Inhibition of stretch-activated channels during eccentric muscle contraction attenuates p70$^{S6K}$ activation.

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Abstract

Eccentric contractions (EC) are known to result in muscle hypertrophy, potentially through activation of the Akt-mTOR-p70S6k signaling pathway. Previous work has also demonstrated that EC result in the opening of stretch-activated channels (SAC) and inhibition of these channels resulted in an attenuation of the EC to induce muscle hypertrophy. The purpose of this study was to test the hypothesis that a known intracellular pathway directly associated with muscle hypertrophy is coupled to the opening of stretch activated ion channels (SAC). Specifically we measured the activation of the Akt, GSK-3β, p70S6k, and ribosomal protein S6 following a single bout of EC in the rat tibialis anterior (TA) muscle. The TA muscles preformed four sets of six repetitions of eccentric contractions. In-vivo blockade of SAC was performed by a continuous oral treatment with streptomycin in the drinking water (4g/l) or by i.v. infusion of Gadolinium (Gd3+) 80µmol/kg. EC increased the degree of Akt and p70S6k phosphorylation in the TA muscle, while in animals where SAC had been inhibited there was a reduced capacity for EC to induce Akt or p70S6k phosphorylation. Accompanying this reduced activation of Akt and p70S6k, was a failure to phosphorylate GSK-3β or S6 when SAC were inhibited. The results from these data indicate the necessity of functional SAC for the complete activation of Akt and p70S6k pathway in response to EC.
Introduction

Changes in contractile activation of skeletal muscle can induce specific changes in skeletal muscle gene expression and also result in changes in protein metabolism. In recent years, a multitude of studies have identified that muscle contraction can activate molecular signaling molecules that regulate important genomic and metabolic events in muscle (2, 7). Although, it appears well accepted that exercise or muscle contraction does in fact activate these mechanisms, the upstream mechanisms that induce the activation of these signaling proteins still remains undefined.

The initiation of protein synthesis is a key contributor to exercise-induced muscle hypertrophy (24, 25). Both eccentric and concentric contractions have been shown to induce protein synthesis, however eccentric muscle contractions (EC) appear to be more effective at enhancing protein synthesis (24, 25). Recent literature has suggested that the Akt-mTOR-p70\textsuperscript{s6k} signaling pathway is a major regulator in the initiation of protein synthesis in skeletal muscle (13). In fact, Bodine et al. (4) found that overexpression of Akt in muscle increased individual muscle fiber size, while Ohanna et al. (22) found that genetic removal of the p70\textsuperscript{s6k} gene resulted in smaller skeletal muscle mass. Baar and Esser (3) first identified that activation p70\textsuperscript{s6k} may be an important contributor to muscle growth after EC. These data have been confirmed now in other models of muscle growth (6). Although, these data have begun to elucidate the mechanisms that regulate muscle growth, the manner by which muscle contraction induces the activation of Akt or p70\textsuperscript{s6k} still remains undefined.

Stretch-activated channels (SAC) were initially described in skeletal muscle by Franco and Lansman (10, 11). Specifically, the channels appear to be permeable to both
Na⁺ and Ca²⁺ (10, 11) and blocked by both Gd³⁺ and streptomycin (12). Previously, McBride et al. (20) found that inhibition of SAC prevented prolonged membrane depolarization associated with eccentric contractions, and blocking SAC following EC restored the RMP toward control values. The inhibition of the SAC also attenuated muscle hypertrophy induced by eccentric contractions (EC) (19). Further, it has also recently been suggested by Yeung et al. (27, 30) that inhibition of SAC can attenuate muscle damage in mouse models of Duchenne’s muscular dystrophy by inhibiting Ca²⁺ entry through SAC. Although, it appears that SAC play an important role in contraction-induced muscle growth, the mechanism by which SAC may induce muscle growth remains undefined.

Here we sought to determine if EC induced-activation of SAC contributed to the activation of signaling mechanisms previously shown to stimulate muscle growth. Specifically, we attempted to determine if pharmacological inhibition of SAC during ECs altered contraction-induced activation of the Akt-p70s6k signaling pathway.
Methods

Animals. Female Sprague-Dawley rats, 3 mo of age, with body weights ranging from 240 to 260 g, were used. All animal care and use protocols were approved by the Institutional Animal Care and Use Committee of California State University, Bakersfield, and were consistent with National Institutes of Health guidelines. Animals were housed in a temperature-controlled room (19-21°C) with a 12:12-h light-dark cycle. Rats were provided unlimited access to standard rat chow and water.

