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

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Spangenburg, Espen E., and Todd A. McBride. Inhibition of stretch-activated channels during eccentric muscle contraction attenuates $p70^{S6K}$ activation. *J Appl Physiol* 100: 129–135, 2006. First published September 22, 2005; doi:10.1152/japplphysiol.00619.2005.—Eccentric contractions (EC) are known to result in muscle hypertrophy, potentially through activation of the Akt-mammalian target of rapamycin-p70 S6 kinase ($p70^{S6K}$) 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 EC-induced 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 SAC. Specifically, we measured the activation of the Akt, GSK-3β, $p70^{S6K}$, and ribosomal protein S6 following a single bout of EC in the rat tibialis anterior (TA) muscle. The TA muscles performed four sets of six repetitions of EC. In vivo blockade of SAC was performed by a continuous oral treatment with streptomycin in the drinking water (4 g/l) or by intravenous infusion of 80 μmol/kg gadolinium (Gd^{3+}). EC increased the degree of Akt and $p70^{S6K}$ phosphorylation in the TA muscle, whereas in animals in which SAC had been inhibited, there was a reduced capacity for EC to induce Akt or $p70^{S6K}$ phosphorylation. Accompanying this reduced activation of Akt and $p70^{S6K}$ 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 $p70^{S6K}$ pathway in response to EC.

skeletal muscle; hypertrophy; Akt; membrane; ions

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 remain 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-mammalian target of rapamycin-p70 S6 kinase ($p70^{S6K}$) 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, whereas Ohanna et al. (22) found that genetic removal of the $p70^{S6K}$ gene resulted in smaller skeletal muscle mass. Baar and Esser (3) first identified that activation $p70^{S6K}$ 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 $p70^{S6K}$ 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 Ca^{2+} (10, 11) and blocked by both Gd^{3+} and streptomycin (12). Previously, McBride et al. (20) found that inhibition of SAC prevented prolonged membrane depolarization associated with EC, and blocking SAC following EC restored the resting membrane potential (RMP) toward control values. The inhibition of the SAC also attenuated muscle hypertrophy induced by EC (19). Furthermore, 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 Ca^{2+} 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 whether EC-induced activation of SAC contributed to the activation of signaling mechanisms previously shown to stimulate muscle growth. Specifically, we attempted to determine whether pharmacological inhibition of SAC during ECs altered contraction-induced activation of the Akt-$p70^{S6K}$ 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.

EC. Animals were anesthetized (60 mg/kg ketamine and 12 mg/kg Rompum) and subsequently performed EC on a pulley device similar to the one described by Wong and Booth (26). The rat was placed in

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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 g of weight were added to the pulley device, providing some added resistance for the concentrically contracting posterior plantar flexor muscles of the distal hindlimb. The 75 g do 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 plantar flexors (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 1-ms 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 nonexercised contralateral control. When training of a single limb with this exercise model over a 16-wk 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-h time point when the muscles were harvested for molecular analysis. We have measured a significant depolarization out to 24 h post-EC with this exercise paradigm (20). The muscles were weighed and immediately freeze clamped in metal tongs cooled in liquid N_2. Frozen muscles were stored at −80°C until processed for protein extraction.

Inhibition of SAC. Before subjecting animals to a single acute bout of EC or measuring contractile function, either they were treated with streptomycin in their drinking water ($n = 6$), or they received Gd^{3+} by intravenous 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+}. Gd^{3+} (80 μmol/kg) was dissolved in normal saline and delivered to the animal by a 2-ml intravenous infusion via the jugular vein. Animals were anesthetized (60 mg/kg ketamine and 12 mg/kg Rompum) before 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 min 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 h following the EC ($n = 6$). The animals remained anesthetized until the completion of all procedures. There was a 0.5-h 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 with nontreated exercised muscles (Table 1).

Muscle contractile function following Gd^{3+} intravenous 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. Before contractile function was measured, 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 ~0.1- to 0.3-ms duration set to optimal length. Maximum isometric twitch tension, time to peak twitch tension, twitch half-relaxation time, rate of twitch relaxation, isometric tension at 100 Hz, maximum isometric tetanic tension, and the maximum rate of force development during a tetanus at 330 Hz were recorded at 35 ± 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 ± 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 Gd^{3+} was administered as described above. Contractile measurements were then performed on the left (treated) TA muscle following a 0.5-h delay between the end of the infusion and the initiation of contractile measurements.

