Inhibition of cross-bridge formation has no effect on contraction-associated phosphorylation of p38 MAPK in mouse skeletal muscle

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Mammalian muscle cells exhibit a remarkable adaptive capacity to modify phenotype in response to environmental and metabolic stress. Cardiac, smooth, and skeletal muscles all respond to increased mechanical loading by hypertrophic growth (i.e., increased fiber diameter) (3, 4, 6, 13, 28, 29, 31, 36, 37). Additional adaptations to functional demands are based on the nature and duration of the imposed load. Mitochondrial density, metabolic enzymes, and tissue capillarity all increase when muscles are subjected to elevations in the duty cycle for extended periods (16). Conversely, a decrease in the duty cycle results in adaptive changes in phenotype in the opposite direction (4). Despite the extensive catalog of information on the genetic and morphological changes that occur through changes in activity or loading, the intracellular signals and the transduction pathways that couple them to transitions in phenotype are still not well understood.

Several signaling cascades involving families of mitogen-activated protein kinases (MAPKs) are responsive to mechanical stress in smooth, skeletal, and myocardial muscle (1, 9, 17, 23, 24, 26, 30, 36, 38, 39, 42, 43). These include extracellular signal-regulated kinase (ERK)1/2, c-Jun NH2-terminal kinase (JNK), and p38 MAPK and are known to be involved in cell proliferation, differentiation, apoptosis, and adaptations to stress (7, 17). Several transcription factors key to muscle phenotype transition are downstream of p38 MAPK, including cyclic AMP response element-binding protein, myocyte enhancer factor 2C, and peroxisome proliferator-activated receptor-γ coactivator-1α, with the latter having been shown to stimulate mitochondrial biogenesis and fast-to-slow phenotypic transition in skeletal muscle (41, 45). Thus MAPK are putative activators of genes important in both hypertrophic and phenotypic transformations in muscle in response to changes in mechanical stress.

The relationship between skeletal muscle force production and MAPK activation has been studied extensively in rodent (11, 17, 26, 38–40) and human tissues (1, 30, 36). Phosphorylation of MAPKs increases as a result of increased active (11, 17, 26, 34, 38–40) and passive tension (5, 11, 26, 40). A positive correlation of peak tension with phosphorylation has been demonstrated for JNK, ERK1/2, and p38 MAPK when eccentric > isometric > concentric > passive tension (listed in decreasing order of activation), with regard to the type of mechanical stress used and the extent of MAPK phosphorylation (11, 15, 22, 26, 34, 38, 40). However, these studies failed to separate the effects of force production from other factors involved in muscle contraction.

To determine whether the force of contraction is mechanistically linked to MAPK phosphorylation, isolated superfused muscles from adult mice were electrically stimulated in the presence and absence of a specific inhibitor of actomyosin ATPase [N-benzyl-p-toluene sulfonamide (BTS)] to eliminate force development (10). In the absence of BTS, the phosphorylation of p38 MAPK in stimulated muscle was 2.5- to threefold that in nonstimulated muscle. In the presence of BTS, Ca2+ handling appeared relatively unchanged, but force production was inhibited by nearly 95%. However, there was still a two- to threefold increase in p38 MAPK phosphorylation. In contrast, electrical stimulation did not change phosphorylation of ERK1/2 relative to nonstimulated muscles. Ca2+ handling
was not inhibited by BTS in these preparations. These observations demonstrate that it is not cross-bridge formation per se but a process associated with actively contracting muscle that is the signal for the mechanosensitivity of p38 MAPK. Interestingly, isometric contractions capable of activating p38 MAPK had no effect on ERK1/2, suggesting that normal bouts of physiological activity are not sufficient to activate this kinase.

MATERIALS AND METHODS

Materials. Chemicals and reagents were purchased from Sigma chemical and were of the highest grade available unless otherwise noted. BTS was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in cell culture grade DMSO. Rhod-2 acetoxymethyl ester (AM) was purchased from Molecular Probes (Corvallis, OR) and suspended before each experiment in cell culture grade DMSO immediately before muscle cell loading. Antibodies were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise noted.