Eccentric muscle contraction: Animals were anesthetized (60 mg/kg ketamine and 12 mg/kg Rompum) and subsequently performed eccentric contractions (EC) on a pulley device similar to the one described by Wong and Booth (26). The rat was placed in the prone position on the supporting platform of a pulley apparatus designed to stabilize the leg and allow full ankle rotation. The hind foot was attached directly to a plate connected to the lever arm of the pulley system, and the ankle was stabilized with the foot at 90° with respect to the lower leg (neutral position). During the exercise protocol 75 grams of weight was added to the pulley device providing some added resistance for the concentrically contracting posterior plantar flexor muscles of the distal hind limb. The 75 grams does not substantially slow down the rate of contraction, or limit the range of motion of either the plantar flexor or dorsiflexor muscle groups. Great care was taken to ensure that the knee, ankle and foot remained in alignment during the movement of the foot lever, and that plantar flexion occurred through the full range of motion. Two monopolar stainless steel needle electrodes were inserted percutaneously near the sciatic notch to stimulate the sciatic nerve. Stimulation of the sciatic nerve above the branch point of the tibial and peroneal nerves caused the plantarflexors (triceps surae) to contract.
concentrically, resulting in stretching of the dorsiflexors, which were simultaneously activated. The dorsiflexors thus contracted eccentrically and lengthened, in opposition to the stronger ankle extensors. Stimulation consisted of 100-Hz stimulus trains with a 1ms stimulus duration and a train duration of 2.5 s (25). The exercise paradigm consisted of four sets of six repetitions with a 20-s rest between repetitions and a 5-min rest between sets. Repeated bouts of this contraction paradigm are known to result in muscle hypertrophy (3, 19). During each procedure, only the right leg was stimulated to produce EC of the tibialis anterior (TA) muscle, and concentric contractions of the soleus muscle. The left leg served as a non-exercised contralateral control. When training of a single limb with this exercise model over a 16 week period, Wong and Booth (26), did not measure a difference in the muscle wet weights between the contralateral control muscles from trained rats and those of age matched sedentary controls. It would therefore appear that the contralateral control muscles do not receive a systemic stimulus for hypertrophy that would complicate our results. Muscles that were used for protein measurements and western blot analysis were harvested from the anesthetized rats 2 h postexercise. The RMP remains depolarized at the 2 hour time point when the muscles were harvested for molecular analysis. We have measured a significant depolarization out to 24 hours post EC with this exercise paradigm (20). The muscles were weighed and immediately freeze clamped in metal tongs cooled in liquid N2. Frozen muscles were stored at -80° C until processed for protein extraction.

Inhibition of stretch activated channels. Prior to subjecting animals to a single acute bout of EC, or measuring contractile function they were treated with either streptomycin in their drinking water (n = 6), or they received gadolinium (Gd3+) by i.v. infusion (n = 18).
Animals were treated with streptomycin in their drinking water (4 g/l) to provide continuous in-vivo blockade of SAC. The streptomycin and Gd\(^{3+}\) treatments or doses are based on RMP work that was originally done in-vitro following either a single or multiple bouts of EC (19, 20). Treatment was initiated 6 days before the exposure to EC, and continued through the completion of the experiments (19, 20). Three separate groups of animals were treated with Gd\(^{3+}\). 80µmol/kg Gd\(^{3+}\) was dissolved in normal saline and delivered to the animal by a 2 ml i.v. infusion via the jugular vein. Animals were anesthetized (60 mg/kg ketamine and 12 mg/kg Rompum) prior to the infusion and a small incision was made to expose the jugular vein. A saline filled catheter was introduced into the jugular vein and secured in place. Blood was pulled back into the catheter to ensure an open line and Gd\(^{3+}\) was infused over 4 minutes followed by a saline flush. Following the Gd\(^{3+}\) infusion the catheter remained in place during the remainder of the experiments until the completion of either the contractile measurements (n=6), RMP measurements (n=6), or the removal of muscle tissue for analysis 2 hours following the EC (n=6). The animals remained anesthetized until the completion of all procedures. There was a 0.5 hour delay between the end of the infusion and the initiation of the EC protocol or the initiation of the contractile measurements. Confirmation of SAC blockade for both streptomycin and Gd\(^{3+}\) was determined by measurement of RMP following EC, and compared to non-treated exercised muscles (Table 1).

