction was not significant (P = 0.07). MCT1, used as a sarcolemmal marker, remained unchanged in the SLP fraction at both stimulation durations. The DHPR content in SLP was also unaltered in Stim compared with Con, at least during the initial 15 min of stimulation. Neither MCT1 nor DHPR was detectable in the En fraction.

Tyrosine phosphorylation state of the Na⁺-K⁺-ATPase. No changes in the tyrosine phosphorylation state were observed in the α₁ in Hom at either 15 or 90 min of contractile activity (Fig. 7). In contrast, α₂ was increased by 51% but only at 15 min of stimulation. By 90 min of stimulation, the tyrosine phosphorylation was unchanged from control.

[^H]ouabain binding. B_max in Hom at was found to increase by 21 and 16% in the Stim compared with Con limbs at 15 and 90 min of stimulation, respectively (Fig. 8). No difference was observed in B_max between 15 and 90 min of stimulation.

DISCUSSION

In this study, we have gained several new insights governing the regulation of soleus muscle Na⁺-K⁺-ATPase activity during repetitive contractile activity. As hypothesized, we have found that contractile activity resulted in a sustained increase in V_max in whole muscle homogenates at both 15 and 90 min of stimulation. The increase in V_max was not accompanied by any changes in the K⁺ affinity of the enzyme, as measured by the K_m and n_H. As might be expected, we have also observed that the increase in V_max was restricted specifically to the SLP fraction, because no changes were observed in the other fraction examined, namely the En, with repetitive contraction. The increase in V_max in the SLP, particularly at 15 min, appears to involve both translocation and increases in the intrinsic activity of the α-isofom, and in particular, the α₂-isofom. The α₂-isofom displayed both an increased content (SLP) and an increased tyrosine phosphorylation (Hom). Moreover, at 15 min of stimulation, α₂ in the En fraction was reduced.

It is well known that increases in the catalytic activity of the Na⁺-K⁺-ATPase occurs with the onset of contractile activity to accommodate the need for active transport of Na⁺ out of the cell and K⁺ into the cell (7). What is particularly unique about our study is that we were able to detect the increase in the activity of the enzyme in vitro in soleus muscles that had been repetitively stimulated. The increase in V_max in the SLP, particularly at 15 min, appears to involve both translocation and increases in the intrinsic activity of the α-isofom, and in particular, the α₂-isofom. The apparent contradiction can be explained by the balance of regulatory factors affecting V_max behavior of the enzyme. It is known that a complex of factors serve to increase catalytic activity during repeated contractions (7), whereas factors also exist that can inhibit activity (25). Accordingly, the changes in V_max may be intimately associated with the specifics of the exercise protocol. In addition, the fiber-type composition of the muscle may be important. In this study, we report the increase in V_max in the soleus with repetitive activity.
white gastrocnemius, no changes in $V_{\text{max}}$ were observed during the 90-min stimulation protocol (unpublished observations).

In this study, we have used 3-O-MFPase activity as a measure of the overall catalytic activity of the enzyme (20). However, 3-O-MFPase, which uses the artificial substrate 3-O-MFP, is a measure of the phosphatase activity of the enzyme (29). The 3-O-MFP substrate substitutes for the aspartylphosphate intermediate of the ATPase to represent the terminal step in ATP hydrolysis (20). The possibility exists that the 3-O-MFPase may not represent the catalytic capacity of the enzyme as measured by direct measurements of ATP hydrolysis (12).

We have also used the vanadate-facilitated $[^3\text{H}]\text{ouabain}$-binding technique to assess the active state of the enzyme (30). Although this measure is typically used to assess $\text{Na}^+\text{-K}^+$-ATPase activity and $\text{Na}^+\text{-K}^+$-ATPase, $\alpha_1$-isoform with stimulation ($B$) in Hom, SLP, and endosomal (En) fractions. Values are means ± SE. For $\alpha_1$, $n = 8$ for Hom, SLP, and En. C15, control; S15, 15 min of stimulation; C90, 90-min control; S90, 90 min of stimulation. Samples (20 μg protein) were loaded onto 7.5% polyacrylamide gels, electrophoresed and immunoblotted to detect presence of $\alpha_1$. A brain (Br) standard (5 μg) was used as a positive control. Results were normalized to Br and expressed as a percentage of the respective control limbs. MW, molecular weight standard. *Significantly different from corresponding Con ($P < 0.05$).