Animals and isolated muscle preparations. All mouse care and experimental protocols were approved by the All University Committee on Animal Use and Care at Michigan State University. Adult male Swiss Webster mice (Harlan, Indianapolis, IN) were maintained in a controlled environment with a 12:12-h light-dark cycle and food and water administered ad libitum. Harvesting of intact muscles was controlled to ensure that they were isolated and superfused at 37°C, the force of contraction was measured between ligatures in situ (typically 16–18 mm) before being adjusted to their optimal resting length (L₀) using the length-tension relationship. Electrical stimulation was delivered via two platinum plate electrodes adjacent to the muscle and generated using a Grass S88 Stimulator (Grass Instruments, Quincy, MA). Force was recorded using an analog-to-digital converter (ADC) (model AT MIO16E; National Instruments, Austin, TX) controlled by commercially available software (LabScribe/NI; Woxor, Dover, NH). Analysis of mechanical transients was performed using a custom algorithm for physiological data developed in our laboratory using a Matlab programming environment (Mathworks, Natick, MA). To test the effects of BTS on force production, 25, 50, 75, or 150 μM BTS was added to the bath immediately after a short series of five control twitches at 1 Hz. At 10-min intervals, a short series of five twitches were obtained to determine the degree of inhibition by BTS at each concentration level.

To test the effects of force generation on MAPK phosphorylation, isometric contractions were induced at 10 Hz for a period of 15 min. The contralateral EDL muscles were used as controls by fixing them at resting length and incubating them in Ringer solution in a separate organ bath for an equivalent period of time. The degree of MAPK phosphorylation in the absence of force production was tested as follows. Forty min before the stimulation protocol, 75 μM BTS was added to the bath and brief test twitches confirming 95–97% inhibition of initial force were performed, followed by the same 15-min stimulation protocol used in control experiments. After these stimulation protocols, the muscles were removed from their tendons, weighed, flash frozen, and stored at −80°C until further analysis.

Ca²⁺ measurements. The relative changes in Ca²⁺ handling in the presence of BTS were measured using fluorescence microscopy. Measurements were performed using a Nikon TE2000U inverted microscope outfitted with a dual channel model 814 photomultiplier detection system (Photon Technology International, Lawrenceville, NJ) coupled to a model D-104 grating photometer (DeltaRAM; Photon Technology International). Excitation was set at 550 nm and emission was set at 570 nm using a model C3286 filter cube (Chroma Technology, Rockingham, VT) specific to rhod-2 and a long working distance lens objective (×10 Plan Fluor). Measurements were obtained for isolated EDL muscles fixed horizontally at resting length and positioned with a micrometer as done in the organ bath experiments. Each organ bath was constructed of Perspex with provisions for superfusion using a Bioptechs microcirculation pump (Bioptechs, Butler, PA), with stimuli delivered via bath-applied platinum electrodes using a Grass model S48 stimulator (Astro-Med). Muscles were mounted in the bath at both ends, one on a stainless steel post fixed to a micromanipulator and the other on an isometric force transducer (Kent Scientific, Torrington, CT). Force was recorded digitally using an analog channel on the DeltaRAM ADC so that force and fluorescence measurements were synchronized. The analog signal was recorded on an Astro-Med DASH II thermal array recorder. Temperature was controlled using a model TS-4 LPD stage heater and a model PTU-3 controller (Physitemp, Clifton, NJ). Muscles were loaded with rhod-2 AM for 30 min at 30–35°C, and, once this step was completed, muscles were stimulated and Ca²⁺ and force were recorded concurrently. Muscles were subsequently treated with 75 μM BTS and Ca²⁺, and force was assayed again after 30 min.