**Muscle contractile function following Gd\(^{3+}\) i.v. treatment.** The animals were anesthetized (ketamine 60 mg/kg and Rompum 12 mg/kg), and the Gd\(^{3+}\) was delivered via jugular vein as described above. The rat was placed on a warming pad to maintain body temperature. Prior to measuring contractile function each TA muscle was exposed
separately and the distal tendon of the TA was isolated and attached to a force transducer (Grass-FT-03) with silk suture (2-0). The leg was fixed in place at the knee and ankle, with the force transducer attached to a micromanipulator. Each TA was stimulated directly by a platinum plate electrode at supramaximal voltage with approximately 0.1-0.3 ms duration set to optimal length (Lₒ). Maximum isometric twitch tension (Pₜ), time to peak twitch tension (Tₚ), twitch half relaxation time (T₁/₂R), rate of twitch relaxation (dR/dT), isometric tension at 100Hz, maximum isometric tetanic tension (Pₒ), and the maximum rate of force development during a tetanus at 330 Hz (dPₒ/dT) were recorded at 35°C ± 0.5°C. Output voltages from the force transducer were amplified and recorded on an analog to digital acquisition system (Powerlab, ADInstruments). Muscle temperature was monitored, and maintained at 35 °C ± 0.5°C by radiant heat. With the catheter in place all contractile measurements were first recorded from the right (control) TA with no additional treatment. Following completion of the control measurements the Gd³⁺ was administered as described above. Contractile measurements were then performed on the left (treated) TA muscle following a 0.5 hour delay between the end of the infusion and the initiation of contractile measurements.

Electrophysiology. Resting membrane potentials (RMPs) were obtained from both control and exercised tibialis anterior (TA) muscles in situ from a separate group of rats from those used for the western blot analysis. The rats remained anesthetized following EC with the recordings taking place within 1-2 hours post-exercise. Recordings were obtained using standard glass microelectrode techniques (20). Electrodes were filled with 3M KCl and had tip resistances of approximately 20-30 MΩ. A Grass platinum reference electrode was placed in the proximal end of the TA. Animals were placed on a thermal
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pad to maintain body temperature (37°C-39°C). The rat and pad were arranged on a metal frame. The hind limb was stabilized by clamping the leg at the knee and ankle, using metal clamps attached to the frame. Control and exercised TA muscles were exposed and cleared of the outer layers of connective tissue. The muscles were incubated with 50 µL of type IV collagenase 12 mg/ml (Sigma) for 20 minutes prior to making recordings. Intracellular recordings were obtained from exercised TA and the contralateral control TA directly following EC and the collagenase treatment. A minimum of 25 fibers were sampled in each muscle.

*Muscle protein-extraction and concentration measurements:* The muscle tissue was homogenized on ice in buffer containing 50 mM Hepes (pH 7.4), 0.1% Triton –X100, 4mM EGTA, 10mM EDTA, 15 mM Na₄P₂O₇•H₂O, 100 mM β-glycerophosphate, 25 mM NaF, 50µg/ml leupeptin, 50 µg/ml pepstatin, 40 µg/ml aprotinin, 5mM Na₃VO₄, and 1 mM PMSF. After homogenization, the samples were stored at -80°C. The protein concentration of the samples was determined in triplicate via the Bradford procedure (Bio-Rad Protein Assay, Hercules, CA).

*SDS-PAGE, western blotting, and immunodetection:* Homogenates of the muscle were solubilized in loading buffer (2.5 mM Tris•HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.025% bromophenol blue) and boiled at 98°C for 5 min as previously described (21). 50-100 µg of total protein was then loaded (µg/sample/lane) onto 10% SDS-PAGE gels. All gels were run at 150 V for 1 hour to separate proteins. The gels were then transferred onto PVDF membranes (Millipore, Billerica, MA) at 50 V for 1 hr at 4°C in transfer buffer (25 mM Tris-base, 192 mM glycine, and 20% methanol). To confirm successful transfer of protein and equal loading of lanes the membranes were
stained with Ponceau S (data not shown). After successful transfer, the membrane was placed in blocking buffer (5% non-fat dry milk in TBS-T (0.1% Tween-20) for 1 hr at room temperature, serially washed (3 x 5 min) and incubated with primary antibody in dilution buffer (5% BSA in TBS-T) overnight at 4°C. After another serial wash with TBS-T (3 x 5 min), the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr followed by another serial wash with TBS-T (3 x 5 min). Enhanced chemiluminescence reagent (Pierce, Rockford, Ill.) was used to detect the HRP activity by exposure to Kodak-XAR5 autoradiographic film for the appropriate durations to keep the integrated optical densities (IOD) within a linear and non-saturated range for all bands of each membrane. The IODs were quantified using ImageQuant densitometry software (Molecular Dynamics, Sunnyvale, CA).

