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Estradiol modulates myosin regulatory light chain phosphorylation and contractility in skeletal muscle of female mice

Shaojuan Lai,[1,3] Brittany C. Collins,[1] Brett A. Colson,[2] Georgios Kararigas,[4] and Dawn A. Lowe[1]

[1]*Programs in Rehabilitation Sciences and Physical Therapy, Department of Physical Medicine and Rehabilitation, Medical School, University of Minnesota, Minneapolis, Minnesota;* [2]*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota;* [3]*College of Biological Engineering, Henan University of Technology, Zhengzhou, Henan, China; and* [4]*Institute of Gender in Medicine, Charite University Hospital, and German Centre for Cardiovascular Research (DZHK), Partner Site, Berlin, Germany*

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Lai S, Collins BC, Colson BA, Kararigas G, Lowe DA. Estradiol modulates myosin regulatory light chain phosphorylation and contractility in skeletal muscle of female mice. *Am J Physiol Endocrinol Metab* 310: E724–E733, 2016. First published March 8, 2016; doi:10.1152/ajpendo.00439.2015.—Impairment of skeletal muscle function has been associated with changes in ovarian hormones, especially estradiol. To elucidate mechanisms of estradiol on skeletal muscle strength, the hormone's effects on phosphorylation of the myosin regulatory light chain (pRLC) and muscle contractility were investigated, hypothesizing an estradiol-specific beneficial impact. In a skeletal muscle cell line, C_2C_{12}, pRLC was increased by 17β-estradiol (E_2) in a concentration-dependent manner. In skeletal muscles of C57BL/6 mice that were E_2 deficient via ovariectomy (OVX), pRLC was lower than that from ovary-intact, sham-operated mice (Sham). The reduced pRLC in OVX muscle was reversed by in vivo E_2 treatment. Posttetanic potentiation (PTP) of muscle from OVX mice was low compared with that from Sham mice, and this decrement was reversed by acute E_2 treatment, demonstrating physiological consequence. Western blot of those muscles revealed that low PTP corresponded with low pRLC and higher PTP with greater pRLC. We aimed to elucidate signaling pathways affecting E_2-mediated pRLC using a kinase inhibitor library and C_2C_{12} cells as well as a specific myosin light chain kinase inhibitor in muscles. PI3K/Akt, MAPK, and CamKII were identified as candidate kinases sensitive to E_2 in terms of phosphorylating RLC. Applying siRNA strategy in C_2C_{12} cells, pRLC triggered by E_2 was found to be mediated by estrogen receptor-β and the G protein-coupled estrogen receptor. Together, these results provide evidence that E_2 modulates myosin pRLC in skeletal muscle and is one mechanism by which this hormone can affect muscle contractility in females.

estrogen; estrogen receptor; kinase; post tetanic potentiation; RLC

OVARIAN HORMONE DEFICIENCY is related to muscle dysfunction and loss of strength. For example, declines in strength are accelerated around the time of menopause (35, 41, 51), and skeletal muscles of postmenopausal women on estrogen-based hormone therapy are stronger than those not on hormone therapy (10, 17, 49). In mouse models, contractile characteristics are impaired in muscles of ovariectomized compared with sham-operated mice (16, 38, 39, 53, 65). It was suggested (41) and subsequently demonstrated that ovarian hormones influence muscle strength by directly affecting the function of contractile proteins (43, 66), particularly the structure and function of myosin (38, 39). Among the ovarian hormones, 17β-estradiol (E_2) appears to be the key hormone, because it reverses muscle weakness and contractile protein dysfunction caused by ovariectomy. Specifically, reduced muscle and contractile protein functions were restored when E_2 was administered to ovariectomized mice (16, 38, 53). In a chemical model of premature aging, of which the ovaries of young adult mice were locally and specifically induced to be senescent, E_2 treatment improved some muscle contractile functions (18). Although these studies have demonstrated that E_2 impacts muscle and myosin functions, the molecular mechanisms by which this hormone affects the structure and function of myosin, and thus strength, remain to be elucidated.

E_2 affects multiple signaling pathways via both genomic and nongenomic manners. While two estrogen receptors (ERs), ERα and ERβ, had originally been shown to exist in skeletal muscle, a third ER, the G protein-coupled receptor (GPER), has also been identified in skeletal muscles (2, 5). An important molecular event in these signaling pathways mediated by E_2 involves the downstream regulation of transcription by the ERs. For example, ERs have been implicated in the regulation of gene transcription in myoblasts, including kinases that regulate GLUT4, myogenin, and myosin heavy chain expression (12). E_2 is also an important molecule activating several kinases and kinase cascades, such as phosphatidylinositide 3-kinase/Akt (PI3K/Akt), MAPK, and AMP-activated protein kinase (AMPK), in a multitude of cells and tissues including skeletal muscle (46, 48, 68). These cellular processes result in altered kinase activity and thus protein phosphorylation. Since protein phosphorylation is one critical regulatory event that can affect muscle contractility (e.g., Ref. 55), we postulated that E_2 exerts its impact on muscle and myosin functions by regulating protein phosphorylation events, particularly those occurring on contractile proteins such as myosin.

Myosin belongs to a superfamily of motor proteins and in striated muscle consists of two heavy chains and two pairs of light chains, the essential light chain and the regulatory light

Address for reprint requests and other correspondence: D. A. Lowe, MMC 388, 420 Delaware St. SE, Minneapolis, MN 55455 (e-mail: lowex017@umn.edu).

chain (RLC). While the essential light chain is considered to play a role in stabilizing the overall structure of myosin, the RLC acts as a lever arm on the α-helical neck region of the myosin heavy chain to transmit free energy of ATP hydrolysis down the neck domain to amplify movements of myosin and force production during contraction (44). Ser^{15} of the RLC can be phosphorylated by myosin light chain kinase (MLCK) (59), and other kinases, such as Rho-associated kinase (ROCK) and ERK1/2, may also phosphorylate serine or threonine sites of the RLC (1, 20). When RLC is not phosphorylated, the light chain domain of myosin is positioned close to the thick filament backbone (67, 71). However, when phosphorylated, the light chain domain projects from the thick filament backbone so that myosin heads are better positioned to bind actin thin filament and initiate the power stroke (6, 28, 36). Thus, by undergoing phosphorylation, RLC modulates myosin structure, molecular force generation, and power output of striated muscle (6, 69). RLC phosphorylation (pRLC) is such a critical mechanism in the heart that specific mutations that disrupt the phosphorylation of RLC in cardiac myosin reduce force and cause cardiomyopathy (15, 40, 55).

In mouse cardiomyocytes, E_2 plays a role in regulating pRLC, which correlates with contractility of those cells (24, 25). Here, we hypothesized that E_2 improves skeletal muscle and myosin functions by increasing pRLC. We examined the related cellular pathways mediated by E_2 on pRLC, as well as the effects on force generation. We present data demonstrating that E_2 mediates phosphorylation of the myosin RLC in skeletal muscle through ERβ and GPER and several kinases, including PI3K, MAPK, and Ca^{2+}/calmodulin-dependent protein kinase II (CamKII). Our results show that the biochemical effect of phosphorylating RLC by E_2 in skeletal muscle has a physiological impact on contractility.