Tissue preparation and Western blot analysis. Muscles were homogenized in isotonic saline buffer (25 mM Tris-HCl, pH 7.6, and 138 mM NaCl) containing 10 mM dithiothreitol (DTT); 10% (vol/vol) glycerol; phosphatase inhibitors 20 mM sodium pyrophosphate (NaP₂), 50 mM NaF, 25 mM β-glycerophosphate, and 1 mM sodium orthovanadate (Na₃VO₄); protease inhibitors 0.01% leupeptin, 0.05% PMSF, and 0.01% pepstatin; and detergent [1% (wt/vol) sodium dodecyl sulfate (SDS)]. The protein concentration of the homogenates was determined using the Bio-Rad DC protein reagent kit. Equal amounts (40 μg) of homogeneate proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) through an 8% (wt/vol) polyacrylamide gel, and then they were electrophoretically transferred onto a nitrocellulose membrane.

Western blot analysis was accomplished by blocking the membrane for 1 h with isotonic saline buffer containing 3% (wt/vol) bovine serum albumin, 5% (wt/vol) nonfat dry milk, and 50 mM NaF (blocking buffer), followed by an overnight incubation at 4°C with a rabbit antibody that specifically detects either the phosphorylated isoforms of p38 (Thr180/Tyr182) or total endogenous isoforms of p38. Primary antibodies were diluted (1:1,000) in blocking buffer. Goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was used to detect bound primary antibody. The antibody-HRP conjugate was diluted (1:2,000) with isotonic saline buffer containing 5% (wt/vol) nonfat dried milk. Bound second antibody was detected using the Phototope-HRP Western blot chemiluminescence kit (Cell Signaling Technology). Chemiluminescent signals were captured on Amersham ECL Hyperfilm.

RESULTS

Force production in isolated muscles. In EDL muscles isolated and superfused at 37°C, the force of contraction was 9.2 ± 1.3 g (n = 13) when stimulated with 5-s bursts at 1 Hz.
These EDL muscles ranged in weight from 7 to 10 mg wet wt (tendon free). Force was inhibited by addition of BTS to the Ringer solution. BTS is a cell-permeable reversible inhibitor of actomyosin ATPase that interferes with Pi release and stabilizes the ADP-S1 complex (10). Addition of BTS causes not only inhibition of force generation but also a proportionate decrease in ATPase activity (35). In saponin-treated muscle fibers from toadfish, BTS has been shown to potently inhibit muscle force (44). To determine the optimum concentration for isolated muscle preparations, BTS was administered at several concentrations and the effects in relation to time course were studied. Concentrations of 25, 75, and 150 μM BTS were independently tested, and the time course of inhibition was determined from residual force production at 10-min intervals for a total of 30 min (Fig. 1). The residual force values were concentration dependent, with 25, 75, and 150 μM BTS resulting in 22, 5, and 1% force remaining after 30 min. However, the time required for the maximal effects of inhibition to be realized was 30 min independent of the BTS concentration used and remained stable as long as the inhibitor was present in the bath (data not shown).

The relative effects of BTS on Ca\(^{2+}\) handling were also assayed using fluorescence microscopy of the same muscle pre- and posttreatment with 75 μM BTS. Figure 2 shows that Ca\(^{2+}\) transient was relatively unaffected by BTS, yet force was completely inhibited. Untreated muscles stimulated at 0.25 Hz (Fig. 2, top) served as controls for Ca\(^{2+}\) and force production for EDL at 37°C. In contrast, force was completely lost in muscles that were treated for 30 min with 75 μM BTS, but the fluorescence indicator for cytosolic Ca\(^{2+}\) was relatively unaffected in the untreated muscles (Fig. 2, bottom).

Force production during 10-Hz continuous stimulation was 0.65 ± 0.28 kg/g wet wt (n = 4) (Fig. 3). Stimulation of muscles at this frequency for 15 min resulted in a rapid and marked decrease in force production to <15% of initial force within 6 min but decreased more slowly over the remainder of the time course. End force production was 5% of the initial force measured (Fig. 3). In contrast, pretreatment with BTS resulted in an initial force that was 5% of control during the first minute of stimulation at 10 Hz. End stimulation values were <1% of initial control values. Thus 75 μM BTS decreased P\(_i\) and the cumulative tension-time integral over 15 min. Figure 3 (inset) graphically depicts these differences.