Electrophysiology. RMPs were obtained from both control and exercised 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 h postexercise. Recordings were obtained by using standard glass microelectrode techniques (20). Electrodes were filled with 3 M KCl and had tip resistances of ~20–30 MΩ. A Grass platinum reference electrode was placed in the proximal end of the TA. Animals were placed on a thermal pad to maintain body temperature (37–39°C). The rat and pad were arranged on a metal frame. The hindlimb 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 min before recordings were made. 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.

Table 1. *Mean of the RMP values measured in vivo immediately after exposure to the exercise*

TA Muscle	Treatment	RMP	SE
Cont	Nontreated	83.2	0.73
Ex	Nontreated	70.8*	0.73
Cont	Streptomycin	81.5	0.41
Ex	Streptomycin	79.2*†	0.52
Cont	Gd^{3+}	82.3	0.27
Ex	Gd^{3+}	77.6*†	0.56

Values are means and SE in −mV. The stretch-activated channel-blocked groups either received streptomycin treatment for 6 days before their exposure to the exercise (Ex) or an acute Gd^{3+} treatment by intravenous infusion. The mean value represents measurements from 6 muscles in all groups with a minimum of 25 recordings from each muscle. Nontreated and streptomycin data were previously published (19) and are shown to confirm that Gd^{3+} did result in the maintenance of resting membrane potential (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. TA, tibialis anterior; Cont, control. *$P < 0.05$ compared with contralateral nonexercised control; †$P < 0.05$ compared with nontreated Ex.

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 X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \cdot H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, 40 μg/ml aprotinin, 5 mM Na_3VO_4, 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). Total protein (50–100 μg) was then loaded (μg per sample per lane) onto 10% SDS-PAGE gels. All gels were run at 150 V for 1 h to separate proteins. The gels were then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) at 50 V for 1 h 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% nonfat dry milk in TBS-T (Tris-buffered saline-0.1% Tween-20)] for 1 h at room temperature, serially washed (3 × 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 × 5 min), the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h followed by another serial wash with TBS-T (3 × 5 min). Enhanced chemiluminescence reagent (Pierce, Rockford, IL) was used to detect the horseradish peroxidase activity by exposure to Kodak-XAR5 autoradiographic film for the appropriate durations to keep the integrated optical densities within a linear and nonsaturated range for all bands of each membrane. The integrated optical densities were quantified by using ImageQuant densitometry software (Molecular Dynamics, Sunnyvale, CA).

Antibodies. The primary antibodies phospho Ser^{473}-AKT (1:1,000 dilution), AKT (1:1,000), phospho Ser^{9}-GSK-3β (1:1,000), GSK-3β (1:2,500), phospho Thr^{389}-$p70^{S6K}$ (1:500), $p70^{S6K}$ (1:500), phospho $Ser^{235/236}$-S6 (pS6–235/236) (1:1,000), and phospho $Ser^{240/244}$-S6 (pS6–240/244) were purchased through Cell Signaling Technologies (Beverly, MA). Anti-rabbit and anti-mouse secondary antibodies (1:2,000) were purchased from Cell Signaling Technologies. 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 by 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 by using a one-way analysis of variance for multiple comparisons followed by Tukey's post hoc test. A P value of <0.05 was considered significant.