MATERIALS AND METHODS

Reagents. Sources for reagents were: 17β-E_2 (Sigma, St. Louis, MO); ML-7 (Tocris Bioscience, Bristol, UK); Screen-Well kinase inhibitor library (Enzo Life Sciences, Farmingdale, NY); phosphatase inhibitor cocktail II and III (P5726 and P0044, respectively, Sigma); Complete, Mini, EDTA-free protease inhibitor cocktail (Roche; Sigma); antibodies to ERα and ERβ (F3-A and 68-4, respectively; Millipore, Billerica, MA), GAPDH (ab9485; Abcam, Cambridge, MA), phosphorylated RLC and RLC (ab2480 and ab48003, respectively, Abcam); control siRNA, ERα siRNA, ERβ siRNA, and GPER siRNA (Dharmacon, Layfayette, CO); HiperFect transfection reagent (Qiagen, Valencia, CA); DMEM (GIBCO, Grand Island, NY); fetal bovine serum and penicillin-streptomycin (Invitrogen, Grand Island, NY); TRIzol Reagent (Thermo Fisher Scientific, Grand Island, NY). All components of the Krebs-Ringer bicarbonate buffer for the in vitro muscle preparation were from Sigma.

Cell culture. C_2C_{12} muscle cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, at 37°C in 5% CO_2. One day before experiments, cells were plated onto six-well dishes and 24 h later were 70–80% confluent. Prior to E_2 treatment, cells were washed once with 1× PBS and incubated in DMEM containing no serum (serum deprivation) for 30 min. 17β-E_2 dissolved in isopropanol or vehicle control (i.e., isopropanol only) was added to cells and cells were incubated for 30 min in the incubator. Cells were then immediately lysed in ice-cold NP-40 lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1% NP-40), containing protease inhibitor and phosphatase inhibitor II and III cocktails. This lysate was centrifuged and the supernatant used for Western blotting.

For siRNA knockdown experiments, cells were plated onto six-well plates and transfected with control siRNA or ER isoform-specific siRNA using HiperFect transfection reagent according to the manufacturer's fast-forward protocol. Cells were lysed 48 or 72 h after transfection in NP-40 lysis buffer as described above.

For kinase inhibitor experiments, C_2C_{12} cells were plated onto six-well plates at a cell confluence of 70–80%. Seventy of the eighty kinase inhibitors from the kinase inhibitor library were tested (10 that were not tested were those known to be nonspecific). Each inhibitor was individually dissolved in DMSO as a 10 mM stock solution. Inhibitor + E_2 or DMSO + E_2 were applied to cells for a final concentration of 10 μM inhibitor (27) and 1 μM E_2. The selective MLCK inhibitor ML-7 was also tested at 40 μM. The final concentration of DMSO in the cell culture medium was ≤0.5% (vol/vol). Cells were incubated for 30 min at 37°C with 5% CO_2. Cells were lysed in NP-40 as described and then used for Western blotting to determine pRLC and total RLC. All cell experiments were carried out in phenol red-free medium and done in triplicate.

Animals. Female C57BL/6 mice aged 3–4 mo were purchased from the National Institute on Aging or were bred on site. Mice underwent bilateral ovariectomy surgeries (OVX) or sham operations (Sham), which consisted of the same procedures as the OVX except that the ovaries were not removed. For some experiments, subgroups of OVX mice immediately received E_2 treatment (OVX+E_2) via 60-day time release pellets, as described previously (38). Muscles from mice were studied 4–6 wk postsurgery, except for a subgroup of Sham and OVX mice that were studied 5 mo after surgeries in one experiment (detailed in legend of Fig. 3). On the day of euthanasia, mice were anesthetized by injection of pentobarbital sodium (100 mg/kg body mass ip), and muscles were dissected. Uteri were dissected and weighed as an indicator of successful ovariectomy surgery or E_2 replacement [mean ± SD uterine mass for Sham, OVX, OVX+E_2: 107.4 (37.2), 14.0 (3.7), and 136.3 (83.6) mg, respectively]. While under anesthesia, mice were euthanized by exsanguination. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and complied with the American Physiological Society guidelines.

In vitro contractility and posttetanic potentiation. Extensor digitorum longus (EDL) or soleus muscles were dissected and mounted in a 1.5-ml bath assembly containing Krebs-Ringer bicarbonate buffer maintained at 25°C. The proximal tendon was connected by 5-0 silk suture to the arm of a muscle lever system (300B-LR; Aurora Scientific, Aurora, ON, Canada), while the distal tendon was tied by suture and stabilized so that no movement occurred. Muscles were preincubated for 30 min prior to any contractile activity. During this time, optimal muscle length was maintained by keeping resting tension at 0.4 or 0.5 g for EDL and soleus muscles, respectively (63, 64). For experiments investigating MLCK's involvement in RLC phosphorylation by E_2, the selective MLCK inhibitor ML-7 at 25 μM for EDL and 2 μM for soleus or an equal volume of ethanol vehicle was added to the Krebs buffer during the 30-min preincubation. A subgroup of muscles was incubated with ML-7 for 30 min, and then 1 μM E_2 was added for 30 min and posttetanic potentiation (PTP) was measured. For experiments testing the acute presence of E_2 on PTP of EDL muscles, 1 μM E_2 was added to the Krebs buffer during the 30-min preincubation prior to PTP contractions.

The PTP protocol for EDL muscles was adapted from Stull et al. (70). Following the 30-min preincubation, a baseline isometric twitch was elicited by stimulating the muscle with a 0.5-ms pulse at 150 V (S48 with SIU5D stimulus isolation unit; Grass Technologies, Warwick, RI). Three twitches were performed with 1-min rest in between each pulse. The muscle was then stimulated at 150 Hz and 150 V for 2,000 ms to produce a prolonged, isometric tetanic contraction. Fifteen seconds following the tetanic contraction, the posttetanic twitch was elicited. PTP was calculated as the percent increase in force from the baseline twitch (the greatest of the three initial twitches) to the posttetanic twitch. The maximal rate of force devel-

opment ($+dP/dt$) from these same twitches were determined, and the percent increase was calculated from the baseline to the posttetanic twitch.

For soleus muscle, there is minimal potentiation of twitch force (<10%) elicited by the EDL PTP protocol or variations of that protocol (50, 60), and in preliminary experiments we found the same. Because it would be difficult to detect attenuation from this already low level of potentiation, we developed a tetanic potentiation protocol for soleus muscles based on the concept *1*) of staircase potentiation (60), only using a higher stimulation frequency to elicit tetanic rather than twitch contractions; *2*) that high stimulation frequency is needed to phosphorylate RLC in soleus muscle (37); *3*) that soleus muscle is fatigue resistant and can endure repetitive, long-duration stimulations during the potentiating contractions without run down; and the knowledge *4*) that stronger stimulus factors such as concentric force and work modulate the extent of potentiation (69). Thus, for soleus muscle the potentiating contractions were elicited through a series of maximal isometric tetanic contractions, again following a 30-min preincubation. Specifically, soleus muscles were stimulated at 120 Hz and 150 V for 400 ms every 2 min until the peak force produced during those contractions plateaued, which typically occurred at eight contractions. Tetanic potentiation (increase in P_o) was calculated as the percent increase in force from the first to final tetanus.

EDL and soleus muscles were quickly removed from the bath assembly after potentiation protocols and frozen within 20 s of the final contraction (69). Muscles were stored at −80°C until examined by Western blot for total RLC and pRLC.

Western blots. Mouse EDL or soleus muscles were homogenized in NP-40 lysis buffer containing protease inhibitor and phosphatase inhibitor II and III cocktails, using bullet blender according to the manufacturer's instructions (Midwest Scientific, St. Louis, MO). Homogenates were centrifuged at 664 *g* for 10 min at 4°C to remove connective tissue. The supernatant containing myofilaments was collected, and protein concentrations were determined. Loading buffer was added to these protein lysates or to the C_2C_{12} cell lysates and were loaded on SDS-PAGE gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes and probed for ERα, ERβ, or pRLC for either 2.5 h at room temperature or overnight at 4°C. Membranes were washed, incubated with a secondary antibody for 1 h, washed, and then scanned and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Blots initially probed for pRLC or ER were stripped and reprobed for total RLC or GAPDH, respectively. Under circumstances when stripping was not possible or successful, duplicate gels of the same lysate were run, e.g., one gel for pRLC and the other gel for total RLC.