Control muscles generated a total of 16 ± 0.8 kg·s/s/mg of muscle during the 15 min of 10-Hz stimulation. In treated muscles, the cumulative tension-time integral was only 2.1 ± 0.1 kg·s/s/mg of muscle (n = 4), a reduction of 88% compared with control muscles.

Phosphorylation of p38 MAPK. Western blot analysis of phosphorylated p38 MAPK and total p38 MAPK were performed on resting and stimulated muscles in the absence and presence of 75 μM BTS. Cellular expression of total p38 MAPK for EDL muscles did not differ between treatments (Fig. 4, top). However, phosphorylation of p38 MAPK was highly dependent on contractile activity. The ratio of phosphorylated p38 MAPK to total p38 MAPK was 0.7 ± 0.18 (n = 4) in resting muscles incubated for the entire time course. This value was not significantly different when measured in freshly dissected muscles, demonstrating that the incubation at resting length had no effect on phosphorylation in isolated superfused preparations (data not shown). Muscles stimulated for 15 min at 10 Hz showed a dramatic increase in the phosphorylation ratio to a value of 2.3 ± 0.4 (n = 4) relative to nonstimulated contralateral control muscles (Fig. 4).

Treatment of muscles with 75 μM BTS to inhibit force production caused a nonsignificant decrease in the phosphorylation ratio of p38 MAPK in resting muscles relative to nontreated resting controls (0.4±0.1). Taken together with the observation that total p38 MAPK does not change, these results show that the mechanism underlying the stimulation of p38 MAPK phosphorylation is mediated not by pharmacological action of BTS but through muscle stimulation itself. Surprisingly, when BTS-treated muscles were stimulated, the phosphorylation ratio of p38 MAPK increased to nearly the same extent measured in stimulated but untreated muscles (2.0 ± 0.4). This dramatic decrease in the amount of force generated in treated muscles with no change in phosphorylation suggests that the mechanism responsible for stimulating the phosphorylation of p38 MAPK is not mediated by mechanotransduction as previously suggested (26, 40).

The potential that relatively small changes in force production were sufficient to activate p38 MAPK could not be excluded by the BTS experiments because of the residual amount (<5%) of force over the time course. If p38 MAPK activation were exquisitely sensitive to contraction-induced mechanical stress, even the small amounts of force generated by BTS-treated muscles might be sufficient to stimulate phosphorylation. To test this hypothesis, untreated muscles were stimulated at 10 Hz to generate an amount of force equivalent to that generated by BTS-treated muscles. Because a single twitch generated ~0.9-g force, nontreated muscles were stimulated for ~150 contractions in the short-stimulation protocol. Figure 2 (inset) shows that nontreated muscles stimulated for a short duration generated slightly more force than BTS-treated EDL muscles within 15 min. Despite the greater force produced with the short-stimulation protocol, there was not a significant increase in p38 MAPK phosphorylation. This was true whether muscles were frozen immediately after the last contraction (15 s) or were allowed to rest for the entire 15-min time course and then frozen (data not shown). If p38 MAPK signaling were as sensitive as hypothesized, the degree of phosphorylation in the nontreated muscles subjected to the short-term stimulation would be identical to that in the BTS-treated muscles under the same experimental conditions. In
fact, the phosphorylation ratio was not significantly different from that of resting muscles (Fig. 5), demonstrating that force production was not the physiological signal that activated p38 MAPK in response to muscle contractile activity.

A correlative relationship between mechanical activity in muscle and ERK1/2 phosphorylation has also been reported in isolated muscles from other rodents using tetanic and/or eccentric contractions (11, 26, 34, 38–40). The conditions in the experiments described are a continuous train of isometric contractions sufficient to activate p38 MAPK; however, such conditions are insufficient to activate ERK1/2. Figure 6 shows the results of Western blot experiments comparing resting control muscles with muscles stimulated at 10 Hz for 15 min. There was no difference in the phosphorylation ratio, demonstrating that contraction, at least in the range of forces described in the present report, are insufficient to stimulate the phosphorylation of ERK1/2.