**Antibodies:** The primary antibodies phospho Ser\(^{473}\)-AKT (1:1000 dilution), AKT (1:1000), phospho Ser\(^{9}\)-Glycogen Synthase Kinase-3β (GSK-3β) (1:1000), GSK-3β (1:2500), phospho Thr\(^{389}\)-p70S6 Kinase (p70\(^{S6K}\)) (1:500), p70\(^{S6K}\) (1:500), phospho Ser\(^{235/236}\)-S6 (pS6-235/236) (1:1000), and phospho Ser\(^{240/244}\)-S6 (pS6-240/244) were purchased through Cell Signaling Technologies, Inc. (Beverly, MA). Anti-rabbit and anti-mouse secondary antibodies (1:2000) were purchased from Cell Signaling Technologies, Inc. (Beverly, MA). Antibody specificity was verified by molecular weight, positive controls (where possible) and lack of secondary antibody signal in the absence of the primary antibody.

**Statistics:** All data are expressed as means ± SE. For immunoblotting procedures statistical significance was determined using a two-way analysis of variance for multiple comparisons followed by a Holm-Sidak post hoc test. For all contractile and RMP
measures statistical significance was determined using a one-way analysis of various for multiple comparisons followed by a Tukey’s post hoc test. A P value of <0.05 was considered significant.

Results

To verify that use of streptomycin and Gd\(^{3+}\) were effective methods for inhibiting SAC, RMP were determined in TA muscles that performed a single bout of EC and compared to contralateral non-exercised muscles. These measurements were compared to RMP values previously recorded in exercised and control muscles of non-treated rats (20). Non-treated TA muscles that underwent EC demonstrated significant depolarization of the RMP (-70.8 ± 0.73 mV) compared with contralateral control muscles (-83.2 ± 0.73 mV) (Table 1). Streptomycin treatment prevented a similar reduction in resting membrane potential of the TA muscle after completion of the EC (-79.2 ± 0.52 mV) when compared to the RMP measured in TA muscle that underwent EC in the non-treated animals. However, the addition of streptomycin did not completely prevent the EC-induced reduction in RMP when compared to the non-exercised contralateral control leg in the streptomycin treated group (-81.5 ± 0.41 mV). Animals acutely treated with Gd\(^{3+}\) in-vivo by i.v. infusion also demonstrated preservation of the resting membrane potential of the TA muscle after completion of EC (-77.6 ± 0.56 mV) when compared to exercised muscles in non-treated animals. However, the addition of Gd\(^{3+}\) did not completely prevent the EC-induced reduction in RMP when compared to the non-exercise contralateral control leg (-82.3 ± 0.27 mV). It should also be noted that streptomycin and Gd\(^{3+}\) treatment did not alter the RMP of non-exercised contralateral
control muscles when compared to non-exercised muscles of the non-treated group (Table 1).

Previously, it has been demonstrated that streptomycin treatment used in the same manner as here did not affect muscle force production (19, 20). We have extended these findings to show that the Gd$^{3+}$ treatment also did not result in any alteration in force production as measured by the maximum twitch (Pt), 100 Hz stimulus, or the peak tetanic tension (Po) (Table 2).

Phosphorylation of Akt was measured through immunoblotting procedures to determine if inhibition of SAC prevented EC-induced activation of Akt. In the non-treated animals, a small significant increase (24%) in Akt phosphorylation was detected after one bout of EC when compared to the contralateral control TA muscle (Figure 1A). However, in the streptomycin treated animals, no significant differences were detected in Akt phosphorylation levels between the TA muscle that underwent EC and the contralateral control. No changes were detected in the total amount of Akt expressed in the TA muscle between any groups.

Serine residue 9 on glycogen synthase kinase-3β (GSK-3β) is a known substrate of Akt (15). Inhibition of GSK-3β occurs through phosphorylation of serine residue 9 and this inhibition is associated with muscle growth (23). Here, we find that phosphorylation of GSK-3β, specifically on serine 9 is significantly increased by 15% with EC when compared to the contralateral control leg (Figure 2A). Interestingly, EC also significantly increased the total content of GSK-3β by 17% when compared to the contralateral control leg. No significant increases were detected in GSK-3β phosphorylation in the streptomycin treated animals after EC, however there were still
significant increases in total content of GSK-3β in the streptomycin treated animals after EC. Since, total expression of GSK-3β increased after the bout of EC we also quantified the ratio of the phosphorylated form versus the non-phosphorylated form and found that no statistical difference was apparent in the control versus streptomycin treated group (data not shown).