The Abcam RLC and pRLC antibodies we used were generated against myosin RLC isoforms found in smooth and cardiac muscle and nonmuscle cells, according to Abcam product information. In preliminary experiments, we tested these antibodies, as well as several other commercially available RLC antibodies, for cross-reactivity to skeletal muscle RLC. For this, we used purified myosin (33) and permeabilized, isolated skeletal muscle fibers from rabbit and mouse muscle (7). Figure 1 shows cross-reactivity of the Abcam RLC and pRLC antibodies to skeletal muscle RLC similar to that reported by others (20), and also shows sensitivity to phosphorylated and dephosphorylated conditions (7, 58). Because there is expected to be minimal to no smooth or cardiac RLC in purified skeletal muscle myosin or in single skeletal muscle fibers, the strong bands detected are skeletal muscle RLC and demonstrate specific cross-reactivity of these antibodies. Furthermore, we had previously confirmed that this standard protocol for rendering permeabilized skeletal muscle with either dephosphorylated or phosphorylated RLC and the Western blot pRLC antibody detection method yielded results consistent with pRLC detection methods using isolated striated muscle fibers (6, 58).

qPCR. RNA from C_2C_{12} cells was isolated using TRIzol Reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA according to directions in the VILO cDNA Synthesis Kit. Relative quantitation of the GPER transcript was determined using SYBR Green Master Mix and the following primers for GPER (forward TTCCTCACCTGGATGAGCTT; reverse CCTGACATCAGCAAAGCAGA).

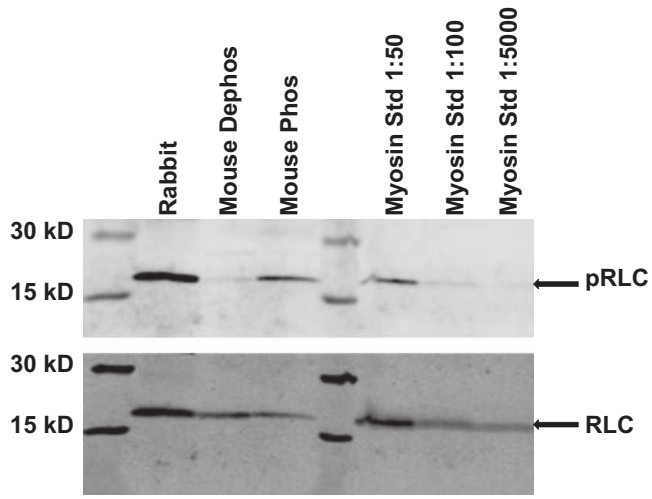


Fig. 1. Immunoblots showing cross-reactivity of anti-myosin light chain antibodies (Abcam, ab2480 and ab48003) to skeletal muscle phosphorylated myosin regulatory light chain (pRLC) and RLC, respectively. Protein samples, 30 μg each, are from single permeabilized skeletal muscle fibers of rabbit (*lane 2*) and mouse (*lanes 3–4*). The sample in *lane 3* was dephosphorylated and in *lane 4* phosphorylated, as described in Ref. 58. *Lanes 6–8* contain dilutions of purified myosin (3.4 mg/ml) from rabbit psoas muscle.

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were done by Student's *t*-tests or one-way ANOVAs. Two-way ANOVAs were used to determine the effect of E_2 vs. vehicle on PTP and pRLC of OVX vs. Sham muscles. When a significant main effect or interaction was detected, Holm-Sidak post hoc tests were done. All statistics were performed using SigmaStat 12.0 (Systat Software, San Jose, CA) with significance accepted at $P < 0.05$.

RESULTS

RLC phosphorylation is affected by E_2 in skeletal muscle cells and skeletal muscle. To determine if RLC phosphorylation in skeletal muscle cells is responsive to E_2, C_2C_{12} cells were treated with various concentrations of E_2 for 30 min. As shown in Fig. 2*A*, phosphorylation of the pRLC was increased by E_2 treatment in a concentration-dependent manner ($P = 0.005$). Total RLC remained unchanged across the E_2 concentrations ($P = 0.180$).

To support this finding with in vivo data, pRLC was measured and found to be different in skeletal muscles from female mice with and devoid of ovarian hormone production ($P = 0.014$; Fig. 2*B*). pRLC was ~35% lower in EDL muscles from OVX mice compared with Sham mice; however, in vivo treatment of OVX mice with E_2 restored pRLC levels to those of Sham mice. Therefore, the decrement in RLC phosphorylation can be attributed to the hormone E_2. Similar results were found in soleus muscles, with pRLC in OVX mice being ~50% lower than that from Sham and OVX+E_2 mice ($P \leq 0.016$). Total RLC content did not differ among muscles from these three groups of mice for either EDL or soleus muscle ($P \leq 0.143$). Together, these in vitro cell culture and in vivo muscle data demonstrate that RLC phosphorylation in skeletal muscle is affected by the surrounding E_2 levels.

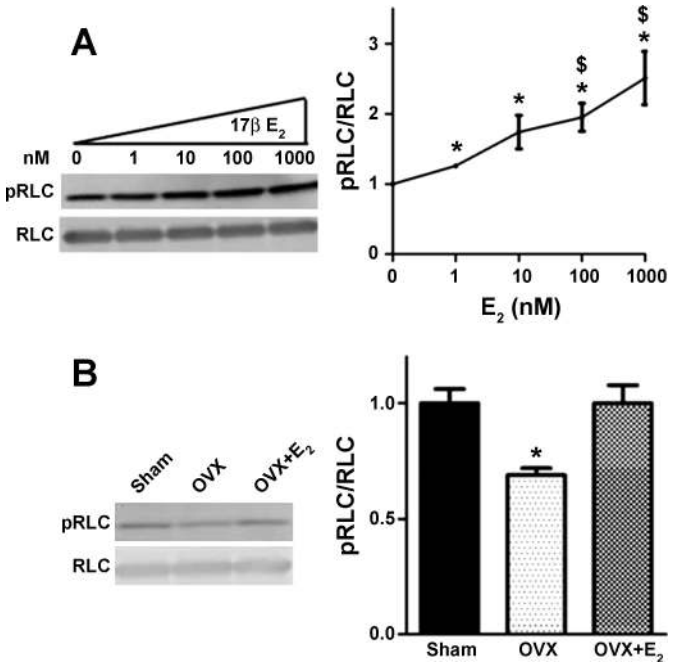


Fig. 2. 17β-Estradiol (E_2) increases phosphorylation of the myosin RLC (pRLC) in C_2C_{12} cells and skeletal muscle. *A*: exogenously added E_2 increased pRLC in C_2C_{12} cells. pRLC was determined relative to total RLC by Western blot analyses and normalized to 0 nM E_2 control condition ($n = 3$ per concentration). *B*: pRLC in EDL muscle from estrogen-deficient mice (OVX) was low compared with those from mice with normal E_2 levels (Sham and OVX+ E_2; $n = 6–7$ per group; muscles studied 4–6 wk postsurgery). pRLC was determined relative to total RLC by Western blot analyses and normalized to Sham control condition. Total RLC content was not affected by E_2 in either experiment. Data are means ± SE. *Significantly different from control (0 nM in A; Sham and OVX+E_2 in B); $Significantly different from 1 nM.