DISCUSSION

The present study has two main findings. First, p38 MAPK phosphorylation occurred in electrically stimulated muscles in vitro even in the near absence of force generation, demonstrating that mechanical signaling per se was not responsible for activating p38 MAPK (Fig. 4). Second, there was no change in ERK1/2 phosphorylation in response to electrical stimulation, demonstrating that p38 MAPK and ERK1/2 responded differently to isometric contractions (Fig. 6). Despite the well-documented direct correlation of phosphorylation of p38 MAPK with force production (11, 17, 26, 27, 29, 30, 34, 39), our present findings suggest that force production per se is not responsible for p38 MAPK phosphorylation in response to electrical stimulation.

These studies relied on the use of BTS as a specific inhibitor of force. BTS is an aryl sulfonamide that has been shown to weaken S1 subfragments of myosin with F-actin in in vitro motility assays. Kinetic analysis of the action of BTS using skinned fiber preparations has shown that it decreases the rate of Pi release as well as the dissociation of S1 and ADP (35). The concomitant ATPase activity is directly inhibited by the decreased release of P_i (35). The molecular action of BTS is highly specific and has been shown to minimally affect Ca^{2+} handling [sarcoplasmic reticulum (SR) ATPase activity] in

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Fig. 2. Typical force and Ca^{2+} kinetics in control and BTS-treated isolated EDL muscles. Top: control muscle force generation and fluorescent indicator (rhod–2 acetoxymethyl ester) for cytosolic Ca^{2+}. Muscles were held at resting length and incubated in Ringer solution at 37°C on the stage of an epifluorescence microscope. Stimuli were delivered at 0.25 Hz. Bottom: same muscle treated with 75 μM BTS for 30 min. Note that the Ca^{2+} fluorescence signal remained unchanged but that force was abolished. The force tracing is deliberately offset for visibility.

Fig. 3. Isometric force production in control (open symbols) and BTS-treated (closed symbols) superfused mouse EDL muscles at 37°C in response to 10-Hz stimulation for 15 min. Force data are presented as g/g tendon-free muscle weight. Inset: cumulative tension-time integral (TTI) produced during the entire 15-min stimulation period presented as kg·s/mg of tendon-free muscle wet wt for control and BTS-treated EDL muscles. The shaded bar at right represents the cumulative TTI for an untreated EDL muscle stimulated for only 150 contractions, meant to simulate the low TTI in BTS-treated muscles.
intact frog fibers (10) or saponin-treated fish fibers (44). Therefore, the use of BTS allows the putative signal of force production to be separated from other signals generated by muscle stimulation.

**MAPK phosphorylation and mechanical signaling.** Mechanical stress is a change in force per cross-sectional area, whereas mechanical strain is a change in force per change in length. Muscles contract in vivo, with small changes in muscle length (low component of mechanical strain) depending on pennation, but force per cross-sectional area increases substantially during contraction (high component of mechanical stress) (12, 18, 20). Hence, in the isometric contraction model used in the present study, mechanical stress was the dominant physical force. In contrast, contractions with concurrent increases in length (eccentric contractions) have a larger strain component than isometric contractions do and can cause substantial damage to the sarcolemmal membrane and the SR (14, 21). Thus, for studies of mechanical signaling, increases in the proportion of mechanical strain to overall muscle loading may result in substantial muscle damage (2, 25). On the basis of these facts, interpretation of the intracellular signaling by MAPKs in exercising muscle may be confounded by underlying membrane and organelle damage. By using isometric contractions, we designed the present study to minimize mechanical strain (or any damage) under physiological conditions.

The results of the present study show two important findings with respect to mechanical stress in isometric contractions. First, Fig. 4 shows that p38 MAPK responded to contractile activity because there was robust phosphorylation in stimulated muscles relative to the contralateral resting controls. However, the inhibition of force production through a loss in cross-bridge formation from BTS administration (Fig. 3) did not attenuate the phosphorylation response (Fig. 4). This supports the argument that p38 MAPK activation is not under mechanical strain control in this model system. Consequently, any effect of BTS on p38 MAPK phosphorylation (Fig. 4) cannot be attributed to the change in mechanical stress or strain. Second, the phosphorylation of p38 MAPK was not affected by isometric contractions, offering evidence for the specificity of this signaling pathway to mechanical stress. In contrast, contractions with concurrent increases in length (eccentric contractions) can cause substantial damage to the sarcolemmal membrane and the SR (14, 21).

