The Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\)-subunit isoform modulates contractility in the perinatal mouse diaphragm


1Department of Molecular and Cellular Physiology and 2Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Submitted 11 May 2004; accepted in final form 6 July 2004

Radzyukevich, Tatiana L., Amy E. Moseley, Daniel A. Shelly, Gregory A. Redden, Michael M. Behbehani, Jerry B. Lingrel, Richard J. Paul, and Judith A. Heiny. The Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\)-subunit isoform modulates contractility in the perinatal mouse diaphragm. *Am J Physiol Cell Physiol* 287: C1300–C1310, 2004. First published July 14, 2004; doi:10.1152/ajpcell.00231.2004.—This study uses genetically altered mice to examine the contribution of the Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\)-catalytic subunit to resting potential, excitability, and contractility of the perinatal diaphragm. The \(\alpha_2\)-protein is reduced by 38% in \(\alpha_2\)-heterozygous and absent in \(\alpha_2\)-knockout mice, and \(\alpha_1\)-isoform is upregulated 1.9-fold in \(\alpha_2\)-knockout. Resting potentials are depolarized by 0.8–4.0 mV in heterozygous and knockout mice. Action potential threshold, overshoot, and duration are normal. Spontaneous firing, a developmental function, is impaired in knockout diaphragm, but this does not compromise its ability to fire evoked action potential trains, the dominant mode of activation near birth. Maximum tetanic force, rate of activation, force-frequency and force-voltage relationships, and onset and magnitude of fatigue are not changed. The major phenotypic consequence of reduced \(\alpha_2\) content is that relaxation from contraction is 1.7-fold faster. This finding reveals a distinct cellular role of the \(\alpha_2\)-isoform at a step after membrane excitation, which cannot be restored simply by increasing \(\alpha_1\) content. Na\(^+\)/Ca\(^2+\) exchanger expression decreases in parallel with \(\alpha_2\)-isoform, suggesting that Ca\(^2+\) extrusion is affected by the altered \(\alpha_2\) genotype. There are no major compensatory changes in expression of sarcoplasmic reticulum Ca\(^2+\)-ATPase, phospholamban, or plasma membrane Ca\(^2+\)-ATPase. These results demonstrate that the Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-isoform alone is able to maintain equilibrium K\(^+\) and Na\(^+\) gradients and to substitute for \(\alpha_2\)-isoform in most cellular functions related to excitability and force. They further indicate that the \(\alpha_2\)-isoform contributes significantly less at rest than expected from its proportional content but can modulate contractility during muscle contraction.

Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\) catalytic subunit; heterozygous mice; knockout mice; resting potential

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\(\beta\)-subunit (~35 kDa) that targets the \(\alpha\)-subunit to the plasma membrane and may medulate substrate affinity (30). \(\gamma\)-Subunits (or FxYD-domain proteins) also exist, which can interact preferentially with specific \(\alpha\beta\)-isozymes to regulate their catalytic properties. Multiple isoforms exist for \(\alpha\), \(\beta\), and \(\gamma\)-subunits, and numerous studies suggest that they play tissue-specific roles.

Skeletal muscles express at least two major \(\alpha\)-subunit isoforms that each show a distinct developmental and muscle type-specific expression and subcellular localization. The \(\alpha_1\)-isoform is expressed throughout muscle development, whereas the \(\alpha_2\)-isoform is expressed later and becomes the predominant subunit of adult muscle (22, 44). Human skeletal muscle also expresses the \(\alpha_2\)-isoform (41). The mouse diaphragm expresses the \(\alpha_1\)- and \(\alpha_2\)-isoforms; during development, the \(\alpha_2\)-isoform is expressed when the t tubules and dihydropyridine receptors appear (9). The \(\alpha_2\)-isoform is expressed at higher abundance in fast glycolytic compared with slow oxidative muscles, reaching up to 87 and 65% of total \(\alpha\) content in the mouse extensor digitorum longus (EDL) and soleus (SOL), respectively (Refs. 9, 22, and 53 and Shelly, unpublished results). The \(\alpha_1\)-isoform is localized on the surface sarcolemma, and the \(\alpha_2\)-isoform is on both the surface and the t tubules at the triads in various adult muscles and species, including mice (56).

The functional roles of the \(\alpha\)-isoforms in skeletal muscle are not completely understood. Because of its ubiquitous tissue distribution, the \(\alpha_1\)-isoform is widely thought to perform a housekeeping role in maintaining equilibrium ion gradients; however, limited studies exist that address this hypothesis in skeletal muscle in the absence of \(\alpha_2\). In addition, a dynamic role of the \(\alpha_1\)-isoform in restoring ion gradients during contraction is inferred from the 20-fold stimulation of Na\(^+\)-K\(^+\) transport in contracting muscles, a rate that utilizes the total muscle Na\(^+\)-K\(^+\) transport capacity (7, 13, 27). There is less consensus on proposed unique role(s) for \(\alpha_2\)-isoform in skeletal muscle. In addition to restoring ion gradients and excitability, it is proposed to modulate Ca\(^2+\) signaling and thereby contractility. The EDL and SOL of mice with genetically reduced \(\alpha_2\)-isoform content produce greater force (22), and this is thought to occur through an association of \(\alpha_2\)-containing pump units with the Na\(^+\)/Ca\(^2+\) exchanger in a submembrane domain, where their combined activities can modulate dynamic Ca\(^2+\) signaling from the sarcoplasmic reticulum (SR). A similar role for \(\alpha_2\)-isoform is proposed to explain the altered
contractility of heart and smooth muscles and the altered Ca\(^{2+}\) signaling in astrocytes of mice with genetically reduced \(\alpha_2\)-isoform content (5, 21, 26, 51). In addition to an acute cellular role, the large \(\alpha_2\)-isoform content of skeletal muscle is proposed to maintain systemic K\(^+\) homeostasis under a variety of chronic conditions of altered plasma K\(^+\) or hormonal status (24, 28, 34, 54; however, see also Ref. 37).

