Enhanced Skeletal Muscle Contraction with Myosin Light Chain Phosphorylation by a Calmodulin-sensing Kinase**

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Repetitive low frequency stimulation results in potentiation of twitch force development in fast-twitch skeletal muscle due to myosin regulatory light chain (RLC) phosphorylation by Ca\(^{2+}\)/calmodulin (CaM)-dependent skeletal muscle myosin light chain kinase (skMLCK). We generated transgenic mice that express an skMLCK CaM biosensor in skeletal muscle to determine whether skMLCK or CaM is limiting to twitch force potentiation. Three transgenic mouse lines exhibited up to 22-fold increases in skMLCK protein expression in fast-twitch extensor digitorum longus muscle containing type IIa and IIb fibers, with comparable expressions in slow-twitch soleus muscle containing type I and IIa fibers. The high expressing lines showed a more rapid RLC phosphorylation and force potentiation in extensor digitorum longus muscle with low frequency electrical stimulation. Surprisingly, overexpression of skMLCK in soleus muscle did not recapitulate the fast-twitch potentiation response despite marked enhancement of both fast-twitch and slow-twitch RLC phosphorylation. Analysis of calmodulin binding to the biosensor showed a frequency-dependent activation to a maximal extent of 60%. Because skMLCK transgene expression is 22-fold greater than the wild-type kinase, skMLCK rather than calmodulin is normally limiting for RLC phosphorylation and twitch force potentiation. The kinase activation rate (10.6 s\(^{-1}\)) was only 3.6-fold slower than the contraction rate, whereas the inactivation rate (2.8 s\(^{-1}\)) was 12-fold slower than relaxation. The slower rate of kinase inactivation in vivo with repetitive contractions provides a biochemical memory via RLC phosphorylation. Importantly, RLC phosphorylation plays a prominent role in skeletal muscle force potentiation of fast-twitch type IIb but not type I or IIa fibers.

Repetitive stimulation of fast-twitch skeletal muscle results in enhanced twitch force (1–3). This twitch force potentiation can be observed following a brief (1–2 s) high frequency stimulation sufficient to induce a tetanus (post-tetanic potentiation) or in response to continual low frequency (5–10 Hz) stimulation (staircase effect). The skMLCK\(^{3}\) is a Ca\(^{2+}\)/CaM-dependent protein kinase that phosphorylates the fast-twitch skeletal muscle isoform of the myosin RLC\(_f\) (4). Stimulation of fast-twitch skeletal muscle results in proportional RLC\(_f\) phosphorylation and twitch force potentiation (3, 5). In skMLCK knock-out mice, RLC\(_f\) is not phosphorylated in response to electrical stimulation and is accompanied by an ablation of post-tetanic twitch force potentiation and a markedly reduced staircase effect (6). These results clearly define skMLCK-mediated RLC\(_f\) phosphorylation as the primary biochemical mechanism responsible for twitch force potentiation.

Post-activation potentiation in human skeletal muscle has recently received attention for its potential to affect human performance in strength and endurance exercise (7, 8). RLC phosphorylation increased eccentric contraction-induced injury in skinned fast-twitch fibers (9). Furthermore, single nucleotide polymorphisms for skMLCK were associated with increased sensitivity to exercise injury in humans (10).

The effect of repetitive stimulation to evoke RLC\(_f\) phosphorylation and twitch force potentiation is much more pronounced in fast-twitch versus slow-twitch skeletal muscle. Investigations in mouse (11), rabbit (12), and rat (13) skeletal muscle all show a greater effect of electrical stimulation on the extent of RLC phosphorylation in fast-twitch versus slow-twitch muscle. When detectable, stimulation-induced RLC phosphorylation in slow-twitch muscle required higher frequencies and longer stimulation times than did fast-twitch muscle, conditions that typically lead to fatigue (11–13). Likewise, twitch force potentiation was more robust in fast- versus slow-twitch muscle (13, 14). Reduced RLC phosphorylation may be due to lower skMLCK and greater myosin phosphatase activities in slow-twitch muscle (13). In skinned fast-twitch skeletal muscle and cardiac muscle fibers, RLC phosphorylation results in force potentiation (15). Therefore, overexpression of skMLCK in slow-twitch muscle is predicted to result in significant RLC phosphorylation, thereby producing twitch force potentiation.