E_2-mediated RLC phosphorylation impacts muscle contractility. To determine the extent to which RLC phosphorylation mediated by E_2 results in a functional consequence, EDL muscles were dissected from Sham and OVX mice and studied in vitro for PTP (Fig. 3, *A* and *B*). Peak force of the unpotentiated, baseline twitches were not different between Sham and OVX mice (56.2 ± 2.3 and 54.1 ± 2.7 mN, respectively, $P = 0.405$). Potentiated twitches generated 25–50% more force than unpotentiated twitches; however, the extent of PTP depended on the presence of E_2 (Fig. 3*C*; interaction $P = 0.046$). In particular, PTP of vehicle-incubated EDL muscles was more than two times greater in those from Sham than OVX mice (51.6 ± 6.7 vs. 24.1 ± 7.1% increase, $P = 0.009$; Fig. 3*C*), indicating that the chronic deficiency of circulating E_2 negatively impacts muscle force potentiation. Acute exposure of muscles from OVX mice to E_2 in the bath resulted in greater PTP ($P = 0.036$; Fig. 3*C*). EDL muscles that were chronically exposed to endogenous E_2 (Sham mice) had relatively high PTP regardless of vehicle or E_2 in the bath.

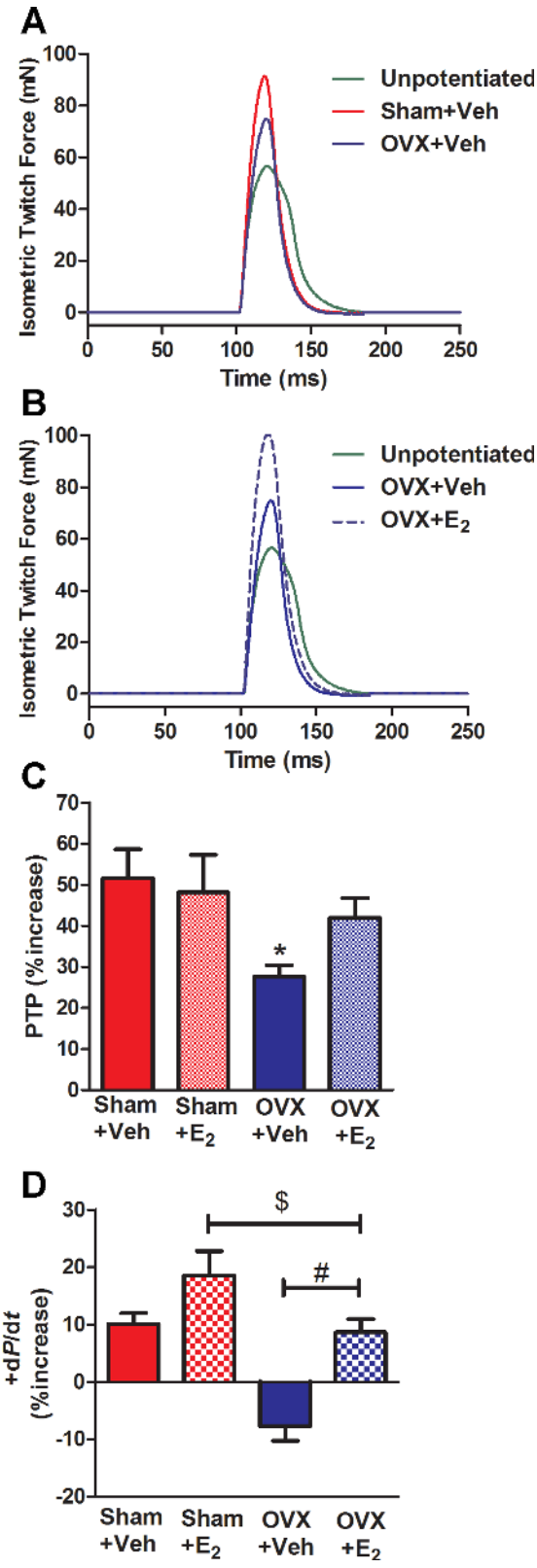
The maximal rate of force development ($+dP/dt$) by EDL muscles during unpotentiated twitches did not differ between Sham and OVX mice (6.41 ± 0.44 and 6.73 ± 0.58 N/s, respectively, $P = 0.656$). Relative to unpotentiated twitches, muscles from Sham mice developed force faster during the potentiated twitches compared with those from OVX mice (Fig. 3*D*; main effect of mouse group, $P < 0.001$). Notably, the increase in $+dP/dt$ was greater in E_2- vs. vehicle-incubated EDL muscles (main effect of treatment, $P < 0.001$) regardless of whether the muscle came from a mouse that had a sham or ovariectomy surgery (interaction $P = 0.183$). These results are consistent with enhanced contractility with chronic and acute exposure to E_2, and diminished contractility with deficiency of E_2. For each measure (i.e., PTP, $+dP/dt$, and pRLC, below), there was no difference between mice that were ovariectomized at 4–6 wk and those that were ovariectomized for 5 mo; $P \leq 0.527$).

Previous studies have shown that RLC phosphorylation is the primary mechanism for PTP (59). To confirm that the E_2-mediated PTP effects in EDL muscles were related to RLC phosphorylation, those muscles were analyzed for pRLC content. Similar to the E_2 effects on PTP, the level of pRLC in EDL muscles depended on the presence of E_2 (interaction $P = 0.048$; Fig. 4). pRLC of vehicle-incubated EDL muscles from OVX mice was about one-half of that from Sham mice. Acute exposure of muscles from OVX mice to E_2 rescued pRLC (Fig. 4). In muscles from Sham mice, acute exposure to E_2 did not affect pRLC. Thus, these biochemical events parallel the physiological effects indicating that E_2's impact on PTP is consistent with an E_2-induced effect on phosphorylation of the RLC.

RLC phosphorylation by E_2 is mediated by ERβ and GPER in muscle cells. Next, we aimed to elucidate which receptor is responsible for mediating the phosphorylation of RLC by E_2. To do this, siRNA strategy was applied to C_2C_{12} cells treated with E_2. Transfection of siRNA for ERα significantly reduced ERα protein to 34% compared with control siRNA ($P = 0.006$; Fig. 5*A*). However, pRLC of cells exposed to E_2 was not affected ($P = 0.868$; Fig. 5*A*). In contrast, siRNA specific for ERβ not only resulted in decreased ERβ expression to ~70% of its control, but it also attenuated E_2-induced pRLC by a similar extent ($P \leq 0.041$; Fig. 5*B*). Because we were not able to identify a quality commercially available antibody that is specific for GPER, successful knockdown of GPER by siRNA was confirmed by measuring its mRNA level by qPCR. siRNA specific for GPER reduced its mRNA level to 47% of control with a simultaneous reduction on pRLC ($P \leq 0.023$; Fig. 5*C*). Together, these data suggest that E_2-induced phosphorylation of the RLC in skeletal muscle cells is mediated through ERβ and GPER, but not ERα.