The tissue and subcellular distribution of the \(\alpha_2\)-isoform, in particular its selective expression in electrically excitable cells (skeletal muscle, heart, nerve) or the cells that surround them (adipocytes, astrocytes), suggested to us that it could modulate excitability in skeletal muscle. In addition, the findings that the \(\alpha_2\)-isoform can modulate contractility in adult heart and skeletal muscles without change in the resting Na\(^+\) and K\(^+\) concentrations (22, 26) suggested that the \(\alpha_2\)-isoform may play a greater role in active compared with resting muscle. The activity of the Na\(^+/\)Ca\(^{2+}\) exchanger (NCX), which is present in the t tubules (47), is low in resting muscle but becomes measurable during tetanic activity to extrude Ca\(^{2+}\) (4). The localization of the \(\alpha_2\)-isoform in the t tubules, where it can clear the activity-dependent K\(^+\) and Na\(^+\) and control the Na\(^+\) gradient that drives Na\(^+\)/Ca\(^{2+}\) exchange, is optimal for modulating both excitability and contractility.

This study uses gene-targeted mice that lack one or both copies of the Na\(^+/\)K\(^+\)-ATPase \(\alpha_2\)-subunit gene (26) to examine the contributions of the \(\alpha_1\) and \(\alpha_2\)-isoforms to the excitability and contractility of skeletal muscle. We tested the hypotheses that the \(\alpha_1\)-isoform alone is able to maintain the equilibrium Na\(^+\) and K\(^+\) gradients in resting muscle and that \(\alpha_2\)-isoform can modulate excitability and contractility in active muscles. We measured resting potentials, action potentials, and contractile force during tetanic and fatiguing stimulation in the adult muscles without change in the resting Na\(^+\) and K\(^+\) concentrations (22, 26).

Contractility measurements. Isometric contractility was measured as described previously (39). Diaphragm strips were attached to triangular clips and mounted to a force transducer in a constant-temperature sealed chamber with freshly circulating Krebs solution containing (in mM) 154 Na, 4.7 K, 2.5 Ca, 1.2 Mg, 17 Cl, 25 HCO\(_3\), 1.2 HPO\(_4\), 11 glucose, and 0.026 EDTA equilibrated with 95% O\(_2\)-5% CO\(_2\) at 37°C. Optimal resting tension at the outset was determined empirically to be 3 mN by adjusting resting tension from zero until maximal twitch force was achieved. Diaphragm strips were field stimulated using platinum wire electrodes with capacitor discharges of equal but alternating polarity (2–80 Hz at 6–20 V, 2 ms duration). Fatigue-producing stimulation was performed by applying 1-s duration at supramaximal voltage (16–18 V) at a frequency (60 Hz) to the diaphragm every 0.45 s. Force was recorded using a differential capacitor force transducer (Harvard Apparatus, South Natick, MA) connected to a data-acquisition system (BioPac System, Goleta, CA). Force was normalized to the average cross-sectional area of the E18.5 diaphragm strip and expressed as a percentage of maximal tetanic force. Cross-sectional areas were measured from fixed sections using a Nikon light microscope at \(\times100\).

Western blot analysis. Microsomal membrane samples were prepared from E18.5 diaphragms and resolved by SDS-PAGE as described previously (9). Proteins were electrophoresed for 0.3–1.5 h on 10% precast polyacrylamide gels containing (in mM) 154 Na, 4.7 K, 2.5 Ca, 1.2 Mg, 17 Cl, 25 HCO\(_3\), 1.2 HPO\(_4\), 11 glucose, and 0.026 EDTA equilibrated with 95% O\(_2\)-5% CO\(_2\) at 37°C. Optimal resting tension at the outset was determined empirically to be 3 mN by adjusting resting tension from zero until maximal twitch force was achieved. Diaphragm strips were field stimulated using platinum wire electrodes with capacitor discharges of equal but alternating polarity (2–80 Hz at 6–20 V, 2 ms duration). Fatigue-producing stimulation was performed by applying 1-s duration at supramaximal voltage (16–18 V) at a frequency (60 Hz) to the diaphragm every 0.45 s. Force was recorded using a differential capacitor force transducer (Harvard Apparatus, South Natick, MA) connected to a data-acquisition system (BioPac System, Goleta, CA). Force was normalized to the average cross-sectional area of the E18.5 diaphragm strip and expressed as a percentage of maximal tetanic force. Cross-sectional areas were measured from fixed sections using a Nikon light microscope at \(\times100\).

METHODS

Muscle preparation. All handling and use of animals complied with the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati. Embryos were obtained at E18.5 by cesarean section from \(\alpha_2\)-HET females mated to \(\alpha_2\)-HET males (26). The surgery was performed immediately after euthanasia by CO\(_2\) asphyxiation, followed by cervical dislocation. The pups were killed by cervical dislocation, followed by decapitation. The whole diaphragm with ribs attached was removed. Tail clips were taken for genotyping by Southern blot analysis (26). Measurements were performed blind before the genotype of the muscle was known.

Electrical recording. The diaphragm was fixed to a Syngard (Dow Corning, Midland, MI) mount on the chamber bottom. Resting potentials and action potentials were measured from superficial fibers, diameter 15–20 \(\mu\)m, in the central region of the muscle with the use of borosilicate glass filament microelectrodes (World Precision Instruments, Sarasota, FL). Electrodes were filled with 3 M KCl (25–40 mM). Membrane potentials were measured using an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) in current-clamp mode. Resting potentials were read directly from the voltage output. We accepted all resting potential values that remained stable and did not depolarize on impalement. To elicit action potentials, a stimulus was delivered extracellularly with the use of a small-tip concentric bipolar electrode (FHC, Bowdoinham, ME) positioned near the muscle surface. Action potentials were captured digitally on tape and analyzed using the Axotape program (Axon Instruments). Resting potentials and single action potentials were recorded using a standard Krebs-Ringer bicarbonate buffer composed of (in mM) 154 Na, 5 K, 2 Ca, 1 Mg, 138 Cl, 25 HCO\(_3\), 1 HPO\(_4\), and 11 glucose at 22°C. Trains of action potentials were recorded using a Cl\(^-\)–free buffer composed of (in mM) 166 Na, 5 K, 1 Ca, 2 Mg, 2 Cl, 78 SO\(_4\), 25 HCO\(_3\), 1 HPO\(_4\), and 11 glucose with 50 \(\mu\)M dantrolene at 29°C. The solutions were bubbled with 95% O\(_2\)-5% CO\(_2\) at pH 7.4.