CaM is limiting for activation of targeted enzymes in cells in culture (16) as well as smooth muscle (17). Thus, reduced skMLCK activation due to limiting Ca\(^{2+}\)/CaM availability may affect the achievable extent of RLC phosphorylation in fast-twitch fibers and slow-twitch fibers in particular.

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\(^{3}\) The abbreviations used are: MLCK, myosin light chain kinase; skMLCK, skeletal muscle MLCK; smMLCK, smooth muscle MLCK; RLC, regulatory light chain of myosin; RLC\(_f\), fast-twitch RLC; RLC\(_c\), slow-twitch RLC; CaM, calmodulin; FRET, fluorescence resonance energy transfer; MYPT, myosin phosphatase-targeting subunit; EDL, extensor digitorum longus; HSA, human skeletal \(\alpha\)-actin; MOPS, 4-morpholinepropanesulfonic acid.
Expression of a skMLCK CaM Sensor in Skeletal Muscle

We generated transgenic mice that express an skMLCK CaM biosensor in both fast-twitch and slow-twitch skeletal muscle to determine 1) whether skMLCK is a limiting factor for RLC phosphorylation and twitch force potentiation in fast-twitch muscle, 2) whether elevating skMLCK levels in slow-twitch muscle is sufficient to enhance RLC phosphorylation and twitch force potentiation, and 3) whether CaM is rate-limiting to frequency-dependent activation of skMLCK in the intact muscle.

EXPERIMENTAL PROCEDURES

Generation of HSA-skKCS Mice—The pcDNA-skKCS plasmid was constructed by subcloning into pcDNA3.1 a 4.6-kb cDNA fragment encoding rabbit skMLCK fused to an MLCK CaM biosensor (18) followed by the human growth hormone polyadenylation sequence to form the plasmid pcDNA-skKCS. The MLCK CaM biosensor consists of an MLCK CaM binding site flanked by enhanced yellow fluorescent protein and enhanced cyan fluorescent protein. The human skeletal α-actin (HSA) promoter (19, 20) was subsequently subcloned into pcDNA-skKCS to generate the HSA-skKCS plasmid. HSA-skKCS was linearized by Pmel digestion. HSA-skKCS mice were generated at the University of Texas Southwestern Transgenic Core Facility by pronuclear injection of the linearized transgene construct followed by implantation of oocytes into pseudo-pregnant ICR mice. Founder mice were identified by PCR using primers directed against the rabbit skMLCK construct. Three independent lines were maintained on a C57BL/6 background. Mice from all lines appeared normal and showed no obvious phenotype. All animal experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee.

Electrical Stimulation of Isolated Skeletal Muscle—EDL and soleus muscles were mounted to Grass FT03 force transducers between platinum electrodes and were immersed in continuously gassed physiological salt solution. Muscles remained quiescent for 30 min and were frozen immediately or following 3 or 15 s of 10-Hz electrical stimulation as described (6). Measurement of force development was recorded on a Powerlab 8/SP data acquisition unit (AD Instruments, Colorado Springs, CO). In both wild-type and transgenic soleus muscle, increased twitch force occurred rapidly within the first 0.5 s due to partial fusion of contractile force with 10-Hz electrical stimulation. Therefore, twitch force was normalized to the twitch occurring at 0.5 s (i.e. sixth twitch). Because of the potential for overexpressed green fluorescent protein containing proteins to interfere with muscle contraction by interacting with myosin (21), we determined that there was no difference in the initial twitch force properties without RLC phosphorylation of EDL muscles from wild-type or transgenic (line 3) mice.