RESULTS

To verify that use of streptomycin and Gd^{3+} was an effective method for inhibiting SAC, RMP were determined in TA muscles that performed a single bout of EC and compared with contralateral, nonexercised muscles. These measurements were compared with RMP values previously recorded in exercised and control muscles of nontreated rats (20). Nontreated 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 RMP of the TA muscle after completion of the EC (−79.2 ± 0.52 mV) compared with the RMP measured in TA muscle that underwent EC in the nontreated animals. However, the addition of streptomycin did not completely prevent the EC-induced reduction in RMP compared with the nonexercised contralateral control leg in the streptomycin-treated group (−81.5 ± 0.41 mV). Animals acutely treated with Gd^{3+} in vivo by intravenous infusion also demonstrated preservation of the RMP of the TA muscle after completion of EC (−77.6 ± 0.56 mV) compared with exercised muscles in nontreated animals. However, the addition of Gd^{3+} did not completely prevent the EC-induced reduction in RMP compared with the nonexercise 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 nonexercised contralateral control muscles compared with nonexercised muscles of the nontreated 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, 100-Hz stimulus, or the peak tetanic tension (Table 2).

Phosphorylation of Akt was measured through immunoblotting procedures to determine whether inhibition of SAC prevented EC-induced activation of Akt. In the nontreated animals, a small, significant increase (24%) in Akt phosphorylation was detected after one bout of EC compared with the contralateral control TA muscle (Fig. 1*A*). 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 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 compared with the contralateral control leg (Fig. 2*A*). Interestingly, EC also significantly increased the total content of GSK-3β by 17% compared with the contralateral control leg. No significant increases were detected in GSK-3β phos-

Table 2. *TA muscle force production in Gd^{3+}-treated and nontreated contralateral control muscles measured in vivo*

Group	Muscle Weight, mg	P_t, g	P_o, g	P_o 100 Hz, g
Gd^{3+} in vivo contractile	583.5±19.0	224.2±6.0	950.0±27.9	898.2±28.5
Control contractile	566.2±15.5	213.4±5.9	1,016±21.3	952.8±18.1

Values are means ± SE. Force production was first measured in the nontreated contralateral control muscles followed by an intravenous infusion of Gd^{3+} and the measurement of force in the treated muscles. The mean value represents measurements from 6 muscles in all groups. P_t, maximum isometric twitch tenion; P_o, maximum isometric titanic tension.

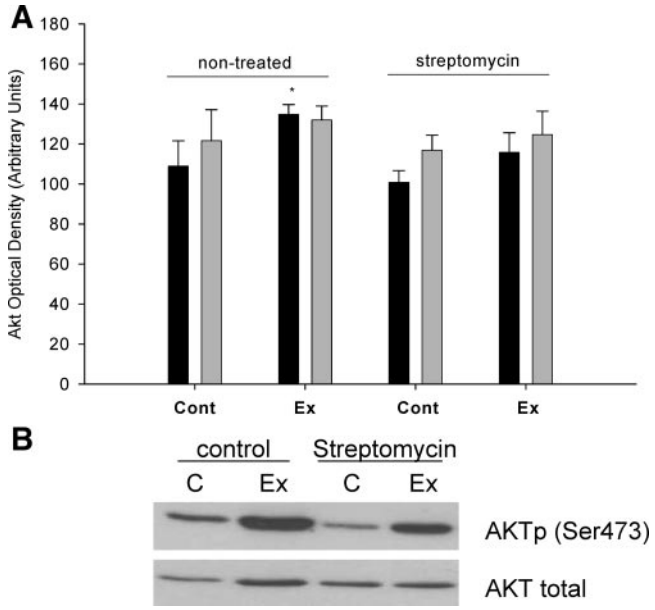

Fig. 1. Akt activation in the tibialis anterior (TA) muscle after exposure to eccentric contraction (EC). *A*: quantification of phosphorylation levels of Akt (Ser-473) and total Akt content in the TA muscle after a bout of EC [exercise group (Ex)]. Inhibition of stretch-activated channels (SAC) was accomplished through streptomycin treatment for 6 days before their exposure to the EC. Solid bars, phosphorylated form of the protein; shaded bars, 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. *B*: example blots of typical phosphorylation levels of Akt (AKTp) (Ser-473) and total Akt content in the TA muscle after a bout of EC. Cont and C, control.

phorylation 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 vs. the nonphosphorylated form and found that no statistical difference was apparent in the control vs. streptomycin-treated group (data not shown).