Contractility measurements. Isometric contractility was measured as described previously (39). Diaphragm strips were attached to triangular clips and mounted to a force transducer in a constant-temperature sealed chamber with freshly circulating Krebs solution containing (in mM) 154 Na, 4.7 K, 2.5 Ca, 1.2 Mg, 17 Cl, 25 HCO\(_3\), 1.2 HPO\(_4\), 1.2 SO\(_4\), 11 glucose, and 0.026 EDTA equilibrated with 95% O\(_2\)-5% CO\(_2\) at 37°C. Optimal resting tension at the outset was determined empirically to be 3 mN by adjusting resting tension from zero until maximal twitch force was achieved. Diaphragm strips were field stimulated using platinum wire electrodes with capacitor discharges of equal but alternating polarity (2–80 Hz at 6–20 V, 2 ms duration). Fatigue-producing stimulation was performed by applying 1-s duration at supramaximal voltage (16–18 V) at a frequency (60 Hz) to the diaphragm every 0.45 s. Force was recorded using a differential capacitor force transducer (Harvard Apparatus, South Natick, MA) connected to a data-acquisition system (BioPac System, Goleta, CA). Force was normalized to the average cross-sectional area of the E18.5 diaphragm strip and expressed as a percentage of maximal tetanic force. Cross-sectional areas were measured from fixed sections using a Nikon light microscope at \(\times100\).
The α1-subunit (9). The E18.5 isoform mRNA and protein, respectively; the lesser extent, the Na$_2$-subunit isoform comprises 62 and 67% of total Na$_2$-isoforms, 40% for brain. and KO mice at E18.5, grouped in 10-mV intervals. Resting potentials range from -11 to -78 mV and, importantly, show a similar distribution among genotypes. Variability in resting potentials can result from fiber-to-fiber differences as well as from microelectrode impalement, especially in fibers with diameters as small as 15 μm. This result indicates that the range and distribution of resting potentials arise from intrinsic fiber-to-fiber differences and/or the inherent limits of the technique and not from other potential consequences of the altered genotype.

Given this spread, an objective criterion is needed to determine the meaningful values. Table 2 compares mean resting potentials obtained from all fibers or from a subset of fibers that were able to generate either evoked or spontaneous action potentials. Both groups are included because fibers that do not fire action potentials may have been damaged or, alternatively, because fibers could have similar resting potentials but altered excitability as a secondary consequence of the altered genotype. Resting potentials in all groups and all genotypes are similar but distribute over a more limited range in the subset of excitable fibers. There is a statistically significant depolarization of 4.0 mV between α2-isoform KO and WT fibers in the

Table 1. Na$^+$-K$^+$-ATPase α-isoforms and Ca$^{2+}$ handling proteins in mouse diaphragm

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>α2-HET</th>
<th>α2-KO</th>
<th>Adult WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$-K$^+$-ATPase-α1</td>
<td>100</td>
<td>147±18 (4)*</td>
<td>194±31 (4)*</td>
<td></td>
</tr>
<tr>
<td>Na$^+$-K$^+$-ATPase-α2</td>
<td>62±11 (5)*</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SERCA1</td>
<td>100</td>
<td>125±22 (5)</td>
<td>76±10 (8)*</td>
<td></td>
</tr>
<tr>
<td>SERCA2</td>
<td>100</td>
<td>152±24 (4)</td>
<td>82±14 (5)</td>
<td>181±18 (4)*</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>100</td>
<td>108±30 (4)</td>
<td>119±24 (4)</td>
<td>217±22 (4)*</td>
</tr>
<tr>
<td>PMCA</td>
<td>100</td>
<td>143±12 (4)*</td>
<td>98±33 (4)</td>
<td>102±22 (5)</td>
</tr>
<tr>
<td>NCX</td>
<td>48±6 (3)*</td>
<td>54±7 (3)*</td>
<td>20 (1)</td>
<td></td>
</tr>
</tbody>
</table>

Values are percent protein in perinatal wild-type (WT) diaphragm, expressed as means ± SE, with n (no. of Western blots from independent samples) in parentheses. E18.5, embryonic age of 18.5 days; HET, heterozygous; KO, knockout; SERCA, sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase; PMCA, plasma membrane Ca$^{2+}$-ATPase; NCX, Na$^+$/Ca$^{2+}$ exchanger; ND, not detected. *Statistical significance compared with perinatal WT.

Fig. 1. Top three blots: representative Western blot of Na$^+$-K$^+$-ATPase α-subunit isoform expression in the perinatal diaphragm of wild-type (WT), α2-isoform heterozygous (HET), and α2-isoform knockout (KO) mice at embryonic age (E) 18.5. Microsomal membrane samples (20 μg for α1- and α2-isoforms, 40 μg for α3-isoform) were loaded in duplicate and probed with isoform-specific antibodies as described in METHODS. Bottom blot: absence of α3-isoform was confirmed by positive and negative controls; it is absent in the kidney, which does not express the α3-isoform, and present in the brain, which normally expresses the α3-isoform. A value of 40 μg of protein was loaded for KO diaphragm and kidney and 5 μg for brain.

Fig. 2. Distribution of resting potentials in diaphragm skeletal muscle fibers of WT, α2-HET, and α2-KO mice at E18.5. Measurements are grouped in 10-mV intervals (resting potential ± 5 mV). A minimum of 200 fibers of each genotype were sampled, from a total of 41 diaphragm muscles, distributed by genotype: WT (n = 14 muscles), α2-HET (n = 16), and α2-KO (n = 11).
total set and 1.8 mV in the subset of excitable fibers. These resting potential values are consistent with values expected in diaphragm fibers just before birth. The resting potential increases rapidly near birth; it reaches −50 to −65 mV in mouse diaphragm at 2–3 days postnatal (40) and averages −63 mV from birth to 3 days in rat diaphragm (14). This result demonstrates that muscle fibers of the perinatal diaphragm are able to maintain near-normal resting potentials, even in the complete absence of the Na⁺-K⁺-ATPase α₂-subunit.