Western Blot Analysis—Muscle proteins precipitated in 10% trichloroacetic acid were solubilized in urea sample buffer for separation of phosphorylated and non-phosphorylated RLC by urea/glycerol-PAGE (22). Proteins from EDL muscle were transferred to polyvinylidene difluoride membranes and probed with a polyclonal primary antibody (6). Because soleus muscle contains both RLCf and slow-twitch RLC (RLCt), two-dimensional electrophoresis was performed for the determination of RLC phosphorylation in soleus muscle. Urea/glycerol-PAGE was followed by SDS-PAGE on 15% resolving gels. Proteins were transferred and analyzed as described above. CaM was also analyzed by urea/glycerol-PAGE using a monoclonal CaM antibody with purified CaM as a standard. SDS-PAGE (7.5% resolving gels) was used for Western blotting of skMLCK using a goat polyclonal skMLCK antibody (23) and a rabbit polyclonal myosin phosphatase-targeting subunit (MYPT) 1/2 antibody (Epitomics). Membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody, and proteins were visualized using ECL Plus detected on the Storm 640.

Kinase Activity in Skeletal Muscle—A portion of white quadriceps muscle from wild-type or transgenic mice was weighed and homogenized (1:50, w/v) in ice-cold homogenization buffer (50 mM MOPS, 10% glycerol, 1 mM dithiothreitol, 4 mM EGTA, 50 mM KCl, 0.25 mM trans-epoxysuccinyl-L-leucyl-ami-no-(4-guanidino)-butane and 0.25 mM diisopropylfluorophosphate at pH 7.0). Homogenates were subjected to centrifugation at 12,000 × g for 10 min at 4 °C. Supernatant fractions were diluted to 1:2000–1:10,000 in 10 mM MOPS, 1 mM dithiothreitol, and 1 mM EGTA at pH 7.0. Aliquots of the supernatant fractions were used for measurements of kinase activity at 30 °C by 32P incorporation into purified RLC in the presence and absence of Ca2+/CaM where reactions were linear with time and proportional to extract dilution (13, 24).

Kinase Activity and FRET Analysis in COS Cells—COS cells were maintained at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium containing 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1 mM sodium pyruvate, and 10% fetal bovine serum. Cells were plated in 35-mm dishes, and 24 h later, cells were transfected using Lipofectamine 2000 with 3 μg of plasmid DNA containing the skMLCK/CaM sensor sequence under control of the cytomegalovirus promoter. Extracts were prepared from cells 48 h after transfection, and kinase activity was determined with standard Ca2+-methanesulfonate solutions to achieve specific free Ca2+ concentrations as described (24). Additional aliquots of kinase extract were used for fluorescence resonance energy transfer (FRET) analysis using an excitation wavelength of 430 nm and emission wavelengths of 480 and 525 nm on a PerkinElmer Life Sciences 650-105 fluorescence spectrophotometer. Free Ca2+ concentrations were identical to those used for kinase activity measurements. In the absence of Ca2+, excitation of enhanced cyan fluorescent protein at 430 nm in CaM sensor skMLCK resulted in peak fluorescence emission at 525 nm from enhanced yellow fluorescent protein due to FRET. Upon Ca2+/CaM binding, FRET decreased with an increased emission at 480 nm and decreased emission at 525 nm. The emission spectrum of the skMLCK CaM sensor in the absence and presence Ca2+/CaM was similar to that previously reported for the sensor moiety linked to the smooth muscle MLCK (17, 23). The catalytic properties of the CaM sensor skMLCK were similar to wild-type skMLCK, which is similar to the comparison of smooth muscle MLCK with and without the biosensor (17, 23). Thus, the addition of the sensor moiety did not significantly change the catalytic properties of the kinase.
Measurement of skMLCK Activation by Electrical Stimulation in Intact Skeletal Muscle—Activation of skMLCK was measured in HSA-skMLCK (line 3) lumbar muscles from the hind paw by FRET of the CaM biosensor using procedures similar to those described for kinase activation in bladder smooth muscle (17). Lumbar muscles were mounted on a fluorescence myograph (Scientific Instruments, Heidelberg, Germany) in a cylindrical quartz cuvette and perfused with physiological salt solution. The muscle was excited at 436 nm, and emitted light was detected by two photomultipliers using 480- and 535-nm filters. The extent of skMLCK activation was determined from the 480/535-nm emission ratio (subjected to a 10-Hz low pass filter) of intact muscles electrically stimulated at 0.2–300 Hz. In a subset of experiments, muscles were permeabilized following electrical stimulation (10 and 150 Hz) with 100 μM β-escin in the presence of 5 mM CaCl₂ and 4 μM CaM, and the 480/535 nm ratio was continuously recorded digitally on a Powerlab 8/SP. Frequency-dependent activation of skMLCK was expressed as a percentage of the maximal Ca²⁺/CaM response. For rate constant studies, the emission ratio was measured without electronic filtering, and data were acquired at 10 kHz for muscles stimulated at 150 Hz for 0.5 s. Each muscle was stimulated a total of 30 times at 3-min intervals. Acquisitions were averaged after alignment to the initial stimulus pulse using a signal-averaging program written for Matlab. Rate constants were estimated by linear regression of points on the steepest rising and falling aspects. For graphic presentation, data were smoothed using Sigma Plot.