E_2-induced RLC phosphorylation is mediated by MLCK in EDL but not soleus muscle. Our findings raise the question as to what downstream signaling pathway(s) is/are activated by E_2 and the ERs to phosphorylate RLC. We hypothesized that kinase cascade(s) downstream of ERβ and GPER affect(s) RLC phosphorylation and consequently muscle contractility. As MLCK is known to directly phosphorylate RLC in muscle, we first focused on that kinase in EDL muscle from ovary-intact mice. As shown in Fig. 6*A*, PTP was 51% lower in EDL muscles exposed to 25 μM ML-7, a specific MLCK inhibitor ($P = 0.018$). Exposure of EDL muscle to E_2 after ML-7 incubation did not rescue the inhibitory effect of ML-7. Biochemically, ML-7 decreased pRLC to ~50% of that in the control, vehicle-treated EDL muscles regardless of E_2's presence in the bath ($P = 0.003$; Fig. 6*B*). Thus, these biochemical data are consistent with the physiological results, further supporting the role of pRLC by E_2 in muscle force potentiation. Importantly, these results imply that in EDL muscle the effect of E_2 on phosphorylation of the RLC is dependent on MLCK activity.

Next, we addressed the same question using soleus muscle, because there are known differences in the extent to which MLCK and pRLC affect the potentiation of force between EDL and soleus mouse muscles (50). Using the tetanic potentiation protocol we developed, Fig. 7 shows that tetanic force was potentiated by 18.7 ± 1.6% in soleus muscle (Veh; Fig. 7, *A* and *C*), and RLC was phosphorylated (Fig. 7*D*, Veh). When the same 25 μM concentration of ML-7 was used on isolated soleus muscles in preliminary experiments, tetanic force was substantially impaired. Reducing ML-7 to 2 μM resulted in normal tetanic force generation so that its inhibitory effects on potentiation could be investigated. Relative to vehicle control, tetanic force potentiation in soleus muscle was halved when



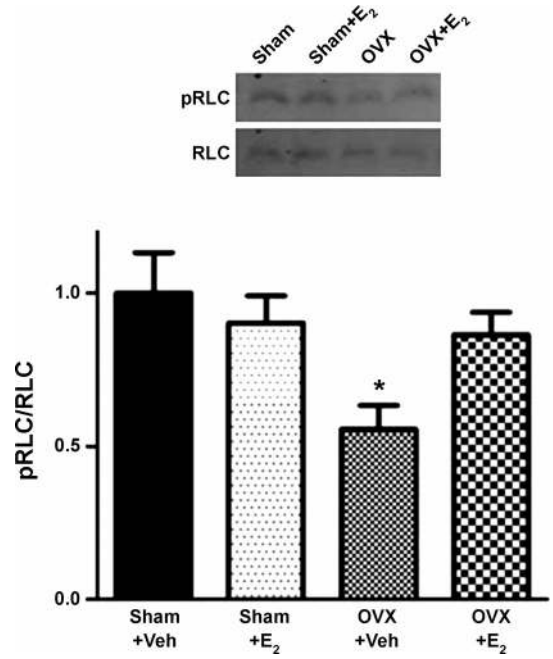


Fig. 4. Phosphorylation of RLC in EDL muscles is proportional to PTP of those same muscles. *Top*: representative Western blots of pRLC and total RLC for EDL muscles from Sham and OVX mice following incubation and contractions in Krebs-Ringer buffer plus vehicle (Veh) or E_2. *Bottom*: *q*uantitative analysis was done by comparing relative pRLC to total RLC. Data are presented relative to the Sham+Veh condition. EDL muscle pRLC was decreased by chronic deficiency of E_2 (OVX+Veh) but was reversed by 30-min exposure to E_2 in the bath (OVX+E_2). The acute presence of E_2 did not affect pRLC of EDL muscles from Sham mice (see Fig. 3 legend for *n*'s). Data are means ± SE. *Significantly different from 3 other groups.

Fig. 3. E_2 improves EDL muscle potentiation of force and maximal rate of force development (+d*P*/d*t*). *A*: representative unpotentiated and potentiated twitch force tracings by isolated EDL muscles from Sham and OVX mice. Potentiated twitch force was greater in EDL muscles of Sham than of OVX mice. *B*: representative twitch force tracings of isolated EDL muscles from OVX mice acutely exposed to vehicle (Veh) or E_2 in the bath. Potentiated twitch force was greater in EDL muscles when E_2 was added to the experimental bath compared with adding only vehicle. *C*: acute exposure of EDL muscles from Sham mice to E_2 in the bath did not affect posttetanic potentiation (PTP). PTP was low in EDL muscles from mice chronically deficient in E_2 (OVX+Veh), which was rescued by 30-min incubation in E_2 (OVX+E_2). *D*: +d*P*/d*t* was faster in EDL muscles chronically exposed to circulating E_2 (Sham mice) and in those acutely exposed to E_2 in the bath; $n = 5–8$ muscles per group for *C* and *D*. Within Sham, $n = 8$ mice 4–6 wk postsurgery and $n = 6$ mice 5 mo postsurgery; within OVX, $n = 8$ mice 4–6 wk postsurgery and $n = 7$ mice 5 mo postsurgery. $P \geq 0.527$ comparing data from 4–6 wk and 5 mo postsurgery groups; therefore, those data were combined (to obtain the 5–8 muscles/group). Data are means ± SE. *Significantly different from 3 other groups; $$E_2$ groups significantly different from Veh groups; #OVX groups significantly different from Sham groups.

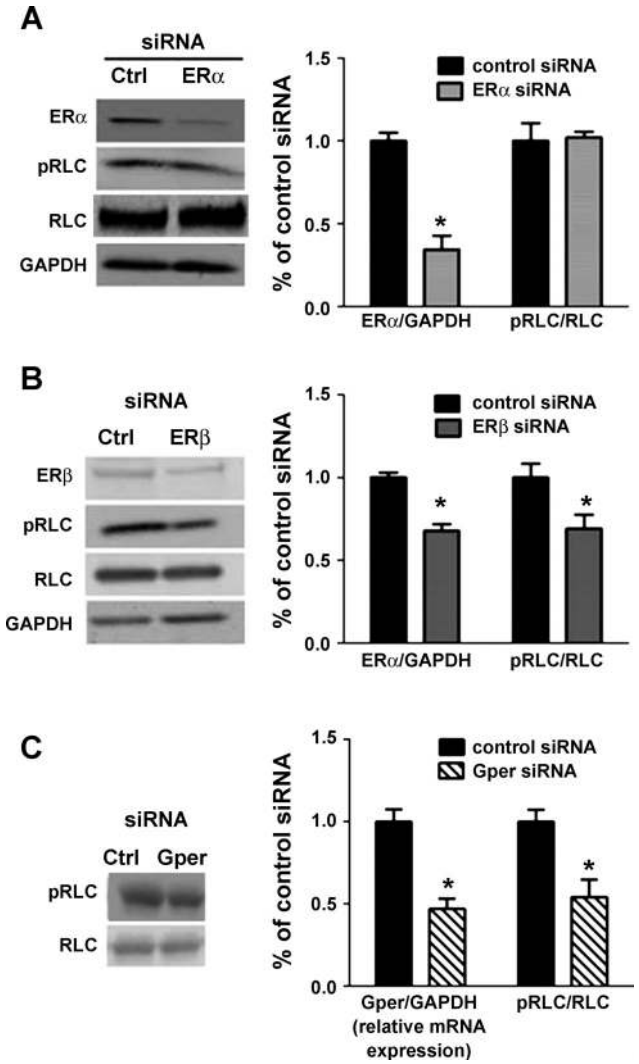


Fig. 5. Modulation of pRLC by E_2 is mediated by estrogen receptor (ER)β and G protein-coupled ER (GPER), but not ERα, in C_2C_{12} cells. *A*: representative Western blots show depletion of ERα by siRNA and the corresponding effect on pRLC. ERα was determined relative to GAPDH and pRLC relative to total RLC. siRNA mediated downregulation of ERα in C_2C_{12} cells but did not affect pRLC. *B*: representative Western blots show reduction of ERβ by siRNA and the corresponding effect on pRLC. ERβ was determined relative to GAPDH and pRLC relative to total RLC. Knockdown of ERβ by siRNA resulted in a decrease in pRLC. *C*: qPCR analysis indicated a significant reduction of GPER mRNA by siRNA, and the corresponding effect on pRLC is shown by Western blot. GPER mRNA was determined relative to GAPDH mRNA. pRLC relative to total RLC protein is shown. Reduced GPER by siRNA resulted in decreased pRLC; n = 3–4 per condition. *Significantly different from corresponding control siRNA.