α₂-Isomorph HET and KO fibers generate normal action potentials and maintain excitability under physiological stimulus conditions. In adult muscle, a steady depolarization of 1–4 mV is not expected to compromise excitation. However, its effect in perinatal muscle, which starts from a less negative resting potential, is not known. Consequently, we examined excitability in all genotypes. We first examined the parameters of single action potentials generated spontaneously by each genotype. Spontaneous electrical and mechanical activity is a hallmark of developing muscle and is technically more feasible to capture in perinatal muscle fibers. Fibers of α₂-isoform HET and KO diaphragms both exhibit spontaneous action potential activity that occurs randomly and is comparable with WT fibers. This activity persists after the addition of curare (25 μM α-tubocurarine chloride), confirming that it originates in the muscle membrane. Threshold potential, overshoot potential, and duration of single action potentials in the α₂-isoform HET and KO are comparable with WT (Fig. 3 and Table 3). The identical overshoot potentials indicate that the Na⁺ current reversal potential (E_Na) is not changed by the altered α₂-isoform expression. The similar threshold potentials and durations indicate that the Na⁺, K⁺, and Cl⁻ currents that underlie the action potential are not substantially different in fibers with reduced or absent α₂-isoform content. In addition, because the Nernst potentials for Na⁺ and K⁺ provide the driving force for the Na⁺ and K⁺ currents during the action potential, this result provides further evidence that their equilibrium ion gradients are not altered, consistent with the small effect of removing α₂-isoform on the resting potential.

The results in Fig. 4 test whether the α₂-isoform HET and KO fibers are able to generate trains of action potentials at physiological frequencies, a requirement for sustained contraction. To capture more events using the same recording time for each genotype, we removed Cl⁻ from the bathing solution. Repetitive firing increases in this condition because the higher membrane resistance destabilizes the membrane potential and because action potential repolarization must proceed without the Cl⁻ currents that normally contribute to restoration of the membrane potential (1). To reduce mechanical activity, we lowered extracellular Ca²⁺ and added dantrolene sodium (50 μM) at 29°C. Dantrolene uncouples excitation-contraction coupling without effect on the resting or action potential (12, 43). These interventions markedly suppress but do not completely eliminate fiber movement. The major difference among genotypes is that the α₂-KO fibers are not able to maintain high-frequency firing during sustained action potential activity. WT and α₂-HET fibers maintain relatively stable intervals of 13–26 ms (50–80 Hz) for up to nine action potentials (Fig. 4D), whereas in α₂-KO fibers, the interval between successive action potentials increases from 25 to 50 ms after four to five action potentials (Fig. 4B), corresponding to a decrease in frequency from 41 to 20 Hz. However, α₂-KO fibers are able to fire evoked trains of action potentials when driven by extracellular stimuli at frequencies up to 86 Hz (Fig. 4C), exceeding the physiological drive from the phrenic nerve (20). This indicates that their lower spontaneous frequency originates in the muscle membrane and, importantly, that it has not compromised the ability of the diaphragm to respond to driven input. Spontaneous activity is gradually replaced by input from the phrenic nerve, which becomes the dominant physiological mode of activation starting from just before birth.

In addition, α₂-KO fibers generate action potential trains with a more constant amplitude and baseline potential (Fig. 4B). This was a consistent observation but could not be quantified because contraction could not be blocked completely. It suggests that perinatal diaphragm fibers without any α₂-isoform are able to maintain normal ion gradients during sustained electrical activity, when the intracellular Na⁺ and extracellular K⁺ are expected to increase dynamically. Taken

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**Table 2. Resting potentials of mouse diaphragm muscle fibers at E18.5**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>α₂-HET</th>
<th>α₂-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>All fibers</td>
<td>−50.0±0.9 (214)</td>
<td>−46.9±0.9 (200)*</td>
<td>−46.0±0.9 (208)*</td>
</tr>
<tr>
<td>Subset</td>
<td>−55.3±0.6 (120)</td>
<td>−54.5±0.6 (103)</td>
<td>−53.5±0.6 (109)*</td>
</tr>
</tbody>
</table>

Resting potentials were computed with the use of either the set of all fibers (range −11 to −78 mV) or a subset of fibers that fired action potentials (range −44 to −66 mV) and are given as means ± SE, with n (no. of fiber samples from a total of 41 diaphragm muscles) in parentheses. *Statistically different from WT.

**Table 3. Parameters of spontaneous single action potentials in mouse diaphragm at E18.5**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>α₂-HET</th>
<th>α₂-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential, mV</td>
<td>−52.1±1.7 (14)</td>
<td>−53.9±0.8 (34)</td>
<td>−53.2±1.2 (30)</td>
</tr>
<tr>
<td>Threshold potential, mV</td>
<td>−42.4±1.4 (12)</td>
<td>−44.0±1.0 (31)</td>
<td>−42.5±1.3 (19)</td>
</tr>
<tr>
<td>Overshoot, mV</td>
<td>+17.4±1.5 (13)</td>
<td>+15.1±1.6 (28)</td>
<td>+14.8±2.2 (18)</td>
</tr>
<tr>
<td>Duration at half-amplitude, ms</td>
<td>1.8±0.1 (16)</td>
<td>1.6±0.1 (33)</td>
<td>1.6±0.1 (23)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with n (no. of fiber samples from each genotype, from a total of 41 diaphragm muscles) in parentheses. No differences are statistically significant.

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![Fig. 3. Representative single action potentials from diaphragm fibers of WT and α₂-KO mice at E18.5.](http://ajpcell.physiology.org/)
together, these measurements show that diaphragm fibers of \( \alpha_2 \)-KO mice show minor impairment of spontaneous action potential firing but are able to maintain excitability under conditions that mimic the physiological pattern of stimulation.