**Fig. 4.** Western blot analysis (top) of phospho-p38 mitogen-activated protein kinases (MAPK) and total p38 MAPK expressed in isolated EDL muscles under experimental conditions. The bar graph (bottom) shows the experimental conditions and a quantitative value for the phosphorylation ratio (phospho-p38/pan-p38). Control muscles were held at resting length but were unstimulated, whereas stimulated muscles were subjected to electrical stimulation at 10 Hz for 15 min. Untreated muscles are represented by stippled bars (left), and the BTS-treated muscles are represented by shaded bars (right) for each condition. Pan (total) p38 MAPK protein levels did not change, regardless of treatment. Stimulation caused a 2.5- to 3-fold increase in phosphorylation with no change in total p38 MAPK. BTS treatment did not alter either resting or stimulated muscle p38 MAPK phosphorylation. Data are presented as means ± SE; n = 4.

**Fig. 5.** Western blot analysis (top) of total phospho-p38 MAPK expression and phospho-p38 MAPK in short-term stimulation protocol. Quantification of the phosphorylation ratio (phospho-p38/pan-p38) is presented in bar graph format (bottom). There is no difference in the phosphorylation of p38 MAPK in short-term stimulated muscles (150 twitches) relative to nonstimulated control muscles.

**Fig. 6.** Western blot analysis of phospho-extracellular signal-regulated kinase (ERK)1/2 in nonstimulated and stimulated EDL muscles. Top blot shows total (pan) ERK1/2 protein levels; bottom blot shows the phosphorylated isoform. The bar graph shows a quantitative value for the phosphorylation ratio (phospho-ERK/pan-ERK). Control muscles were held at resting length but were unstimulated, whereas stimulated muscles were subjected to 10-Hz electrical stimulation for 15 min. Isometric contractions did not change the phosphorylation of ERK1/2.
ment that p38 MAPK, while activated as a consequence of electrical stimulation, does not respond to mechanical loading and that some other aspect of physiological activity associated with contraction, perhaps metabolic in nature, must be responsible. This result conflicts with a previous study of isolated superfused rat EDL muscles in which the investigators concluded that ERK1/2 was sensitive to a metabolic signal, whereas p38 MAPK was sensitive to mechanical force (40). The disagreement of those previous results with those of the present study might be explained by the nature of the contractions performed. Wretman et al. (40) used tetanic contractions with changes in muscle length, eccentric and concentric, resulting in a strain component not present in the experiments described in the present study. Strain-dependent activation of p38 MAPK has been shown in rat EDL muscle. Boppart et al. (5) showed that passive stretch of rat EDL with forces as small as 0.06 N resulted in a fivefold increase in p38 MAPK phosphorylation. Their study also showed similar trends in slow-twitch soleus muscles, however, the relative increases in p38 MAPK phosphorylation were not as dramatic until mechanical strain was \( \geq 0.24 \) N, suggesting fiber-specific effects. However, ERK1/2 phosphorylation with passive stretch was identical independent of muscle fiber-type content. Taken together, these results suggest that ERK1/2 responds to strain in the presence or absence of active force generation and is not fiber type dependent, while p38 MAPK responds to events associated with muscle activation but is not dependent on force production. Consistent with this conclusion is that isometric contractions did not activate ERK1/2 in the present study (Fig. 6).

MAPK phosphorylation and other physiological signaling. During contractile activity, depolarization of the sarcosomal membrane results in activation of the ryanodine receptor, thereby initiating \( \text{Ca}^{2+} \) release from the SR. The rise in cytosolic \( \text{Ca}^{2+} \) immediately activates SR ATPases to pump \( \text{Ca}^{2+} \) back into the SR as well as causes a conformational change in troponin, permitting the attachment of myosin to actin and initiating the myosin power stroke (19). Active SR ATPases and actomyosin ATPases consume ATP, liberating ADP and P\(_i\) (8). Therefore, within the normal contractile cycle, three tenable signals are generated: force, altered \( \text{Ca}^{2+} \) homeostasis, and altered ATP-free energy homeostasis. The present study shows directly that inhibition of force by BTS had no effect on p38 MAPK phosphorylation (Fig. 4), thereby eliminating force production as the signaling mechanism. This implies that either altered \( \text{Ca}^{2+} \) homeostasis or altered ATP-free energy homeostasis should be considered a signal.