exposed to ML-7, down to only 9.8 ± 1.7, (Fig. 7, *B* and *C*; P = 0.010). This inhibitory effect was accompanied by a corresponding reduction in pRLC (P = 0.002; Fig. 7*D*). Exposure of soleus muscle to E_2 following the ML-7 treatment rescued both physiological and biochemical inhibitory effects (Fig. 7, *C* and *D*), suggesting that E_2 augments pRLC in soleus muscle without a dependence on MCLK. Thus, these data provide evidence that phosphorylation of the RLC mediated by E_2 in soleus muscle can occur through another kinase or kinase cascade in addition to MLCK.

Kinase cascades regulating E_2-mediated RLC phosphorylation. To further identify possible kinase/kinase cascades involved in RLC phosphorylation by E_2 beyond MLCK, a library containing inhibitors specific for various known kinases was used. C_2C_{12} cells were treated with E_2 alone or E_2 combined with inhibitor, and the consequent pRLC in the cellular lysate was assessed by Western blot. Results are summarized in Table 1, showing that 11 specific inhibitors attenuated pRLC triggered by E_2. The inhibition ranged from 13 to 38% relative to the cells treated with E_2 alone (control condition). Affected kinases included CamKII and ROCK. Together, these data provide mechanistic insight into other kinase cascades affecting the E_2-induced phosphorylation of the RLC.

DISCUSSION

E_2 impacts skeletal muscle contractility, and here we tested the hypothesis that an underlying mechanism involves E_2-dependent posttranslational modification of a contractile pro-

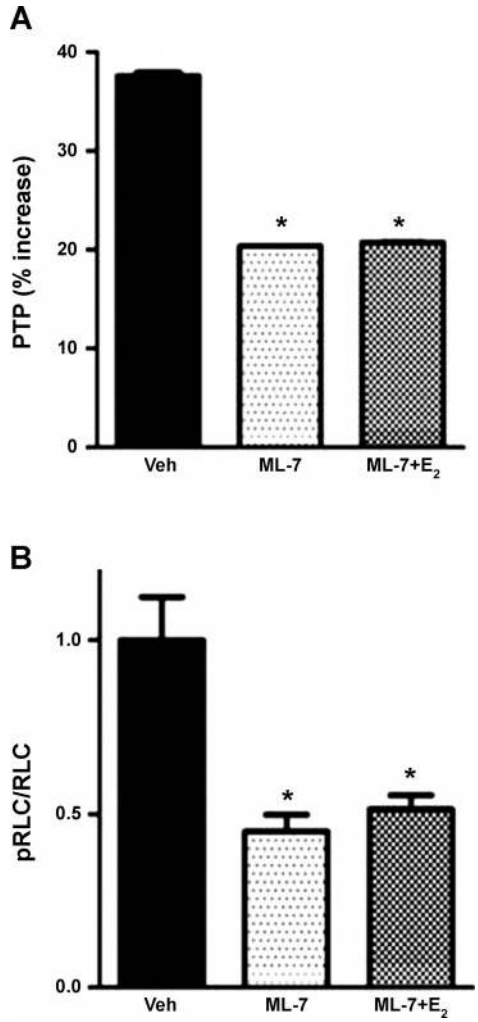


Fig. 6. PTP and corresponding pRLC of EDL muscle by E_2 exposure depend on active myosin light chain kinase (MLCK). *A*: PTP was reduced by half with exposure of EDL muscles to MLCK inhibitor ML-7 in bathing medium. The reduction was not reversed by subsequent exposure to E_2 (ML-7+E_2). *B*: pRLC was decreased by ML-7 and, similarly to PTP, E_2 exposure did not reverse the inhibitory effect. These experiments were conducted on muscles from ovary-intact female mice. Data are means ± SE; n = 6 per condition. Error bars are within mean bars for some data. *Significantly different from Veh.

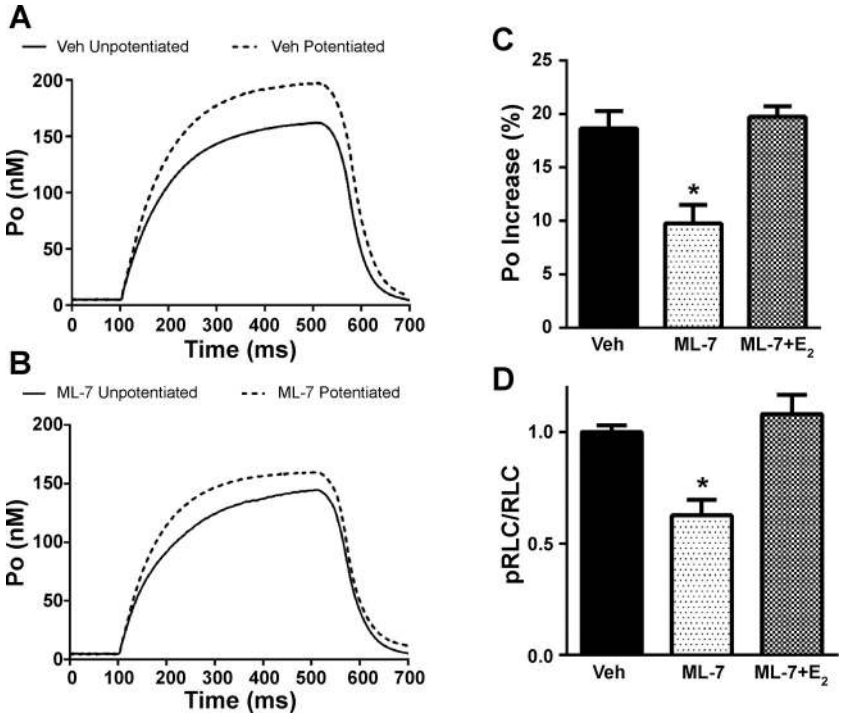


Fig. 7. Potentiation of tetanic force and corresponding pRLC of soleus muscle by E_2 exposure do not require active MLCK. *A*: representative unpotentiated and potentiated isometric tetanic force tracings by isolated soleus muscles in Krebs buffer with vehicle added. Force is potentiated by ~20%. *B*: representative unpotentiated and potentiated isometric tetanic force tracings by isolated soleus muscles in Krebs buffer containing ML-7. Force is potentiated by only ~10% in the presence of the MLCK inhibitor. *C*: tetanic force potentiation (P_o increase) was inhibited by ML-7, and, in contrast to EDL muscle (Fig. 6), the inhibitory effect on force increase was reversed by exposure to E_2. *D*: pRLC was decreased by ML-7 and, consistent with force potentiation, the reduction of pRLC by ML-7 in soleus muscle was rescued by E_2. These experiments were conducted on muscles from ovary-intact female mice. Data are means ± SE; $n = 4$ per condition. *Significantly different from Veh.

tein. Our data support the hypothesis that E_2 induces phosphorylation of the myosin RLC and that in mouse hindlimb muscles there is a physiological consequence of increasing force generation. Our results also indicate that E_2 works through specific kinase signaling pathways involving ERβ and GPER. These data extend previous reports of E_2 affecting skeletal muscle force generation (16, 38, 39, 43, 66) and provide insight into a specific molecular modification of myosin as an underlying mechanism.