RESULTS

Myosin Light Chain Kinase Protein Expression and Enzymatic Activity—Skeletal muscle MLCK expression was determined in different tissues from skMLCK transgenic mice. The transgene for the skMLCK results in a larger molecular mass on SDS-PAGE due to the CaM biosensor. In tissue samples, transgene expression was readily evident in both fast-twitch (EDL and white quadriceps) and slow-twitch (soleus) skeletal muscle (Fig. 1A). Negligible to undetectable amounts of transgene were observed in heart, urinary bladder, liver, and kidney.

Quantitative analysis by Western blotting was performed in EDL and soleus muscles for the three lines of transgenic mice. In EDL muscle, the amount of total skMLCK (endogenous plus transgenic) was 3.8 ± 0.1-, 17.7 ± 2.2-, and 22.5 ± 0.9-fold greater than wild-type EDL expression for lines 1, 2, and 3, respectively (Fig. 1B). The amount of endogenous skMLCK in EDL muscle was similar between wild-type and transgenic mice in line 1. Conversely, endogenous skMLCK was reduced by 75% in line 2 and 78% in line 3. Expression of skMLCK in soleus muscle from wild-type mice was 14% of wild-type EDL expression. Soleus skMLCK expression in transgenic mice was equal to 1.5 ± 0.2-, 11.9 ± 0.8-, and 16.2 ± 1.6-fold of wild-type EDL. Endogenous expression of skMLCK in soleus muscle was similar between wild-type and transgenic mice from line 1, whereas endogenous skMLCK was not detected in transgenic mice for lines 2 and 3. Thus, the three transgenic lines expressed skMLCK in different amounts greater than endogenous skMLCK with comparable amounts in fast- and slow-twitch muscles. The high amounts of transgene expression in lines 2 and 3 resulted in decreased expression of endogenous skMLCK in both fast-twitch and slow-twitch muscles.

Skeletal muscle MLCK enzymatic capacity was determined in extracts prepared from fast-twitch quadriceps muscle from wild-type and transgenic mice by 32P incorporation into purified RLCp. Skeletal muscle MLCK activity was elevated 1.3 ± 0.1-, 12.3 ± 0.9-, and 15.3 ± 2.0-fold in transgenic mice from lines 1, 2, and 3, respectively (Fig. 1C). The results from enzymatic measurements of kinase activities are consistent with the expression amounts measured by Western blotting.
Expression of a skMLCK CaM Sensor in Skeletal Muscle

Calmodulin and MYPT1/2 Expression—Protein expression of CaM was determined in EDL and soleus muscles from wild-type and transgenic mice from line 3 by Western blotting with purified bovine CaM as a standard. In EDL muscle, expression was 0.29 ± 0.02 versus 0.25 ± 0.02 ng of CaM/µg of total protein for wild-type and transgenic mice (line 3), respectively (Fig. 2A). In soleus muscle, expression was 0.12 ± 0.01 versus 0.13 ± 0.02 ng of CaM/µg of total protein for wild-type and transgenic mice (line 3), respectively. Expression of MYPT1 and -2 was also determined in EDL and soleus muscles from wild-type and transgenic mice (line 3). In EDL muscles from both wild-type and transgenic mice, MYPT1 expression accounted for ~90% of total MYPT expression, whereas similar amounts of MYPT1 and -2 were observed in soleus muscles (Fig. 2B). Neither MYPT1 nor MYPT2 was significantly different between wild-type and transgenic mice in either muscle type. Thus, transgenic overexpression of skMLCK did not affect total amounts of CaM or MYPT1/2 in fast- or slow-twitch muscles.