Diaphragm fibers of \( \alpha_2 \)-HET and \( \alpha_2 \)-KO mice show altered contractility. The absence of one copy of the Na\(^+\)-K\(^+\)-ATPase \( \alpha_2 \)-isoform results in increased twitch and tetanic force in the adult EDL muscle (22) and greater contractile force in the heart, which is associated with a larger Ca\(^{2+}\) change during the contraction cycle (26). Total ablation of the \( \alpha_2 \)-isoform also alters agonist-induced contraction in smooth muscle at birth (51). The perinatal diaphragm of \( \alpha_2 \)-KO mice generates normal twitch force in response to a single stimulus (39), but its contractility during sustained activity, the normal physiological mode of this muscle, has not been examined. Figure 5 and Table 4 show that maximum tetanic force in the \( \alpha_2 \)-HET and \( \alpha_2 \)-KO diaphragm is comparable with WT. In addition, the rate of contraction at half-maximum force is not altered, indicating that the speed of contractile activation is not changed. However, relaxation from contraction is significantly faster in the \( \alpha_2 \)-HET and \( \alpha_2 \)-KO fibers. The time to half-relaxation after a maximum tetanic contraction is significantly shorter in the \( \alpha_2 \)-HET and \( \alpha_2 \)-KO diaphragm and speeds relaxation by 1.7-fold (Table 4).

While these measurements included the use of a supramaximal stimulus amplitude and frequency to obtain maximum tetanic force, the frequency or voltage dependence of contraction is an indication of the efficiency of the excitation-contraction coupling process and the degree of fiber recruitment. Figure 6 demonstrates that the frequency dependence of force generation is significantly different between WT and KO genotypes (\( P < 0.001 \)) and that the KO diaphragm generates significantly less force at frequencies from 4 to 14 Hz (\( P < 0.05 \)). This is consistent with the faster relaxation of individual peaks on recordings of tetani in this frequency range (data not shown) and the faster relaxation after a tetanus. Figure 6B demonstrates that the voltage dependence of contraction in the \( \alpha_2 \)-KO diaphragm is similar to WT over a range of voltages (6–20 V). These findings indicate that the efficiency of activation above 14 Hz is not affected by the loss of \( \alpha_2 \)-subunit.

The transport activity of the Na\(^+\)-K\(^+\)-ATPase is absolutely required to maintain contractile force in active muscles (45), especially during sustained activity when the K\(^+\) and Na\(^+\) fluxes can exceed the capacity of the Na\(^+\)-K\(^+\)-ATPase to restore the resting gradients (6). Figure 6C examines the effect of total \( \alpha_2 \)-isoform ablation on the fatigability of the diaphragm. The \( \alpha_2 \)-KO diaphragm is able to maintain force generation significantly better than WT at frequencies of 4–14 Hz.}

Fig. 4. Spontaneous and evoked action potential activity in the diaphragm of WT, \( \alpha_2 \)-HET, and \( \alpha_2 \)-KO mice at E18.5. A and B: representative action potential trains generated spontaneously by WT and \( \alpha_2 \)-KO muscle, respectively. Dashed line indicates 0 mV. C: evoked action potential train recorded from an \( \alpha_2 \)-KO diaphragm in response to a driven stimulus of 86 Hz. D: mean intervals between successive action potentials for \( n = 6 \) (WT), 8 (HET), and 15 (KO) recordings from a total of 8 mice. *Significantly different in KO compared with WT.

Fig. 5. Representative maximum tetanic contractions of WT and \( \alpha_2 \)-KO diaphragm at E18.5. Diaphragm muscle strips were stimulated extracellularly with a supramaximal stimulus of 16 V and 2 ms, applied continuously at 60 Hz.
compared with WT during fatigue-producing stimulation and declines to the same maximum tetanic force as WT (40% of initial). This result indicates that the perinatal diaphragm is able to maintain $K^+$ gradients during sustained activity, without any $\alpha_2$-isoform. It further suggests that the intrinsic, fatigue-resistant phenotype of the diaphragm is not affected by the loss of $\alpha_2$-isoform. Together, the normal contractility of the $\alpha_2$-KO diaphragm under both tetanic and fatigue conditions suggests that removal of the $\alpha_2$-isoform does not alter contractile activation.

Faster relaxation rate of $\alpha_2$-HET and $\alpha_2$-KO muscle does not result from compensatory changes in the expression of Ca$^{2+}$ handling proteins. Relaxation from contraction occurs when cytosolic Ca$^{2+}$ is restored to resting levels. The faster relaxation of the $\alpha_2$-HET and $\alpha_2$-KO diaphragm suggests that Ca$^{2+}$ removal after tetanic contraction may be enhanced when $\alpha_2$-isoform is absent or reduced. One mechanism by which this could occur is if the altered genotype causes compensatory changes in a protein(s) that influences Ca$^{2+}$ clearance after contraction. To test this, we measured the expression of SERCA1 and SERCA2, the Ca$^{2+}$ transporters of the SR, in skeletal muscle; calsequestrin, the Ca$^{2+}$-binding protein of the SR lumen; PLB, a regulator of SERCA2; the PMCA; and the plasma membrane NCX.

In mammalian muscles, the rate of relaxation at early times after contraction is controlled largely by the rate of active Ca$^{2+}$ transport into the SR, mediated by SERCA. After a tetanic contraction, this requires clearing cytosolic Ca$^{2+}$ in the 10 $\mu$M range within 500 ms. Increased SERCA expression is associated with faster relaxation and underlies the faster relaxation of fast compared with slow fiber types (11, 57), whereas reduced SERCA expression is associated with slowed relaxation (38). The adult diaphragm of rat expresses SERCA1 and the muscle-specific SERCA2a, and these represent ~75 and 23% of total SERCA expression, respectively (38). The SERCA isoform content of mouse perinatal diaphragm is not known, but other neonatal muscles express SERCA1b and SERCA2b, splice variants of SERCA1 and SERCA2. The SERCA antibodies used in this study recognize both a- and b-forms.

Figure 7 and Table 1 show that SERCA1 is expressed in the perinatal diaphragm of WT mice at levels comparable with adult. It is unchanged in the $\alpha_2$-HET and reduced in the $\alpha_2$-KO diaphragm, a direction opposite that required to speed relaxation. Because the amount of protein used to detect SERCA was small, we probed 2 $\mu$g each of the same samples with an antibody against calsequestrin as a technical control. Calsequestrin expression is comparable in all genotypes.
PMCA is the primary extrusion pathway for smaller, sustained Ca$^{2+}$ loads. Because of its low activity and high affinity (52), it is widely thought to play a greater role in resting Ca$^{2+}$ homeostasis than in the rapid, dynamic Ca$^{2+}$ loads during the contraction cycle. Expression of PMCA increases 1.4-fold in the α2-HET and is unchanged in the α2-KO. The small increase in the HET cannot explain a dramatically faster relaxation in both genotypes within 500 ms of a tetanus.