Phosphorylation of p70S6k was measured using immunoblotting techniques to determine if inhibition of SAC altered EC-induced activation of p70S6k. EC significantly increased the phosphorylation levels by 93% of p70S6k when compared to the contralateral control TA muscle (Figure 3A). Animals treated with streptomycin retained the ability to increase p70S6k phosphorylation after EC when compared to the contralateral control leg, however this phosphorylation was significantly reduced by 23% when compared to the non-treated EC value. No significant differences were detected in the total amount of p70S6k. To confirm these data, experiments were performed using an alternative SAC inhibitor, Gd³⁺. Here, again we demonstrate a large increase in p70S6k phosphorylation in response to EC when compared to the contralateral control TA muscle. However, when the animals were acutely treated with Gd³⁺, there was a significant 37% reduction in the phosphorylation level of p70S6k in response to EC when compared to non-treated exercised TA muscle (Figure 4A). Although, it should be noted that there was still significant, albeit reduced, phosphorylation of p70S6k in response to EC when compared to the contralateral control TA. No differences in total content of p70S6k were detected in muscles of Gd³⁺ treated rats.

Phosphorylation of serine residues 235/236 and 240/244 on ribosomal protein S6 are major substrates for p70S6k (18). To determine if the reduction in p70S6k
phosphorylation in response to SAC inhibition resulted in reduced p70\textsuperscript{s6k} activity, the phosphorylation status of S6 was measured at serine residues 235/236 and 240/244. Phosphorylation of S6 at serine residues 235/236 was significantly enhanced by 111% in response to EC when compared to the contralateral control TA (Figure 5A). Neither streptomycin nor Gd\textsuperscript{3+} had any effect on the phosphorylation status of residue 235/236 on S6 after EC. With respect to serine residues 240/244, EC significantly enhanced S6 phosphorylation levels by 170% when compared to the contralateral control TA (Figure 5C). However, unlike residues 235/236, streptomycin and Gd\textsuperscript{3+} treatment resulted in significant reductions in the phosphorylation level of residues 240/244 by 30% and 14% when compared to the exercised non-treated TA muscle, respectively. No changes in total S6 expression were detected after Gd\textsuperscript{3+} treatment when compared to the control muscles that underwent the EC, however there were minor significant increases in total S6 expression after the EC in all groups except for the streptomycin treated group (data not shown).

**Discussion**

Here we demonstrate for the first time a potential link between the activation of SAC during EC and downstream intercellular signaling events that are thought to contribute to mechanisms mediating exercise-induced muscle hypertrophy. Our data indicate that repetitive EC reduced the RMP through the activation of SAC, and this was associated with activation of the Akt and p70\textsuperscript{s6k} proteins. When the SAC were inhibited with chronic in situ streptomycin treatment, there was an attenuation of the ability of the
EC to activate Akt and p70s6k. These data were further confirmed by using an acute delivery of Gd$^{3+}$ to inhibit SAC where we again found an attenuation of EC to activate p70s6k. These data would suggest for the first time that in skeletal muscle SAC that are activated during EC may in part stimulate kinase activity of Akt and p70s6k.