Potentiation of Isometric Twitch Force and RLC Phosphorylation in Fast-twitch EDL Muscle—Low frequency stimulation of wild-type EDL muscle from each mouse line resulted in a progressive increase of twitch force. Following 15 s of 10-Hz stimulation, twitch force increased to greater than 50% in wild-type EDL muscle. EDL muscles from line 1 showed a similar staircase effect to wild-type mice (Fig. 3, A and B). Transgenic mice from line 3, the highest expressing line, displayed a more rapid increase in twitch force with significantly greater levels of potentiation at the 3–12-s measurements (Fig. 3, E and F). A similar effect was observed in transgenic mice from line 2 (data not shown). Following the maximal potentiation response, a small degree of fatigue was observed prior to 15 s in transgenic EDL muscles (Fig. 3, E and F). A similar response is observed in wild-type EDL muscles following maximal potentiation at 15 s (data not shown).

Phosphorylation of RLC was similar in EDL muscles in the quiescent state in both genotypes of all three lines. In line 1, there was a marginal but significant increase in RLCr phosphorylation in transgenic versus wild-type mice following 3 s of stimulation of EDL muscles (Fig. 3, C and D). No difference in RLCr phosphorylation between genotypes was observed following 15 s of stimulation. In line 3, RLCr was markedly increased versus wild-type after 3 s (0.36 ± 0.02 versus 0.56 ± 0.01 mol of phosphate/mol of RLCr for wild-type and transgenic mice, respectively) and 15 s of stimulation (0.52 ± 0.02 versus 0.71 ± 0.02 mol of phosphate/mol of RLCr for wild-type and transgenic mice, respectively, Fig. 3, G and H). Values similar to line 3 were observed for line 2 (data not shown). Thus, expression of skMLCK in fast-twitch muscle increases the rates of RLCr phosphorylation and twitch potentiation comparable with the greater amounts of kinase.

Potentiation of Isometric Twitch Force and RLC Phosphorylation in Slow-twitch Soleus Muscle—Maximal twitch potentiation in wild-type soleus muscle ranged from 1.08- to 1.14-fold. No significant difference between wild-type and transgenic soleus muscle contraction responses was observed at any of the indicated time points in transgenic line 1 (data not shown). In line 3, a small but significantly greater twitch potentiation (1.11 ± 0.04 versus 1.23 ± 0.04-fold, p < 0.05) was observed at 12 s (Fig. 3, I and J). In line 2, a similar minimal potentiation response was observed with significant differences observed at 6–12 s (data not shown).

Phosphorylation of RLCr and slow-twitch regulatory light chain (RLCq) was determined in soleus muscles from lines 3. In wild-type soleus muscle, electrical stimulation for 15 s increased RLCr phosphorylation from 0.08 ± 0.02 to 0.17 ± 0.02 mol of phosphate/mol of RLCr (Fig. 3, K and L). No increase in RLCr was observed after 3 s in wild-type mice. RLCr phosphorylation was increased in transgenic versus wild-type soleus after 3 and 15 s of stimulation. The maximal extent of RLCr phosphorylation in transgenic soleus muscles was 0.62 ± 0.05 mol of phosphate/mol of RLCr. RLCr was unaltered by electrical stimulation in wild-type soleus muscle, whereas phosphorylation rates increased in soleus muscles from transgenic animals from 0.10 ± 0.01 and 0.44 ± 0.02 mol of phos-
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FIGURE 3. Twitch force potentiation and RLC phosphorylation in fast- and slow-twitch skeletal muscle. A–D, responses for transgenic (TG) line 1. A, representative force tracings for fast-twitch EDL muscle from wild-type (upper panels) and transgenic (lower panels) mice. B, mean force development values for EDL muscle from wild-type (open circles) and transgenic mice (open circles). C and D, representative Western blot image following urea/glycerol-PAGE (C) and mean values (D) for RLC phosphorylation without electrical stimulation (open bars), or following 3 s (hatched bars) or 15 s (closed bars) of 10-Hz electrical stimulation. WT, wild-type. E–L, responses from transgenic line 3. E, representative force tracings for EDL muscle from wild-type (upper panels) and transgenic (lower panels) mice. F, mean force development values for EDL muscle from wild-type (closed circles) and transgenic (open circles) mice. G and H, representative Western blot image following urea/glycerol-PAGE (G) and mean values (H) for RLC phosphorylation in EDL without electrical stimulation (open bars) or following 3 s (hatched bars) or 15 s (closed bars) of 10-Hz electrical stimulation. I, representative force tracings for slow-twitch soleus muscle from wild-type (upper panels) and transgenic (lower panels) mice. J, mean force development values for soleus muscle from wild-type (closed circles) and transgenic mice (open circles). K, representative Western blot image following two-dimensional urea/glycerol-PAGE and SDS-PAGE for RLC and RLC phosphorylation in soleus muscle from wild-type and transgenic mice following 15 s of electrical stimulation at 10 Hz (arrowheads indicate phosphorylated forms of RLC). L, mean values for RLC and RLC phosphorylation without electrical stimulation (open bars) or following 3 s (hatched bars) or 15 s (closed bars) of electrical stimulation at 10 Hz. Values represent mean ± S.E. (n = 5–11 for B–J, n = 3–6 for I); *, p < 0.05; **, p < 0.0001 versus wild-type.