Phosphorylation of $p70^{S6K}$ was measured by using immunoblotting techniques to determine whether inhibition of SAC altered EC-induced activation of $p70^{S6K}$. EC significantly increased the phosphorylation levels by 93% of $p70^{S6K}$ compared with the contralateral control TA muscle (Fig. 3*A*). Animals treated with streptomycin retained the ability to increase $p70^{S6K}$ phosphorylation after EC compared with the contralateral control leg; however, this phosphorylation was significantly reduced by 23% compared with the nontreated EC value. No significant differences were detected in the total amount of $p70^{S6K}$. To confirm these data, experiments were performed using an alternative SAC inhibitor, Gd^{3+}. Here again we demonstrate a large increase in $p70^{S6K}$ phosphorylation in response to EC compared with the contralateral control TA muscle. However, when the animals were acutely treated with Gd^{3+}, there was a significant 37% reduction in the phosphorylation level of $p70^{S6K}$ in response to EC compared with nontreated exercised TA muscle (Fig. 4*A*). However, it should be noted that there was still significant, albeit reduced, phosphorylation of $p70^{S6K}$ in response to EC compared with the contralateral control TA. No differences in total content of $p70^{S6K}$ were detected in muscles of Gd^{3+}-treated rats.

Phosphorylation of serine residues 235/236 and 240/244 on ribosomal protein S6 are major substrates for $p70^{S6K}$ (18). To determine whether the reduction in $p70^{S6K}$ phosphorylation in response to SAC inhibition resulted in reduced $p70^{S6K}$ 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 compared with the contralateral control TA (Fig. 5*A*). Neither streptomycin nor Gd^{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% compared with the contralateral control TA (Fig. 5*C*). However, unlike residues 235/236, streptomycin and Gd^{3+} treatment resulted in significant reductions in the phosphorylation level of residues 240/244 by 30 and 14% compared with the exercised nontreated TA muscle, respectively. No changes in total S6 expression were detected after Gd^{3+} treatment compared with 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 $p70^{S6K}$ proteins. When the SAC were inhibited

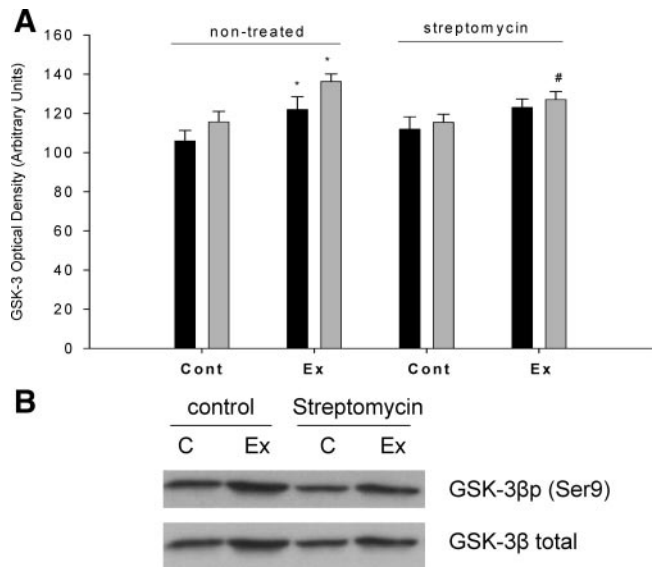

Fig. 2. GSK-3β inactivation in the TA muscle after exposure to EC. *A*: quantification of phosphorylation levels of GSK-3β (Ser-9) and total GSK-3β content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days before their exposure to the EC. Solid bars, phosphorylated form of the protein; shaded bars, 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. *B*: example blots of typical phosphorylation levels of GSK-3β (GSK-3βp) (Ser-9) and total GSK-3β content in the TA muscle after a bout of EC.