Mechano-signal transduction has been suggested for a number of years to contribute to exercise-induced muscle growth (5). The transmission of tension across the cytoskeleton structure of the muscle has the ability to influence changes in gene expression and activation of various signaling mechanisms (5). Previous studies have suggested that the tension applied to the extracellular matrix may be sensed through focal adhesion complexes associated with the muscle cell membrane, thus allowing transmission of the mechanical signal from the external environment to specific internal organelles in the muscle cell (8, 14). Another possible contributor to mechano-signal transduction is through the activation of SAC, which are thought to be expressed in striated muscle (10-12, 19, 20, 27-29). SAC were first described in cultured skeletal muscle cells as mechanosensitive ion channels, which increase their open probability in response to mechanical stress (11). Investigators have suggested that SAC “act as membrane-embedded mechanoelectrical switches” (27, 30) that are critical for a number of cellular processes, such as volume regulation, electrolyte homeostasis and signal transduction (27, 30). SAC can allow passage of various ions, including Ca$^{2+}$, Na$^+$ and K$^+$, whereas others classes of mechanosensitive channels are selectively permeable to K$^+$ or Cl$^-$ (27, 30). SAC described in skeletal muscle appear to be permeable to Ca$^{2+}$ and Na$^+$ ions, and this action is inhibited by both streptomycin and Gd$^{3+}$ (30).
Here we demonstrate, using markedly different pharmacological agents to inhibit SAC, that activation of these channels during EC, is critical for the complete phosphorylation of Akt and p70\(^{S6k}\). The data obtained in this study indicate that SAC contribute to the phosphorylation of Akt and p70\(^{S6k}\), but are not solely responsible for the activation of these proteins. This was apparent in that neither streptomycin nor Gd\(^{3+}\) prevented Akt or p70\(^{S6k}\) phosphorylation, indicating that either SAC blockade was incomplete, or some other factor may be contributing to the activation of these signaling proteins. Our results disagree with recent data suggesting that C2C12 myotubes subjected to multi-axial stretch in the presence of 500\(\mu\)M Gd\(^{3+}\) were able to fully activate p70\(^{S6k}\) (17). There are a few possible explanations for this discrepancy. First, C2C12 myotubes exist in a ‘developmental’ state and therefore may not totally mimic the response adult muscle undergoes during EC. Second, although C2C12 do contain active forms of SAC (9), it is possible that forms expressed by C2C12 myotubes are not the same as adult muscle and therefore C2C12 myotubes may not contain the mechanisms necessary to phosphorylate Akt or p70\(^{S6k}\) through SAC. Although, the data do not agree, the explanation for the difference appears to lie in vastly different models chosen to examine the role of SAC during contraction.

Our data imply that SAC contribute to activation of various signaling proteins that are critical for muscle growth induced by lengthening contractions. Using two markedly different treatment strategies to inhibit SAC, we were able to determine that prevention of the normal change in RMP following EC results in a failure to fully activate Akt and p70\(^{S6k}\). Streptomycin, an aminoglycoside, has been shown to inhibit SAC at concentrations of 50-200\(\mu\)M in cultured cell studies (11). Here, we delivered
streptomycin to animals for 6 days through the drinking water to allow for a chronic inhibition of the SAC. This chronic inhibition of SAC resulted in a failure to fully phosphorylate Akt or p70s6k after EC. Streptomycin has a number of non-specific effects that could affect the interpretation of our results (30). For example, streptomycin has been shown to cause read through on various stop codons on mRNA (27). Therefore, an alternative SAC inhibitor was employed to confirm the data achieved with the streptomycin treatment. Using an acute i.v. infusion of Gd3+ to inhibit SAC before the exercise bout, we again found a failure of EC to fully activate Akt or p70s6k. It should be noted that Gd3+ has other side effects that include inhibition of Cl− channels (27), so although these non-specific effects could affect our interpretation the data still agree with our findings from the streptomycin experiments suggesting that SAC are important to signal transduction during EC. Therefore, it appears it did not matter whether the SAC were acutely or chronically inhibited with respect to the role EC played in phosphorylation of Akt or p70s6k. With the complete agreement of our streptomycin and Gd3+ data, we believe that inhibition of Akt or p70s6k is the result of inhibition of SAC and not a non-specific side effect of the treatment. Although, no significant differences in force production resulted from the pharmacological inhibition of the SAC, there were minor changes in the force production, which may have resulted in changes in phosphorylation of p70s6k or the its downstream substrates. Unfortunately, it is impossible to predict if these minor changes in force production altered p70s6k phosphorylation. When the gene sequence of the SAC is identified it allow investigators a better opportunity to analyze these questions in greater depth.
Although, we found only minor changes in Akt or GSK-3β phosphorylation with muscle contraction, it should be noted that there were surprisingly consistent increases in the native expression of GSK-3β. This is of particular interest considering that this increase occurred only two hours after the exercise bout. These data suggest that GSK-3β expression in muscle is very sensitive to increased muscle loading and may alter the physiological mechanisms of this signaling protein. Previous publications have found that GSK-3β is sensitive to increased mechanical load (6), but this the first publication to demonstrate an increase in GSK-3β expression so quickly after a single bout of exercise. The sensitivity of the native form of GSK-3β to mechanical load warrants further exploration.