potentiation is a well established physiological property of fast-twitch skeletal muscle

that occurs in response to repetitive stimulation (1, 2). Reports that RLC is phosphorylated in response to electrical stimulation (5, 25) and that the magnitude of twitch force potentiation is proportional to the extent of RLC phosphorylation (3, 5, 13) represent early evidence that RLC in skeletal muscle is a biochemical mechanism responsible for frequency-dependent twitch force potentiation. In striated muscle, Ca2+/CaM phosphorylation is primarily mediated through a thin filament regulatory system, troponin-tropomyosin. Although sarcomeric RLC phosphorylation has no effect on actin-activated ATPase activity of striated

Activation of skMLCK in Response to Electrical Stimulation—

We have used the CaM biosensor, which is part of the transgene construct, to measure skMLCK activation in response to repetitive electrical stimulation in intact lumbrical muscles. The utility of this biosensor as an indicator of skMLCK activation was demonstrated in transfected COS cells. Binding of Ca2+/CaM

to the biosensor measured by FRET and kinase activation determined by 32P incorporation into purified RLC showed similar calcium dependence (Fig. 4A). Thus, the change in FRET measures Ca2+/CaM binding coincident to kinase activation. Similar results were reported for a smooth muscle MLCK biosensor (24). The expression of the skMLCK biosensor is readily apparent with fluorescence images of intact muscle (Fig. 4B).

The rate and extent of skMLCK activation was proportional to the electrical stimulation frequency from 0.2 to 150 Hz (Fig. 4, C and D). Normalization of the responses after maximal activation of the CaM biosensor with Ca2+/CaM in permeabilized fibers showed 24 ± 7 and 59 ± 7% activation at 10 and 150 Hz, respectively (Fig. 4E). Stimulation at 50 and 300 Hz resulted in activation similar to that obtained at 150 Hz. Thus, Ca2+/CaM appears to be limiting for skMLCK biosensor activation at high frequencies of contraction.

Maximal activation and inactivation rates were determined for skMLCK in relation to force development and relaxation (Fig. 4F). skMLCK activation occurred rapidly with no apparent latency relative to force development (Fig. 4F, inset). The activation and inactivation rate constants were 10.6 ± 0.2 and 2.8 ± 0.2 s−1, respectively, whereas the rate constants for force development and relaxation were 38.2 ± 1.7 and 33.4 ± 2.3 s−1, respectively.

DISCUSSION

Potential of twitch force is a well established physiological property of fast-twitch skeletal muscle

phosphate/mol of RLC in response to 15 s of stimulation. Furthermore, RLC phosphorylation was significantly greater in soleus muscle from transgenic versus wild-type mice following 3 and 15 s.