Fig. 3. Representative Western blots ($A$) and relative changes in $\alpha_1$-isoform with stimulation ($B$) in Hom, SLP, and endosomal (En) fractions. Values are means ± SE. For $\alpha_2$, $n = 8$ for Hom and $n = 5$ for SLP and En. Samples (20 μg of protein for SLP and Hom and 30 μg protein for En) were loaded onto 7.5% polyacrylamide gels, electrophoresed, and immunoblotted to detect the presence of the $\alpha_2$-isoform. For positive control, 5 μg of Br were used. Results were normalized to Br and then normalized relative to Con limbs. *Significantly different from Con ($P < 0.05$).
ATPase content, based on its binding affinity to the nucleotide site of the α-subunit (30), the property assessed, in effect, represents the active state of the enzyme, which depends on the α-β heterodimer (2, 18). In this study, elevations in $V_{\text{max}}$ were observed at both 15 and 90 min of stimulation in whole muscle homogenates, similar to what was observed for the 3-O-MFPase activity.

Ideally, the measurement of Na$^+$-K$^+$-ATPase activity should correspond to the Na$^+$-K$^+$-ATPase content as assessed by $[^{3}H]$ binding capacity. However, on the basis of estimates of molecular 3-O-MFPase activity, the measured 3-O-MFPase activity is less than 50% of the $[^{3}H]$ouabain values. There could be many reasons for this, which we have addressed in an earlier paper (12). As an example, the Na$^+$-K$^+$-ATPase exists in different locations with different α- and β-isoform combinations, which may result in differences in catalytic activity. The use of the 3-O-MFPase as a measure of hydrolytic activity of the enzyme may be suspect given the affinity of the enzyme for the artificial substrate, the spontaneous hydrolysis that occurs, and, perhaps more importantly, the fact that it is the phosphatase that is assessed (12). One must also remember that the calculations of molecular activity of the enzyme are determined under conditions different from the actual measurements of the Na$^+$-K$^+$-ATPase activity.

To determine whether cellular location was important in the increase in $V_{\text{max}}$ that was observed with contractile activity, we have used differential centrifugation to separate the tissue into two fractions, namely the SLP and En fractions (16). Fuller et al. (16) have shown that, by using a series of selective centrifugations of cardiac tissue homogenates prepared from a hand-held glass tissue grinder, at least 80% of the Na$^+$-K$^+$-ATPase could be captured in the SLP pellet whereas ~20% remained in the En. When this protocol was employed in the present study with the soleus muscle, we found an approximate eight-fold enrichment in $V_{\text{max}}$ in SLP compared with Hom. Moreover, the $V_{\text{max}}$ in the En fraction represented only ~10% of the SLP fraction. This relatively simple isolation procedure circumvents a major criticism of previous isolation protocols, namely the very low amount of the cellular Na$^+$-K$^+$-ATPase enzyme remaining in the enriched sarcolemma fraction (7). Given that the majority of the Na$^+$-K$^+$-ATPase resides in the SLP fraction using the Fuller et al. (16) protocol, and the fact that the tissue sample is restricted to two fractions, namely the SLP and En, one has much more confidence that the experimental manipulation, in our case contractile activity, did not result in a selective sarcolemmal yield. Further support for the fractionation procedure is also provided by other properties analyzed. DHPR, thought to reside exclusively in the T tubules (4), could only be detected in the SLP fraction in both Con and Stim muscle. We have observed that GLUT-4, which is known to exist in both the sarcolemma and intracellular sites in red muscle (27), is more enriched in SLP (64%) compared with En (36%) in Con.

Our results indicate that the increase in $V_{\text{max}}$ observed with contractile activity in Hom occurred specifically in the SLP fraction. Moreover, as with the Hom, the other kinetic properties examined, namely $K_{m}$ and $n_{H}$, were also unaltered in the exercised muscle. No differences were observed in any of the kinetic properties studied between the control and stimulated muscles in the En fraction. Given that the increase in $V_{\text{max}}$ in the SLP in the stimulated muscle was comparable to the Hom, and based on the enrichment that occurred, translocation of the
enzyme apparently resulted. Interestingly, because the $V_{\text{max}}$ of the En was unaltered with stimulation, the process of translocation appears to have resulted in more functional enzyme.

Evidence for translocation of selected subunits with muscle contraction is provided in the quantitative immunoblotting measurements. At 15 min of repetitive activity, an increased $\alpha_2$ content was observed in the SLP, which was accompanied by decreases in the En fraction. There is also evidence for a translocation of the $\alpha_1$-isoform to the SLP at this time because $\alpha_1$ decreased in the En and showed near-significant ($P > 0.08$) increases in the SLP fraction. De novo synthesis of Na\(^+-\)K\(^+-\)ATPase protein does not appear to explain our results because we found no evidence of changes in either $\alpha_1$ or $\alpha_2$ in the Hom at the 15-min time point. Immunoblot measurements of the $\beta_1$-isoform in Hom and SLP and En fractions indicated that neither the content of this subunit nor that translocation of existing subunits was altered with stimulation.