E_2, as well as the estrogenic compound genistein, has previously been shown to enhance pRLC in other cell types including mouse cardiac muscle (24, 54). Consistent with those results, the current study provides evidence that E_2 increased pRLC in a skeletal muscle cell line (Fig. 2*A*). Furthermore, whereas pRLC was decreased by ovarian hormone deficiency in skeletal muscle of OVX mice, E_2 treatment of OVX mice successfully rescued the decrement (Fig. 2*B*). Collectively, these data provide evidence that E_2 enhances phosphorylation of the myosin RLC in skeletal muscle.

In skeletal muscle, pRLC increases the sensitivity of the actin-myosin contractile apparatus to Ca^{2+} activation, thus enhancing myosin motor function and development of force (23, 36, 60). Thus, we speculated that E_2-induced pRLC has a functional impact on contractility. We tested our hypothesis by investigating PTP in isolated skeletal muscles, because pRLC induced the potentiation of force and the rate of force development (14, 59, 60). Indeed, potentiated force and the maximal rate of force development ($+dP/dt$) were lower in EDL muscles from OVX than in Sham mice. These muscles also had low pRLC, suggesting that chronic deprivation of E_2 in skeletal muscle to E_2 perturbs contractility by low pRLC. The OVX-induced decrements on pRLC and PTP were rescued by acutely exposing isolated muscles to E_2 (OVX+E_2; Figs. 3 and 4). The long-term consequence of heightened PTP by E_2-mediated pRLC is not clear, but because PTP regulates mechanical function of contracting skeletal muscle by augmenting Ca^{2+} sensitivity of contractile apparatus to increase the development of force, it has been suggested that overall muscle performance could benefit (14, 60). Theoretically, the nature of E_2 repetitive fluctuations in females could induce an adaptive response that would benefit muscle performance in the long term, although further studies will need to test this theory. Additional physiologically relevant effects of E_2 on muscle contractility include the hormone's impact on the myosin super-relaxed state (SRX) (7), which is also known to be regulated by phosphorylation of the RLC (58), and the insulin signaling pathway to maintain glucose homeostasis (3).

The effects of E_2 on muscle can be mediated by ERs through genomic or nongenomic actions of the hormone (4, 31). The modulation of protein activity in the cytoplasm by nongenomic steroid mechanisms can be very rapid, on the time scale of

Table 1. *Eleven compounds inhibited E_2-induced phosphorylation of the myosin RLC in C_2C_{12} cells of 70 compounds tested*

Compound	Target Kinase	Inhibition of pRLC (%)
PD-98059	MEK	13 ± 3
Tyrphostin 51	EGFRK	14 ± 3
Tyrphostin AG1295	Tyrosine kinase	30 ± 2
SB-202190	p38 MAPK	18 ± 5
PP1	Src	28 ± 3
ML-7	MLCK	30 ± 6
BML-257	Akt	33 ± 7
KN-62	CamKII	29 ± 6
H-8	PKA/PKG	38 ± 6
Wortmannin	PI3K	24 ± 2
Y-27632-2HCL	ROCK	26 ± 6

Data are means ± SE. E_2, 17β-estradiol; pRLC, phosphorylated myosin regulatory light chain. Compounds listed are those that inhibited pRLC >10% relative to E_2 alone (control condition).

seconds to minutes, and the resulting functions are often related to the activation of protein kinase cascades (30). In the current study, phosphorylation of the RLC was increased in C_2C_{12} cells and in isolated muscles within 30 min of E_2 administration (Figs. 2 and 4). Such an acute response suggests that a nongenomic mechanism of kinase activation by E_2 may be involved. However, in some circumstances, signaling pathways for genomic and nongenomic actions of E_2 on target genes can interact (4), and E_2 has been shown to influence the expression of several members of the contractile apparatus in the heart, including myosin heavy chain, via direct gene regulation (25, 26). Therefore, genomic mechanisms should not at this point be excluded, and additional studies are necessary to distinguish genomic vs. nongenomic ER mechanisms of kinase activation in skeletal muscle fibers in relation to phosphorylation of the RLC.

To begin to delineate which ER in skeletal muscle triggers events that phosphorylate the RLC, we used specific siRNA to knock down each ER in C_2C_{12} cells. Results of these experiments demonstrate that pRLC is mediated through ERβ and GPER, but not ERα (Fig. 5). This is partially consistent with a study on vascular smooth muscle cells showing that E_2 regulates pRLC by a specific subset of receptors, in this case through GPER and ERα (19). The difference in ERα vs. ERβ inducing pRLC in different muscle types may be due to a number of factors, including different subcellular localization of ERs, expression levels of ERs, and abundance of signaling components specific to smooth vs. skeletal muscle cells. Future efforts to illustrate ER-mediated pRLC in skeletal muscle should initially be focused on ERβ and GPER based on our siRNA results in C_2C_{12} cells. Also, we previously demonstrated that ERs are expressed in skeletal muscles of OVX mice (2), indicating that reduced kinase signaling is not primarily impacted by the level of ER expression.

MLCK is a key kinase phosphorylating RLC in skeletal muscle (23). In the present study, the E_2-induced increases in pRLC and associated force generation occurred through MLCK in both fast-twitch EDL and slow-twitch soleus muscles, because the MLCK inhibitor ML-7 efficiently blunted these effects (Figs. 6 and 7). Exposure of EDL muscles to E_2 did not affect the inhibition of pRLC and muscle potentiation by ML-7 (Fig. 6), indicating that the EDL muscle relies on MLCK for E_2-induced pRLC. However, in soleus muscles, an E_2-sensitive kinase or kinase pathway, in addition to MLCK, can phosphorylate RLC because E_2 exposure to those muscles increased pRLC and force in the presence of ML-7 (Fig. 7). This result is not surprising, as genetic deletion of skeletal muscle MLCK in mice only partially abolished pRLC, indicating the presence of other kinases leading to the phosphorylation of RLC, such as the zipper interacting protein kinase (23, 59). The discrepancy between EDL and soleus muscles in the reliance of MLCK on E_2-induced pRLC could be related to different abundances of MLCK and myosin light chain phosphatase proteins and their activities in the two muscles (50), differences in cellular context for ER-mediated signaling pathways, or myosin isoform differences between the two muscles. In regard to the latter, there is fiber type heterogeneity in the mouse soleus muscle as is it comprised of type I and IIa fibers based on myosin heavy chain isoform expressions (13), whereas mouse EDL muscle is more homogeneous, with only type II fibers (52), and it has been demonstrated that potentiation is greater in type II than in type I fibers (60). How the effects of E_2 may differ among fibers with various isoforms of the myosin heavy and light chains is not presently clear, nor is the impact of estrogen status on expression of the various myosin isoforms. There are reports of OVX-related shifts toward slower isoforms/fiber types (11, 22, 42), toward faster isoforms/fiber types (29, 45, 61), and no changes in myosin isoforms/fiber types (9, 39, 42, 47, 66). Importantly, when such shifts are detected they are modest, in the magnitude of 10% or less, and thus likely have little impact on the extent of force potentiation reported here.