Activation of skMLCK in Response to Electrical Stimulation—

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muscle myosins, sarcomeric RLC phosphorylation in both skinned skeletal muscle fibers and skinned cardiac muscle fibers leads to a leftward shift in the force-pCa relationship, thus increasing the calcium sensitivity of the myofilaments (Ca²⁺ sensitization) (4, 26–28). Phosphorylation of RLC increases the cross bridge transition to the strongly bound, force-generating state, whereas slowing the rate of decay of the force-generating state. Repetitive increases in cytosolic Ca²⁺ necessary for contractile responses would also result in Ca²⁺ binding to CaM and Ca²⁺/CaM then binding to skMLCK for activation (29, 30). Based on the known biochemical properties of skMLCK, a quantitative model predicted 1) the importance of the interpulse interval for fractional kinase activation; 2) limiting kinase activity so that RLCf was phosphorylated in seconds, not milliseconds; and 3) the much greater kinase activity relative to the phosphatase activity so that a small fractional activation of kinase resulted in RLCf phosphorylation that was sustained (4, 13).

Recently, we provided genetic support for this model using skMLCK knock-out mice (6). In contrast to wild-type littermates, electrical stimulation was without effect on RLCf phosphorylation in response to electrical stimulation in skMLCK knock-out mice. This loss of RLCf phosphorylation was accompanied by markedly reduced potentiation of twitch force. These results with skMLCK knock-out mice provide direct evidence for the involvement of skMLCK-mediated RLCf phosphorylation to induce potentiation of twitch force in fast-twitch skeletal muscle.

Because phosphorylation of RLCf by skMLCK is dependent on both the catalytic activity of skMLCK as well as the binding of Ca²⁺/CaM to skMLCK, we determined whether either skMLCK or CaM was limiting to the frequency-dependent RLCf phosphorylation and the subsequent potentiation of twitch force. We show that overexpression of skMLCK results in a gain of function at the level of RLCf phosphorylation and twitch force potentiation without a corresponding up-regulation in CaM expression. Therefore, in fast-twitch EDL muscle, the amount of skMLCK appears limiting for RLCf phosphorylation.

Reports of robust frequency-dependent potentiation of twitch force have thus far been restricted to fast-twitch muscle (1). Previously, differences in the magnitude of responses to twitch potentiation and RLC phosphorylation have been reported in rat fast-twitch white (type IIb fibers) versus slow-twitch red (type I fibers) skeletal muscle (13). Greater RLC phosphorylation was suggested to be the consequence of a 14-fold greater kinase to phosphatase activity in fast versus slow muscle. It was also noted that fast-twitch, red (type IIa fibers) contained less skMLCK, resulting in less RLC phosphorylation with electrical stimulation (6). Consistent with this finding, we observed that expression of skMLCK in wild-type mouse soleus muscle was 10% of EDL muscle. Furthermore, twitch force potentiation in wild-type mouse soleus muscle was limited to an 8–14% increase. We considered that the lack of significant potentiation in soleus muscle was due to minimal...
skMLCK-induced RLC phosphorylation and that overexpression of skMLCK in soleus muscle would result in twitch force potentiation similar to that observed in wild-type fast-twitch EDL muscle. Despite achieving transgene expression similar in magnitude in both EDL and soleus muscle, overexpression of skMLCK in soleus muscle did not mediate a robust twitch force potentiation despite a marked frequency-dependent phosphorylation of both RLC\(_f\) and RLC\(_v\) in transgenic soleus muscle. Like slow-twitch skeletal muscle fibers, cardiac ventricular myofibrils also contain RLC\(_v\) (also called ventricular RLC; RLC\(_v\); and MLC2v). Nevertheless, results from studies investigating the effects of RLC\(_v\) phosphorylation on force development in intact cardiac muscle are in stark contrast to those observed in soleus muscle in the current investigation. In skinned cardiac muscle fibers, phosphorylation of RLC\(_v\) results in increased force and rate of cross bridge recruitment at submaximal Ca\(^{2+}\) concentrations (15, 31). Likewise, transgenic mice expressing a non-phosphorylatable RLC\(_v\) exhibit reduced systolic pressure and contractility, indicating that phosphorylation of RLC\(_v\) affects force development in intact cardiac muscle (32).