At 90 min of stimulation, $V_{\text{max}}$ remained elevated. However, at this time point, the role of translocation becomes more complicated. Although we have found increases in $\alpha_2$ in SLP, no reciprocal decrease in this isoform was observed in En. For $\alpha_1$, increases in SLP were also observed at 90-min stimulation that was accompanied by increases in En as well. Because $\alpha_1$ also increased in Hom at this time point, it appears as if more $\alpha_1$ protein is present.

The results of this investigation support the findings of previous studies that have also demonstrated increase intracellular trafficking of the $\alpha$-subunits with muscle contraction. Tsakirides et al. (36), using 60 min of treadmill running, found increases in the plasma membrane content of both the $\alpha_1$- and $\alpha_2$-isoforms in rat tissue samples representing a mixture of both red and white fibers. In contrast to the present study, however, these investigators were not able to detect reciprocal changes in an internal membrane fraction or in other recovered fractions. Tsakirides et al. also found that exercise failed to elicit significant increases in the $\beta_1$-subunit of the plasma membrane fraction in the red muscle samples. As in the present study, given the relatively low abundance of the $\beta_2$-isoform (12), this isoform was not probed for. In white muscles, where $\beta_2$ is more abundant, no significant translocation was observed with exercise (36). In a more recent study employing 5 min of intermittent stimulation, the content of $\alpha_1$ and $\beta_1$ but not $\alpha_2$ was increased in sarcolemmal giant vesicles from rat soleus (23). The effects of exercise on subunit translocation in human muscles have also been studied by using giant sarcolemma vesicles and Western blotting procedures (24). Increases in vesicular membrane content of $\alpha_2$- and $\beta_1$- but not $\alpha_1$-isoform content were observed.

At present, in the vastus lateralis muscle, the origin of the translocated Na\(^+-\)K\(^+-\)ATPase from intracellular sites remains unknown. It has been established by immunocytochemical procedures that the $\alpha_1$-isoform is largely restricted to the sarcolemma whereas the $\alpha_2$-isoform is diffusively distributed.

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**Fig. 6.** Representative Western blots (A) and the relative changes in the monocarboxylate transporter MCT1 (MCT1), glucose transporter (GLUT-4), and dihydropyridine receptor (DHPR) distribution (B) in SLP and En fractions with stimulation. For GLUT-4 and DHPR only 15 min of stimulation were investigated. Values are means ± SE (n = 5). The amount of protein sample for SLP and En was 20 and 40 μg, respectively. The samples were loaded onto 7.5 polyacrylamide gels, electrophoresed, and immunoblotted to detect the presence of MCT1, GLUT-4, and DHPR. MCT1 and DHPR were not detectable in En fraction. Results were normalized relative to control and expressed as a percentage. *Significantly different from Con ($P < 0.05$).
with repetitive stimulation. Others (10, 22, 23), that increases in [3H]ouabain binding occur. We have reported, as have the cell (26, 38). In addition, it is difficult to understand why binding, in itself, induces internalization of the may not be tenable. Evidence exists to indicate that ouabain to the above study was that the [3H]ouabain binding is selective only translocation in exercised rat muscle. An assumption of the muscle contraction, given the acute increases in Na/H1001 ATPase activity that occurred (10). We have reported, as have glut-4, also occurs. Given this evidence, it is difficult to rationalize the results of McKenna et al. (28), who, using PY69 antibody and subject to immunoblot analyses with antibodies against α1 and α2 Flow (F) represents 30 μg of loaded flow through supernatant containing non-tyrosine-phosphorylated α-subunits. Results were normalized relative to 5 μg of Br and then expressed as a percentage of Con. *Significantly different from Con (P < 0.05).

Fig. 7. Representative immunoprecipitations (A) and relative changes in tyrosine phosphorylation state (B) of α1- and α2-isoforms of the Na+−K+-ATPase in Hom of muscles stimulated for 15 and 90 min. Values are means ± SE (n = 5). Hom samples (750 μg protein) were immunoprecipitated with PY69 antibody and subject to immunoblot analyses with antibodies against α1 and α2. Flow (F) represents 30 μg of loaded flow through supernatant containing non-tyrosine-phosphorylated α-subunits. Results were normalized relative to 5 μg of Br and then expressed as a percentage of Con. *Significantly different from Con (P < 0.05).

Throughout the muscle fibers, including the T tubules and possibly caveolae (7).