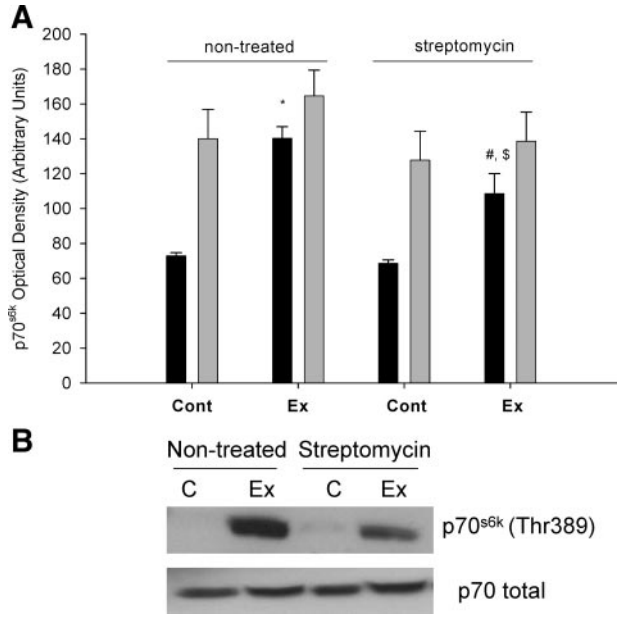

Fig. 3. p70 S6 kinase ($p70^{S6K}$) activation in the TA muscle after exposure to EC. *A*: quantification of phosphorylation levels of $p70^{S6K}$ (Thr-389) and total $p70^{S6K}$ content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days before their exposure to the EC. Solid bars, phosphorylated form of the protein; shaded bars, 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 nontreated 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.

with chronic in situ streptomycin treatment, there was an attenuation of the ability of the EC to activate Akt and $p70^{S6K}$. 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 $p70^{S6K}$. 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 $p70^{S6K}$.

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 switchs" (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 other 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 multiaxial stretch in the presence of 500 μM 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

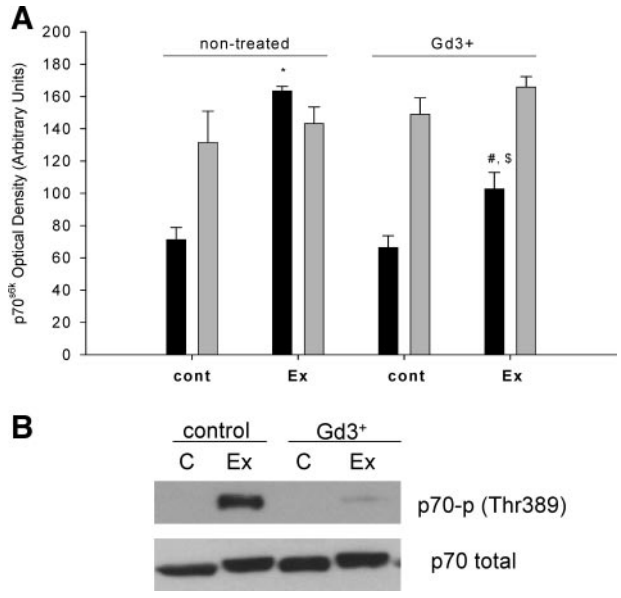

Fig. 4. $p70^{S6K}$ activation in the TA muscle after exposure to EC. *A*: quantification of phosphorylation levels of $p70^{S6K}$ (Thr-389) and total $p70^{S6K}$ content in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through Gd^{3+} infusion 0.5 h before their exposure to the EC. Solid bars, phosphorylated form of the protein; shaded bars, 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 nontreated Ex group. *B*: example blots of typical phosphorylation levels of $p70^{S6K}$ (p70-p) (Thr-389) and total $p70^{S6K}$ content in the TA muscle after a bout of EC.