At this time, we have no clear understanding as to how the SAC may be contributing to the activation of Akt or p70^66k. It is possible that the increased conductance of a particular ion is activating a mechanism necessary to fully phosphorylate these signaling proteins. Yeung et al. (30) have recently demonstrated in muscles taken from mdx mice that there was increased Na^+ and Ca^{2+} flux through SAC after contraction. In addition, this same group found that flux of both of these ions could be inhibited by streptomycin and Gd^{3+} (30). Although we have not identified a mechanism for how these ions may affect the activation levels of Akt or p70^66k, there are examples of possible links for this mechanism in other tissues. For example, it has been suggested that p70^66k activation requires a sequence of conformational changes and phosphorylation reactions, that include a specific initial priming step for complete activation (16). This priming process is dependent on calcium and failure of this priming step to occur results in a global reduction of S6K1 phosphorylation (16). Therefore, it is
entirely possible that ion passage through SAC is the potential priming step in skeletal muscle thereby contributing to the complete activation of Akt or p70s6k during EC.

Skeletal muscle hypertrophy can also result from non-eccentric types of contraction. For example, Adams and Haddad (1) recently demonstrated that both concentric and isometric contractions delivered similar levels of muscle hypertrophy when compared to eccentric contractions. Unfortunately, in skeletal muscle SAC activity appears only to occur during lengthening or stretch-induced contractions (20, 27). This would suggest that the mechanism described here is specific to eccentric or lengthening types of contractions. Thus, at this time it is only possible to conclude that SAC are contributing to signaling mechanisms after eccentric or lengthening muscle contractions.

In conclusion, this study has identified that SAC are an important aspect to EC-induced signaling protein activation. The data suggest that contribution of these SAC is necessary but not sufficient for full exercise-induced activation of Akt or p70s6k. Finally, the data suggest that further studies of these channels in exercise-induced muscle growth is warranted.
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Figure Legends

**Figure 1A-B.** Akt activation in the TA muscle after exposure to eccentric contraction.  
A. Quantification of phosphorylation levels of Akt (Ser-473) and total Akt content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days prior to their exposure to the EC. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *P < 0.05 compared with non-treated control.  
B. Example blots of typical phosphorylation levels of Akt (Ser-473) and total Akt content in the TA muscle after a bout of EC.

**Figure 2 (A-B).** GSK-3β inactivation in the TA muscle after exposure to eccentric contraction.  
A. Quantification of phosphorylation levels of GSK-3β (Ser-9) and total GSK-3β content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days prior to their exposure to the EC. The dark bars refer to the phosphorylated form of the protein, while gray bars refer to native levels of the protein. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *P < 0.05 compared with non-treated-control.  
#P < 0.05 compared with streptomycin treated contralateral control.  
B. Example blots of typical phosphorylation levels of GSK-3β (Ser-10) and total GSK-3β content in the TA muscle after a bout of EC.

**Figure 3 (A-B).** p70^65k activation in the TA muscle after exposure to eccentric contraction.  
A. Quantification of phosphorylation levels of p70^65k (Thr-389) and total p70^65k content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was
accomplished through streptomycin treatment for 6 days prior to their exposure to the EC. The dark bars refer to the phosphorylated form of the protein, while gray bars refer to native levels of the protein. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *$P$ < 0.05 compared with nontreated-control, #$P$ < 0.05 compared with streptomycin treated contralateral control, $\$P$ < 0.05 compared with non-treated Ex group. B. Example blots of typical phosphorylation levels of p70^S6K (Thr-389) and total p70^S6K content in the TA muscle after a bout of EC.

Figure 4 (A-B). p70^S6K activation in the TA muscle after exposure to eccentric contraction. A. Quantification of phosphorylation levels of p70^S6K (Thr-389) and total p70^S6K content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through Gd^{3+} infusion 0.5 hours prior to their exposure to the EC. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *$P$ < 0.05 compared with nontreated-control, #$P$ < 0.05 compared with streptomycin treated contralateral control, $\$P$ < 0.05 compared with non-treated Ex group. B. Example blots of typical phosphorylation levels of p70^S6K (Thr-389) and total p70^S6K content in the TA muscle after a bout of EC.

Figure 5 (A-D). Ribosomal S6 phosphorylation in the TA muscle after exposure to eccentric contraction. A. Quantification of phosphorylation levels of S6 (Ser 235/236) in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days prior to their exposure to the EC or Gd^{3+} infusion 0.5 hrs hours prior to their exposure to the EC. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *$P$ < 0.05 compared with nontreated-control, #$P$ < 0.05 compared with streptomycin treated contralateral control,
@ P < 0.05 compared with Gd\textsuperscript{3+} treated contralateral control. B. Example blots of typical phosphorylation levels of S6 (Ser-235/236) in the TA muscle after a bout of EC.