The plasma membrane NCX in skeletal muscle operates in forward mode during contraction and can contribute to global Ca$^{2+}$ clearance (4). This contribution is small compared with SERCA because of its slow kinetics and low transport capacity (19, 23). NCX expression is low in the perinatal diaphragm of WT mice (Fig 7; note heavy loading required to detect it) and decreases by almost one-half in the α2-HET and α2-KO. A change in this direction is expected to decrease Ca$^{2+}$ clearance across the plasma membrane and promote Ca$^{2+}$ accumulation rather than clearance. Moreover, the resulting Ca$^{2+}$ increase is not expected to change global Ca$^{2+}$ due to the low NCX capacity of skeletal muscle, and this is supported by the finding that peak force is not changed under twitch or tetanic conditions.

DISCUSSION

Na$^{+}$-K$^{+}$-ATPase α1-isoform alone is able to maintain the resting potential and equilibrium ion gradients of perinatal diaphragm. The resting potential is determined largely by the diffusion (Nernst) potentials for K$^{+}$ ($E_K$) and to a lesser extent Na$^+$ ions ($E_{Na}$), which result directly from the gradients set by the Na$^+$-K$^{+}$-ATPase. The finding that resting potentials are not substantially changed in muscles which lack any α2-subunit demonstrates that the α1-isoform alone is able to maintain the equilibrium K$^+$ and Na$^+$ gradients of perinatal diaphragm. This conclusion is further supported by the finding that diaphragms of α2-HET and α2-KO mice generate single action potentials with normal threshold and overshoot potentials, parameters that depend directly on the K$^+$ and Na$^+$ Nernst potentials.

This finding was initially surprising, considering the high α2-isoform expression in WT diaphragm (9, 39). However, measurements in an independent α2-KO mouse model found no difference among the resting potentials of WT, HET, and KO diaphragm (25). In addition, the adult EDL of α2-HET mice shows no change in the resting K$^+$ or Na$^+$ content (22), a result that predicts no change in resting potential. To relate the present finding to this study, we measured resting potentials in adult EDL muscles of α2-HET mice. Resting potentials were $-80.1 \pm 3.8 \text{ mV}$ in WT and $-78.0 \pm 5.5 \text{ mV}$ in HET (Moseley AE, Lingrel JB, and Heiny JA, unpublished data). Thus reducing α2-isoform in adult EDL, where α2-isoform comprises up to 87% of total α-isoform content, does not significantly change the resting potential or resting ion gradients, consistent with the small effect of total α2-isoform ablation on the perinatal diaphragm. The small contribution by α2-isoform to the resting potential is statistically significant, on the basis of over 600 measurements. The absence of a contribution by α2-isoform in the study of Ikeda et al. (25) may be due to the small sample ($n = 4-5$) or to the different KO model. Together, these findings demonstrate in a physiological model that the α1-isoform alone is able to main-

Fig. 7. Expression of Ca$^{2+}$ handling proteins in the perinatal diaphragm of WT, α2-HET, and α2-KO mice at E18.5 and in the adult diaphragm of WT mice. Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) antibodies recognize both α and β splice variants. Microsomal membrane samples loaded per lane: SERCA1 and calsequestrin, 2 μg; SERCA2, 20 μg; all others, 40 μg. Calsequestrin was used as a loading control, and linearity of protein loading and detection were verified for calsequestrin by running immunoblots with multiple sample concentrations. Samples from the following tissues of WT mice were used as positive controls: adult brain for the Na$^+$-K$^{+}$-ATPase α-isoforms, adult diaphragm for SERCA1 and SERCA2, adult heart for phospholamban, Na$^+$/Ca$^{2+}$ exchanger (NCX), and plasma membrane Ca$^{2+}$-ATPase (PMCA) (data not shown).

SERCA2 is not expressed at significant levels in the perinatal diaphragm of WT mice (note increased loading to reach a detectable level), although it is readily detected in the adult, consistent with previous reports (8). There is a tendency for SERCA2 to increase in the α2-HET and to decrease in the α2-KO, but the changes do not reach statistical significance. PLB, a regulator of SERCA2, is also expressed at low levels in the perinatal diaphragm, and its expression in the α2-HET and α2-KO is comparable with WT. Thus both SERCA2 and its regulator are developmentally regulated, and there are no obvious compensatory changes in their expression in the α2-HET or α2-KO. Stimulation of SERCA2 activity by PLB is determined by the PLB-to-SERCA2 ratio and the phosphorylation state of PLB. Given the low SERCA2 expression in the perinatal diaphragm of all genotypes and the opposite change in SERCA2 expression in the KO and HET, it is unlikely that some combination of small changes in SERCA2 and PLB, acting together, could explain an almost twofold faster relaxation, even if all of the available SERCA2 is maximally stimulated. Both SERCA isoforms are also stimulated by Ca$^{2+}$ itself during the contraction cycle; however, the finding that maximum force is not changed implies that the global Ca$^{2+}$ change is comparable in all genotypes. Together, these results suggest that the faster relaxation rate does not result from an increase in SERCA number.

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tain equilibrium ion gradients and the resting potential of diaphragm muscle.

This conclusion does not exclude the possibility that the \(\alpha_1\)-isoform may also play a dynamic role(s) in active muscles, when the Na\(^+\) and K\(^+\) concentrations are expected to diverge from equilibrium. Indeed, our finding that a diaphragm with only \(\alpha_1\)-isoform can maintain action potentials and force under a variety of conditions demonstrates that \(\alpha_1\)-isoform makes an important contribution in active muscle. A role of both \(\alpha\)-isoforms in active muscles is also inferred from the finding that the rate of active Na\(^+\)-K\(^+\) transport increases up to 20-fold in contracting muscles (13, 27), a rate that requires utilization of up to 100% of the total \(\alpha\)-isoform transport capacity (7).