The reason for the lack of twitch potentiation with substantial RLC phosphorylation in soleus muscles from transgenic mice is not clear. C57BL/6 mouse EDL muscle contains 18% type IIa and 70% type IIb fibers (33). In contrast, the mouse soleus muscle contains as little as 6% type IIb fibers with 59% type IIa and 35% type I fibers (34). Thus, the potentiation of contraction is correlated to type IIb fibers. Functional studies relating RLC phosphorylation to isometric twitch force potentiation in type IIa fibers are not reported, although these fibers contain a smaller amount of skMLCK than type IIb fibers, resulting in less RLC phosphorylation with repetitive stimulation (13). Electron micrographs of isolated thick filaments show that the myosin heads undergo a conformational change with phosphorylation that moves them away from the backbone of the thick filament, allowing the myosin head to be in closer proximity to the thin actin filament (35, 36). Changing the spacing between thin and thick filaments by osmotic compression or elongating sarcomere length brings the myosin heads in closer proximity to the actin filament, mimicking the effect of RLC phosphorylation (36). Potentially, the interfilament spacing of type I and type IIa fibers may be sufficiently small for optimal force development even if RLC is not phosphorylated. There may also be differences in RLC and myosin heavy chain interactions of the different myosin isoforms that affect head displacements or other biochemical differences intrinsic to the muscle fiber types.

Over the past decade, FRET has been used to measure many real-time cellular signaling events (37). It has been suggested that numerous CaM-dependent proteins must compete for CaM, which is available in a limited pool in cells in culture (38, 39) and cardiomyocytes (40). Transgenic mice expressing a smooth muscle MLCK (smMLCK) CaM biosensor showed that the kinase was not fully activated under conditions of maximal increases in cytosolic Ca\(^{2+}\) concentrations in smooth muscle cells in bladder tissue (17). The smMLCK transgene was expressed at only 30% of the endogenous kinase and thus served as a reporter for Ca\(^{2+}/\)CaM binding and activation. In the present study, we have examined skMLCK using a similar CaM biosensor to study calmodulin binding and activation of skMLCK in intact skeletal muscle fibers. Results show 60% maximal activation of the kinase. However, because the skMLCK transgene is expressed at 20-fold greater amounts than the endogenous kinase, we conclude that CaM is unlikely to be a limiting factor for skMLCK activation in wild-type skeletal muscle. This conclusion is consistent with enhanced frequency-dependent RLC\(_v\) phosphorylation in EDL muscle in transgenic muscle with unaltered CaM expression.

Computer modeling based on the biochemical Ca\(^{2+}\) binding properties suggests that Ca\(^{2+}\) binds to troponin and CaM at a similar rate (30). Our results show that skeletal muscle force development increases at a faster rate than CaM binding to skMLCK, indicating that conformational changes that are part of the excitation-contraction coupling events distal to Ca\(^{2+}\) binding to troponin occur more rapidly than the formation of the Ca\(^{2+}/\)CaM/skMLCK complex. Rapid kinetic measurements indicate a diffusion controlled, bimolecular association of Ca\(^{2+}/\)CaM with skMLCK (41). Furthermore, the association of Ca\(^{2+}/\)CaM with skMLCK results in rapid activation without an apparent conformational latency. Although Ca\(^{2+}\) dissociates faster from CaM than from troponin (30), the rate of dissociation of Ca\(^{2+}\) from CaM bound to skMLCK is slower, resulting in dissociation of CaM from the kinase at 3 s\(^{-1}\) (42). This biochemical dissociation rate is similar to the rate measured herein in vivo (2.8 s\(^{-1}\)). Importantly, this slow rate of CaM dissociation from skMLCK allows for a longer period of skMLCK activation between Ca\(^{2+}\) transients at low frequencies of contraction. Thus, we provide insights into the mechanism by which RLC phosphorylation leads to a biochemical memory for enhanced twitch force.

In conclusion, skMLCK is limiting for frequency-dependent phosphorylation of RLC in fast- and slow-twitch skeletal muscles. RLC phosphorylation enhances twitch-force potentiation only in fast-twitch EDL muscle, presumably in type IIb fibers. Consistent with a gain of skMLCK function in transgenic skeletal muscle, FRET analysis shows that CaM is only partially limiting to skMLCK activation in transgenic mice and thus unlikely to be limiting under normal conditions in wild-type mice.

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