C. Quantification of phosphorylation levels of S6 (Ser 240/244) in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days prior to their exposure to the EC or Gd\textsuperscript{3+} infusion 0.5 hours prior to their exposure to the EC. The mean value represents measurements from 6 muscles in all groups. Values are means ± SE. *P < 0.05 compared with nontreated-control, #P < 0.05 compared with streptomycin treated contralateral control, @ P < 0.05 compared with Gd\textsuperscript{3+} treated contralateral control, $ P < 0.05$ compared with non-treated Ex group. D. Example blots of typical phosphorylation levels of S6 (Ser-240/244) in the TA muscle after a bout of EC.
Table 1. Mean of the resting membrane potential (RMP) values (all units in –mV) measured in vivo immediately after exposure to the exercise (Ex). The SAC-blocked groups either received streptomycin treatment for 6 days prior to their exposure to the Ex or an acute Gd3+ treatment by i.v. infusion. The mean value represents measurements from 6 muscles in all groups with a minimum of 25 recordings from each muscle. Non-treated and Streptomycin data are previously published (19) and are shown to confirm that Gd3+ did result in the maintenance of RMP after Ex. However, it should be noted that experiments were conducted on additional animals to ensure similar results and the data were pooled with the previously published data. All values are expressed as means ± standard errors of the mean (SEM) *P < 0.05 compared with contralateral non-exercised control, #P < 0.05 compared with nontreated-Ex.

<table>
<thead>
<tr>
<th>TA muscle</th>
<th>Treatment</th>
<th>RMP</th>
<th>SEM</th>
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</thead>
<tbody>
<tr>
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<td>83.2</td>
<td>0.73</td>
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<tr>
<td>Ex</td>
<td>non-treated</td>
<td>70.8*</td>
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<td>Streptomycin</td>
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<td>Ex</td>
<td>Streptomycin</td>
<td>79.2*, #</td>
<td>0.52</td>
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<tr>
<td>Cont</td>
<td>Gd3+</td>
<td>82.3</td>
<td>0.27</td>
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<tr>
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<td>Gd3+</td>
<td>77.6*, #</td>
<td>0.56</td>
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Table 2. TA muscle force production in Gd3+ treated and non-treated contralateral control muscles measured in vivo. Force production was first measured in the non-treated contralateral control muscles followed by an i.v. infusion of Gd3+, and the measurement of force in the treated muscles. The mean value represents measurements from 6 muscles in all groups. All values are expressed as means ± standard errors of the mean (SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>muscle weight (mg)</th>
<th>Pt (g)</th>
<th>Po (g)</th>
<th>Po 100 Hz (g)</th>
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</thead>
<tbody>
<tr>
<td>Gd3+ in-vivo Contractile</td>
<td>583.5 ± 19.0</td>
<td>224.2 ± 6.0</td>
<td>950.0 ± 27.9</td>
<td>898.2 ± 28.5</td>
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<td>Control Contractile</td>
<td>566.2 ± 15.5</td>
<td>213.4 ± 5.9</td>
<td>1016 ± 21.3</td>
<td>952.8 ± 18.1</td>
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Figure 1A

Akt Optical Density (Arbitrary Units)

![Graph showing Akt optical density for control and treated groups with non-treated and streptomycin conditions.](image)

Figure 1B

control         Streptomycin
      C     Ex    C     Ex

AKTp (Ser473)

AKT total
Figure 2A

GSK-3 Optical Density (Arbitrary Units)

non-treated

streptomycin

Figure 2B

control  Streptomycin
C  Ex  C  Ex

GSK-3βp (Ser9)

GSK-3β total
Figure 3A

Cont Ex  Cont  Ex
p70\textsuperscript{s6k} Optical Density (Arbitrary Units)

0 20 40 60 80 100 120 140 160 180 200

non-treated  

streptomycin

Figure 3B

Non-treated  Streptomycin

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>Ex</th>
<th></th>
<th>C</th>
<th>Ex</th>
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</thead>
<tbody>
<tr>
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<tr>
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p70\textsuperscript{s6k} (Thr389)

p70 total
Figure 5A

S6 phosphorylation (Ser 235/236) (Arbitrary Units)

<table>
<thead>
<tr>
<th></th>
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<th>streptomycin</th>
<th>Gd3+</th>
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Figure 5B

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<tr>
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S6p (Ser 235/236)
Figure 5C

S6 Phosphorylation (Ser 240/244) (Arbitrary Units)

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Figure 5D

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S6p (Ser 240/244)