A related conclusion is that the \(\alpha_2\)-isoform contributes significantly less to the resting potential than expected from its proportional expression. Normally, the transport activity of the pump itself, which is electrogenic, makes a direct contribution of up to 16 mV to the resting potential (42, 49). The electrogenic potential adds to the diffusion potentials for K\(^+\) and Na\(^+\) (at constant Na\(^+\) and K\(^+\) concentrations) and is a direct measure of pump activity (2, 31). Our data show that the \(\alpha_2\)-isoform contributes up to 4 mV to the resting potential of embryonic diaphragm in the \(\alpha_2\)-KO and \(\alpha_2\)-HET, a muscle in which \(\alpha_2\)-isoform comprises >70% of total \(\alpha\)-subunit. Recently Krivoi et al. (32), using a range of ouabain concentrations to selectively inhibit \(\alpha_1\)- and \(\alpha_2\)-isoforms, found that \(\alpha_1\)- and \(\alpha_2\)-containing isozymes contribute 15 and 4.5 mV, respectively, to the resting potential of adult rat diaphragm. This close agreement from independent laboratories and models argues strongly that \(\alpha_2\)-isoform contributes significantly less at rest than expected from its pump number.

\(\alpha_2\)-Isoform influences spontaneous action potential activity in the perinatal diaphragm without impairing excitability to nerve input. The lower firing frequency of spontaneously active KO fibers reveals a developmental influence of the \(\alpha_2\)-isoform on electrical activity before birth. This role is apparently minor, because it does not compromise the ability of \(\alpha_2\)-KO fibers to respond to driven input and does not impair developmental progression to any obvious degree, on the basis of multiple indexes of muscle differentiation (39). The basis of the decreased excitability during spontaneous firing is not known. It could arise from a secondary effect of \(\alpha_2\)-isoform ablation on a Na\(^+\) or K\(^+\) channel or a resting ion channel that is needed for repetitive excitation. For example, an increased conductance between action potentials could slow depolarization to threshold and limit action potential frequency.

\(\alpha_2\)-Isoform modulates contractility in the diaphragm. The major phenotypic consequence of \(\alpha_2\)-isoform ablation is a dramatically faster rate of relaxation from tetanic contraction. This result is itself surprising, since it is not common to find a selective effect on relaxation without change in contractile activation or force. All indexes of contractile activation (rate, voltage dependence) and force (twitch and tetanic) are normal in KO, except that the force-frequency relationship is shifted to higher frequencies. A shift to higher frequencies is consistent with the faster relaxation of the KO muscle. This result excludes the possibility, for example, that the change in \(\alpha_2\)-isoform may have altered contractile protein content, which is expected to alter both activation and relaxation. The dramatic and selective effect on relaxation may suggest that Ca\(^{2+}\) clearance across the SR or the plasma membrane is enhanced when \(\alpha_2\)-isoform is reduced or absent. However, when this question was examined carefully, we found no change in Ca\(^{2+}\) handling proteins that can explain the result. SERCA is the major player in clearing the large, dynamic Ca\(^{2+}\) change during the contraction cycle, but there is no evidence for increased expression of SERCA or PLB, its principal modulator. The expression of other Ca\(^{2+}\) handling proteins does not change dramatically, except for NCX expression, which is reduced by about one-half in both HET and KO. Decreased NCX number is expected to promote Ca\(^{2+}\) accumulation rather than clearance. The reduction in NCX number is interesting because NCX number also changed in the heart of \(\alpha_2\)-KO mice (36) and in astrocytes of \(\alpha_2\)-HET and \(\alpha_2\)-KO mice (21), where decreased \(\alpha_2\)-isoform expression is associated with increased NCX number. The significance of these differences is not known but may suggest that control of Na\(^+-\)K\(^+-\)ATPase \(\alpha_2\)-isoform and NCX expression are somehow linked.

When comparing the perinatal diaphragm to other muscles with genetically altered \(\alpha_2\)-isoform expression, a common finding is that muscles with reduced or inhibited \(\alpha_2\)-isoform all show altered contractility, without change in bulk Na\(^+\) or K\(^+\) concentrations. This consistent and striking finding suggests that \(\alpha_2\)-isoform is able to influence dynamic Ca\(^{2+}\) signaling. It is possible that the \(\alpha_2\)-isoform may preferentially provide the Na\(^+\) gradient for Na\(^+\)/Ca\(^{2+}\) exchange in a submembrane domain where their combined activities can modulate Ca\(^{2+}\) signaling. This mechanism is proposed to explain the enhanced contractility of EDL, SOL, and heart muscles of \(\alpha_2\)-HET mice (22); the altered contractility of smooth muscles of \(\alpha_2\)-KO mice (51); and the altered Ca\(^{2+}\) signaling in \(\alpha_2\)-KO astrocytes, in which the Na\(^+-\)K\(^+-\)ATPase \(\alpha_2\)-isoform and the NCX colocalize near junctions with the endoplasmic reticulum (ER) (5, 21, 26, 29, 51).

Although the contribution of NCX to rapid clearance of global Ca\(^{2+}\) is small compared with SERCA, the reduction in NCX number may cause Ca\(^{2+}\) to accumulate locally near the triads, where it can influence Ca\(^{2+}\) signaling from the SR (10, 33). The Na\(^+-\)K\(^+-\)ATPase \(\alpha_2\)-isoform and the NCX are both present in the tubules and triads (47), and there is evidence in heart and skeletal muscle for a microenvironment near the Na\(^+-\)K\(^+-\)ATPase, where the local Na\(^+\) concentration differs from cytosolic levels (50). Up to 85% of the tubule area in skeletal muscle is associated with the SR across a junctional gap of only 12–14 nm (15, 17), where the local Ca\(^{2+}\) in the cleft may reach hundreds of micromolar (46). Thus, although the reduction in \(\alpha_2\)-isoform leads to opposite changes in NCX expression in heart and skeletal muscles, the consequence of this on the junctional Ca\(^{2+}\) change may be an increase in both muscles. This is because NCX can operate in reverse mode during cardiac contraction to supply trigger Ca\(^{2+}\), whereas in skeletal muscle it operates in forward mode to clear Ca\(^{2+}\) (4).