Collectively, these studies are consistent with the notion that repetitive exercise does induce subunit translocation, at least in the α-isofoms, to the plasma membrane. Moreover, we have also been able to provide evidence supporting previous studies (19), namely that a translocation of the glucose transporter, GLUT-4, also occurs. Given this evidence, it is difficult to rationalize the results of McKenna et al. (28), who, using [3H]ouabain binding, have failed to observe evidence for translocation in exercised rat muscle. An assumption of the above study was that the [3H]ouabain binding is selective only to the α-isofoms in the plasma membrane. This assumption may not be tenable. Evidence exists to indicate that ouabain binding, in itself, induces internalization of the α-subunits of the cell (26, 38). In addition, it is difficult to understand why increases in [3H]ouabain binding were not observed with muscle contraction, given the acute increases in Na+−K+-ATPase activity that occurred (10). We have reported, as have others (10, 22, 23), that increases in [3H]ouabain binding occur with repetitive stimulation.

Our results indicate that the increase in Vmax was not compromised when the muscle stimulation was extended to 90 min. As observed at 15 min of stimulation, the increase in Vmax at 90 min appears to be restricted to the SLP fraction because Vmax remained high in this fraction and no changes were observed in the En fraction. It is clear from our immunoblotting results that at 90 min of contractile activity increases in both α1 and α2 content in the SLP fraction occurred. However, in contrast to the shorter period of activity, namely at 15 min, decreases in those isoforms in En were not observed. Our failure to observe a reciprocal decrease in these isoforms, at least for the α1, may be explained at least in part by an increase in cellular content, because α1 increased in the Hom at this time point. As with the 15-min time point, we could find no evidence at 90 min for changes in total cellular content of the β1-isoform for translocation. The results of Tsakirides et al. (36) are of interest in this regard. Although these investigators did not find an increase in total pump content with 60 min of treadmill running in rats, they did report a marked increase in the α1-mRNA but not the α2-mRNA and β1-mRNA levels, in exercised soleus muscles. It must be acknowledged that changes may be easier to detect in purified fractions compared with homogenates given the large differences in protein content.

In this study, we also provide evidence for exercise-induced changes in the intrinsic regulation of Na+−K+-ATPase activity. After 15 min of muscle contraction, tyrosine phosphorylation of the α2-isoform, but not the α1-isoform, was increased. No changes in tyrosine phosphorylation were observed at 90 min in either isoform. It is well known that the Na+−K+-ATPase activity is under complex regulatory control (7). In addition to changes in a number of metabolites, such as Na+ and K+, the phosphorylation-dephosphorylation state of the enzyme also appears important. Our present understanding of the regulation of the phosphorylation status of the Na+−K+-ATPase in skeletal muscle is that both PKA and PKC are involved, probably in an isoform-specific manner (3, 33). Activation of these enzymes results in an increase phosphorylation of either serine or threonine residues (33). There are also reports of increased tyrosine phosphorylation, secondary to increases in insulin (5). Our results, indicating an increase in the phosphorylation status of the α2-isoform, in conjunction with a translocation of the α2 to the SLP, indicate an insulin-like response (5). Interestingly, the response of the soleus to insulin is also to increase Vmax in plasma membrane fractions and transient phosphorylation of

Fig. 8. Changes in maximal [3H]ouabain binding (βmax) in whole muscle homogenates (Hom) in animals stimulated for 15 and 90 min. Values are means ± SE (n = 8). Muscle samples (2 to 8 mg) were incubated with 1 μM of [3H]ouabain as described in METHODS. *Significantly different from Con (P < 0.05).
the α2-subunit isoform. This is similar to our results with contractile activity. Tyrosine phosphorylation was also observed in the α3-subunit, but the increase was minor compared with the change in α2-phosphorylation. Not to be discounted, however, is the potential role of the small regulatory protein phospholemman, which when phosphorylated can have a dramatic effect in altering Na\(^+\)-K\(^+\)-ATPase activity. Because phospholemman also exists in skeletal muscle, its regulatory role in the kinetic responses of the Na\(^+\)-K\(^+\)-ATPase to contractile activity needs to be defined. Unfortunately, the measurement of the phosphorylation states of serine and threonine residues was not possible because the large number of measurements performed consumed all of the homogenate.

In summary, we have shown that, when assessed in vitro, the maximal Na\(^+\)-K\(^+\)-ATPase activity is increased in rat soleus muscle in response to a brief period of contractile activity. The increase in V\(_{\text{max}}\) occurs in the absence of changes in the apparent affinity for K\(^+\). Separation of the tissue sample into two fractions indicated that translocation of α-isofoms and, in particular, the α2-isofom to the sarcomplasm from the endosome was closely related to the increase in V\(_{\text{max}}\). Our results also suggest that tyrosine phosphorylation of the enzyme may also be involved in increasing V\(_{\text{max}}\), at least during the early phase of contractile activity. Collectively, our results indicate that both translocation and regulatory phosphorylation appear to be involved in the increase observed in V\(_{\text{max}}\).

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