In this study, \( \text{Ca}^{2+} \) homeostasis in isolated superfused fast-twitch muscles was investigated using rhod-2 AM as the \( \text{Ca}^{2+} \) indicator. Incubation with BTS resulted in the massive loss of force production (Fig. 1) while having no apparent deleterious effects on \( \text{Ca}^{2+} \) handling (Fig. 2). However, p38 MAPK phosphorylation was still elevated by electrical stimulation. Interestingly, the short-term stimulation protocol (Fig. 3) resulted in no significant increase in p38 MAPK phosphorylation relative to unstimulated controls (Fig. 5). This is consistent with the expectation that the amount of \( \text{Ca}^{2+} \) released as a function of time will scale with the number of twitches produced. Therefore, the rise in intracellular \( \text{Ca}^{2+} \) cannot be eliminated as a signal for contraction-induced phosphorylation of p38 MAPK.

Because there is an energetic cost associated with \( \text{Ca}^{2+} \) handling and force production, it is possible that perturbations in ATP homeostasis could also signal for the phosphorylation of p38 MAPK. In fast-twitch muscles, the estimated cost of force production (actomyosin ATPase activity) is \( \sim 70\% \) of the overall ATP use, with the balance being consumed by \( \text{Ca}^{2+} \) handling (SR ATPase activity \( \geq 20\% \)) and membrane ion pumps (\( \sim 5\% \)) (32). Even though energetic costs were not directly measured in the present study, the use of BTS to inhibit actomyosin ATPase activity implies a reduction in the overall ATP consumption proportionate to the measured decrease in force generation (Fig. 1). However, the energetic cost of \( \text{Ca}^{2+} \) handling and meager force production is still above resting ATPase activities. Our observation that the extent of p38 MAPK phosphorylation was similar in stimulated muscle in the presence and absence of BTS (Fig. 4) implies that in energetic signal-mediated p38 MAPK phosphorylation, either the response to the signal was saturated at very low perturbations of ATP free energy or the signal in both tissues was of similar strength and was not energetically mediated.

One possible signaling mechanism not necessarily accounted for in the present study was the effect of reactive oxygen species (ROS). ROS are generated during intensive contractile activity (33) and stimulate the phosphorylation of ERK1/2 in response to repeated concentric contractions (40). However, Wretman et al. (40) observed that p38 MAPK was not phosphorylated in response to concentric contractions; thus the production of ROS appears to have no role in activating p38 MAPK signaling. Our study showed that p38 MAPK phosphorylation increased 2.5- to threefold in response to chronic isometric contractions (Fig. 4), whereas the phosphorylation of ERK1/2 did not change relative to nonstimulated muscle (Fig. 6). Although we cannot definitively rule out that isometric contractions generate ROS, our observation that ERK1/2 was not phosphorylated, but that p38 MAPK was, most likely rules out ROS as a potential signaling mechanism in isometric contractions.

Concluding remarks. On the basis of the results of the present study, we conclude that p38 MAPK is significantly activated in isolated mouse fast-twitch muscle in response to chronic isometric contractions. The observation that the fold increases in p38 MAPK phosphorylation were similar in stimulated muscle in the absence and presence of BTS indicates that mechanical stress is not the signaling mechanism for activation of p38 MAPK. Furthermore, the observation that ERK1/2 was not phosphorylated in response to chronic isometric contractions suggests that ERK1/2 responds to strain in the presence or absence of active force generation, while p38 MAPK responds to events associated with muscle activation but not force production. These putative signaling mechanisms could include changes in intracellular \( \text{Ca}^{2+} \) and/or changes in ATP-free energy homeostasis.

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