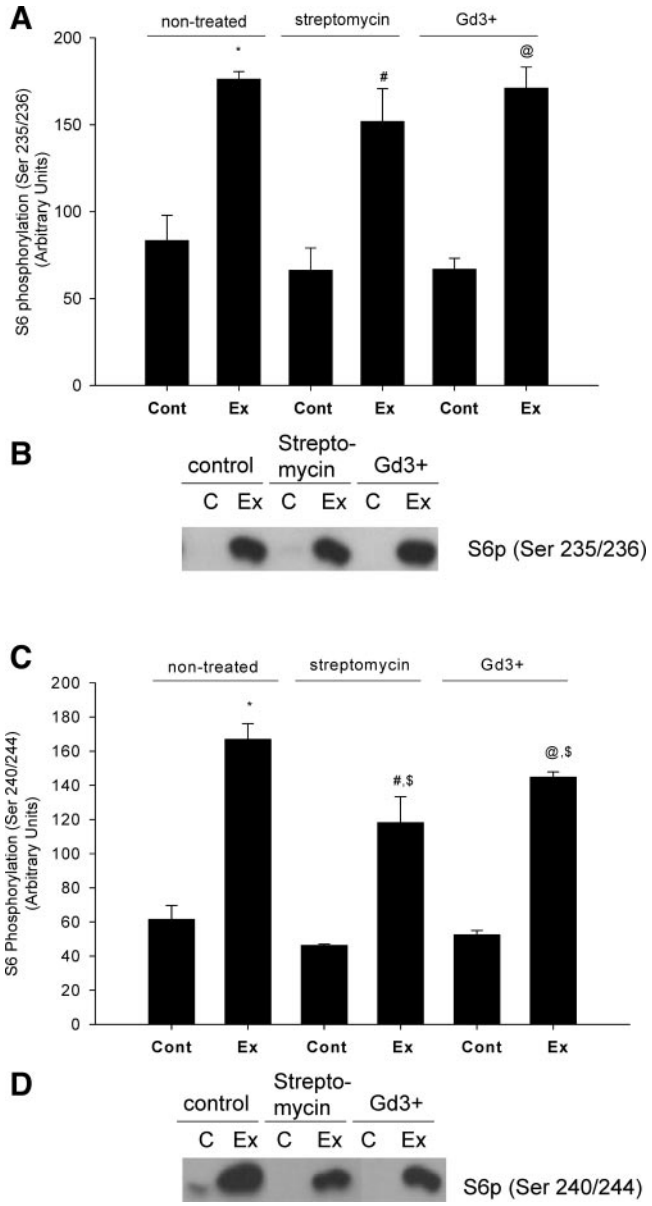

Fig. 5. Ribosomal S6 phosphorylation in the TA muscle after exposure to EC. *A*: quantification of phosphorylation levels of S6 (Ser 235/236) in the TA muscle after a bout of EC (Ex). Inhibition of SAC was accomplished through streptomycin treatment for 6 days before their exposure to the EC or Gd^{3+} infusion 0.5 h before 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^{3+}-treated contralateral control. *B*: example blots of typical phosphorylation levels of S6 (S6p) (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 before their exposure to the EC or Gd^{3+} infusion 0.5 h before 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^{3+}-treated contralateral control; $\$P < 0.05$ compared with nontreated Ex group. *D*: example blots of typical phosphorylation levels of S6 (Ser-240/244) in the TA muscle after a bout of EC.

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 μM 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 $p70^{S6K}$ after EC. Streptomycin has a number of nonspecific 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 intravenous infusion of Gd^{3+} to inhibit SAC before the exercise bout, we again found a failure of EC to fully activate Akt or $p70^{S6K}$. It should be noted that Gd^{3+} has other side effects that include inhibition of Cl^- channels (27), so, although these nonspecific 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 $p70^{S6K}$. With the complete agreement of our streptomycin and Gd^{3+} data, we believe that inhibition of Akt or $p70^{S6K}$ is the result of inhibition of SAC and not a nonspecific 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 $p70^{S6K}$ or its downstream substrates. Unfortunately, it is impossible to predict if these minor changes in force production altered $p70^{S6K}$ phosphorylation. When the gene sequence of the SAC is identified, it allows 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 2 h 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 is 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 $p70^{S6K}$. 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 $p70^{S6K}$, there are examples of possible links for this mechanism in other tissues. For example, it has been suggested that $p70^{S6K}$ 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 $p70^{S6K}$ during EC.

Skeletal muscle hypertrophy can also result from noneccentric types of contraction. For example, Adams et al. (1) recently demonstrated that both concentric and isometric contractions delivered similar levels of muscle hypertrophy compared with EC. 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 EC or lengthening types of contractions. Thus at this time it is only possible to conclude that SAC are contributing to signaling mechanisms after EC 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 $p70^{S6K}$. Finally, the data suggest that further studies of these channels in exercise-induced muscle growth is warranted.

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