Multiple signaling and transport systems that can influence the junctional Ca\(^{2+}\) concentration are localized at the triad junction, including the Na\(^+-\)K\(^+-\)ATPase \(\alpha_2\)-subunit, the PMCA, the dihydropyridine receptor, and the ryanodine receptor (RyR). The overall consequences of reduced \(\alpha_2\)-isoform and altered NCX expression on Ca\(^{2+}\) signaling depend on complex interactions between the local Ca\(^{2+}\) change, Ca\(^{2+}\) release by the RyR, Ca\(^{2+}\) stores within the SR/ER, and Ca\(^{2+}\)-dependent filling of the SR/ER stores, which are not well...
understood. It is possible that an increased junctional Ca\(^{2+}\) change may shorten relaxation by enhancing Ca\(^{2+}\)-dependent inactivation of RyR1. To uncover the mechanism of the modul-
ination of contractility in skeletal muscle by the Na\(^{+}\)-K\(^{-}\)-ATPase \(\alpha_2\)-isoform, an important direction for future expe-
iments will be to determine the activity-related Ca\(^{2+}\) changes, both local and global, and the alterations in NCX function that occur in muscles with altered \(\alpha_2\)-isoform expression.

It is noteworthy that the inherent fatigue resistance of the perinatal diaphragm is not compromised in \(\alpha_2\)-KO fibers. This is consistent with the finding that \(\alpha_2\)-KO fibers fire repetitive action potentials without change in baseline or afterpotential (Fig. 3D) and indicates that they are able to maintain excit-
ability despite the activity-dependent increase in extracellular K\(^{-}\)s, including K\(^{-}\) in the t tubule lumen. This result is puzzling because removal of \(\alpha_2\)-isoform is expected to leave the t
ubes without a functional enzyme, leading to depolarization. It is possible that \(\alpha_1\)-subunit targeting is altered in the \(\alpha_2\)-KO or that the \(\alpha_1\)-subunit distributes over both surface and non-
junctional t tubule membranes. Studies of \(\alpha_1\)-isoform localiza-
tion in WT muscles do not completely exclude the latter possibility, given the small area of nonjunctional membrane (10–15% of t tubule area in adult fast muscles). Moreover, the perinatal diaphragm, a continuously active and highly fatigue-
resistant muscle, is inherently less dependent on active trans-
port to clear dynamic K\(^{-}\) loads in the tubules, because the small fiber size (7- to 10-\(\mu\)m radius, compared with >50 \(\mu\)m in adult) allows for efficient clearance by diffusion alone (1). In addition, its more depolarized resting potential is protective against dynamic changes in \(E_K\) that occur as extracellular K\(^{+}\) rises during repetitive action potential activity. The large dif-
ference between the resting potential and \(E_K\) is the major factor over which an increase in K\(^{+}\) or Cl\(^{-}\) permeability can hyperpolarize the membrane passively (at least up to the limit set by \(E_K\)). Before the concentration gradients and resting \(E_K\) are fully restored by active transport.

Finally, the finding that contractility is altered in the \(\alpha_2\)-HET and \(\alpha_2\)-KO diaphragms addresses underlying questions. Why does the diaphragm express two \(\alpha\)-isoforms? Or, in the absence of \(\alpha_2\)-isoform, can adding more \(\alpha_1\)-isoform pump units restore normal muscle function? Our results show that an increase in \(\alpha_1\)-isoform number alone, up to double that in WT diaphragm, cannot compensate for the lack of \(\alpha_2\)-isoform. Despite their near-normal resting potentials, excitability, and contrac-
tile activation, the \(\alpha_2\)-HET and \(\alpha_2\)-KO diaphragms do not relax normally from contraction.

Perspectives. The distinct expression pattern and subcell-
ular localization of the Na\(^+\)-K\(^{-}\)-ATPase \(\alpha\)-subunit iso-
forms in skeletal muscle suggest that they play distinct cellular roles. Results of this study and previous attempts to uncover their roles using mice with genetically altered \(\alpha_2\)-isoform content can be summarized as follows. 1) Reduction or ablation of \(\alpha_2\)-isoform, the major \(\alpha\)-isoform of differentiated muscle, has only a minor effect on key equi-
librium parameters controlled by the Na\(^+\)-K\(^{-}\)-ATPase (the resting potential and the resting Na\(^{+}\) and K\(^{+}\) gradients). This demonstrates that the Na\(^+\)-K\(^{-}\)-ATPase \(\alpha_1\)-subunit alone plays a large role in maintaining the equilibrium K\(^{+}\) and Na\(^{+}\) gradients, as widely proposed. This conclusion does not exclude additional roles for \(\alpha_1\)-isoform in active muscle. 2) The electrogenic activity of \(\alpha_2\)-isoform contrib-
utes up to 5 mV to the resting potential of skeletal muscle. This is a consistent finding in perinatal and adult skeletal muscles from two independent transgenic models and in the diaphragm of adult rats with pharmacologically inhibited \(\alpha_2\)-isoform activity. This small contribution is strikingly disproportionate to its abundance in skeletal muscles and indicates that the \(\alpha_2\)-isoform contributes less under basal conditions than expected from its pump number. 3) Skeletal muscles with reduced \(\alpha_2\)-isoform content show altered contractility (enhanced force in adult EDL or enhanced relax-
ation in the perinatal diaphragm). This suggests that \(\alpha_2\-
isoform can influence intracellular Ca\(^{2+}\) signaling. The parallel finding that contractility is altered without change in the global Na\(^{+}\) or K\(^{-}\) gradients suggests that this modula-
tion may occur in a microdomain, as proposed for astrocytes and other muscle types. 4) The cellular consequences of genetically reduced \(\alpha_2\)-isoform content (altered contractility and/or altered excitability in development) occur in active muscles. Together with the lack of a major effect of the \(\alpha_2\)-isoform on the resting potential or resting gradients, this suggests that the \(\alpha_2\)-isoform may make a greater contribu-
tion during muscle activity at a step after membrane exci-
tation. A challenge for future research will be to identify the factor(s) that stimulates the activity of each \(\alpha\)-isoform during muscle contraction or other dynamic conditions of altered demand.

REFERENCES


35. Thompson CB, Dorup I, Ahn J, Leong PK, and McDonough AA. Glucocorticoids increase sodium pump α2- and β1-subunit abundance and