E_2 activates several kinases and signaling pathways in various skeletal muscle cell lines (4, 8, 48) and skeletal muscle of rodents (32, 56, 68). In our screen of E_2-responsive kinases using a kinase inhibitor library and C_2C_{12} cells, we identified 11 inhibitors that decreased pRLC triggered by E_2 (Table 1). The corresponding kinases can be profiled according to known kinase cascades, suggesting that E_2-mediated phosphorylation of the RLC may be stimulated through the PI3K/MAPK/Akt/MLCK and CamKII/ROCK kinase pathways (Fig. 8). Our result that E_2 stimulates the MAPK pathway to phosphorylate the RLC is consistent with results from Hamdi and Mutungi (20). Similarly, PI3K and Akt are involved in modulating pRLC mediated by E_2, which is consistent with E_2-mediated PI3K signaling in smooth muscle contraction (19). ROCK has been shown to mediate pRLC by decreasing myosin light chain phosphatase activity and/or directly phosphorylating RLC (1, 54, 57, 62). Our findings demonstrate that inhibition of ROCK led to decreased E_2-induced pRLC by 26%. Cross-talk among signaling pathways may also play a role in regulating pRLC (34). For example, it was suggested that cross-talk exists between the tyrosine kinase pathway and the MLCK pathway (21). By

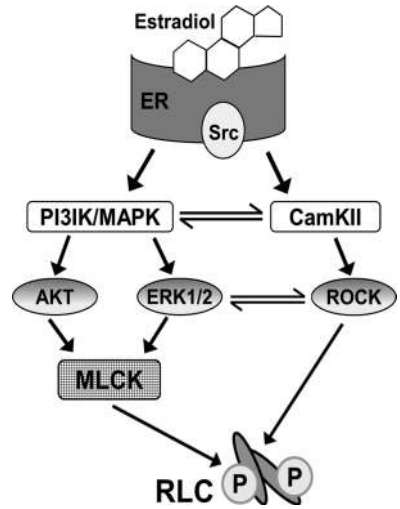


Fig. 8. Schema depicting cellular signaling pathways that potentially stimulate pRLC in skeletal muscle. On the basis of our results from ML-7 experiments with skeletal muscles and kinase inhibitor screening in C_2C_{12} cells, as well as known kinase cascades as reported in the literature, we propose potential pathways involved in E_2-mediated phosphorylation of myosin RLC. All postulated pathways would theoretically start with E_2 binding to ERβ or GPER to activate downstream kinase cascades and culminate in pRLC and enhanced force generation. Src, tyrosine kinase; PI3K, phosphatidylinositide 3-kinases; MAPK, mitogen-activated protein kinase; CamKII, Ca^{2+}/calmodulin-dependent protein kinase II; Akt, protein kinase B; ERK1/2, extracellular signal-regulated kinases; ROCK, Rho-associated protein kinase; MLCK, myosin light chain kinase; RLC, regulatory light chain.

integrating potential RLC kinases from the kinase inhibitor assay into known kinase cascades, the present data suggest that the MAPK, PI3K/Akt, and CamKII cascades are involved in E_2-induced-phosphorylation of RLC. Furthermore, based on our results with ML-7, it can be inferred that soleus muscle utilizes all of the pathways illustrated, whereas EDL muscle appears to rely solely on those converging on MLCK. Additional experiments are required to substantiate the deduction, particularly in additional muscles composed of various fiber types.

In summary, we provide evidence that skeletal muscle responds to E_2 by modulating phosphorylation of the myosin RLC with functional consequences on contractility. E_2-stimulated pRLC in skeletal muscle is mediated by several kinases and appears to involve the receptors ERβ and GPER. The extent to which E_2 influences the phosphorylation of other contractile proteins, such as myosin-binding protein C and troponin I, are important questions yet to be addressed, as most of the kinases we identified for pRLC are not unique to phosphorylation of this protein. What has been determined in this work is that E_2 mediates phosphorylation of RLC and that this appears to be one mechanism by which this hormone exerts its effects on myosin and muscle function. This and additional potential mechanisms are important to elucidate in order to understand the muscle weakness that occurs with hormone deficiency and how those effects translate to long-term functional impact in aging women.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.L., B.C.C., B.A.C., G.K., and D.A.L. conception and design of research; S.L., B.C.C., and D.A.L. performed experiments; S.L., B.C.C., and D.A.L. analyzed data; S.L., B.C.C., B.A.C., G.K., and D.A.L. interpreted results of experiments; S.L. and B.C.C. prepared figures; S.L., B.C.C., B.A.C., G.K., and D.A.L. drafted manuscript; S.L., B.C.C., B.A.C., G.K., and D.A.L. edited and revised manuscript; S.L., B.C.C., B.A.C., G.K., and D.A.L. approved final version of manuscript.

REFERENCES

1. **Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K.** Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 271: 20246–20249, 1996.
2. **Baltgalvis KA, Greising SM, Warren GL, Lowe DA.** Estrogen regulates estrogen receptors and antioxidant gene expression in mouse skeletal muscle. *PLoS One* 5: e10164, 2010.
3. **Barros RP, Gustafsson JA.** Estrogen receptors and the metabolic network. *Cell Metab* 14: 289–299, 2011.
4. **Bjornstrom L, Sjoberg M.** Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 19: 833–842, 2005.
5. **Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ.** Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics* 45: 607–617, 1997.
6. **Colson BA, Locher MR, Bekyarova T, Patel JR, Fitzsimons DP, Irving TC, Moss RL.** Differential roles of regulatory light chain and myosin binding protein-C phosphorylations in the modulation of cardiac force development. *J Physiol* 588: 981–993, 2010.
7. **Colson BA, Petersen KJ, Collins BC, Lowe DA, Thomas DD.** The myosin super-relaxed state is disrupted by estradiol deficiency. *Biochem Biophys Res Commun* 456: 151–155, 2015.
8. **D'Eon TM, Rogers NH, Stancheva ZS, Greenberg AS.** Estradiol and the estradiol metabolite, 2-hydroxyestradiol, activate AMP-activated protein kinase in C2C12 myotubes. *Obesity (Silver Spring)* 16: 1284–1288, 2008.
9. **Eason JM, Schwartz GA, Pavlath GK, English AW.** Sexually dimorphic expression of myosin heavy chains in the adult mouse masseter. *J Appl Physiol* 89: 251–258, 2000.
10. **Finni T, Noorkoiv M, Pollanen E, Ronkainen PH, Alen M, Kaprio J, Kovanen V, Sipila S.** Muscle function in monozygotic female twin pairs discordant for hormone replacement therapy. *Muscle Nerve* 44: 769–775, 2011.
11. **Fonseca H, Powers SK, Goncalves D, Santos A, Mota MP, Duarte JA.** Physical inactivity is a major contributor to ovariectomy-induced sarcopenia. *Int J Sports Med* 33: 268–278, 2012.
12. **Galluzzo P, Rastelli C, Bulzomi P, Acconcia F, Pallottini V, Marino M.** 17β-Estradiol regulates the first steps of skeletal muscle cell differentiation via ERα-mediated signals. *Am J Physiol Cell Physiol* 297: C1249–C1262, 2009.
13. **Glaser BW, You G, Zhang M, Medler S.** Relative proportions of hybrid fibres are unaffected by 6 weeks of running exercise in mouse skeletal muscles. *Exp Physiol* 95: 211–221, 2010.
14. **Grange RW, Cory CR, Vandenboom R, Houston ME.** Myosin phosphorylation augments force-displacement and force-velocity relationships of mouse fast muscle. *Am J Physiol Cell Physiol* 269: C713–C724, 1995.
15. **Greenberg MJ, Kazmierczak K, Szczesna-Cordary D, Moore JR.** Cardiomyopathy-linked myosin regulatory light chain mutations disrupt myosin strain-dependent biochemistry. *Proc Natl Acad Sci USA* 107: 17403–17408, 2010.
16. **Greising SM, Baltgalvis KA, Kosir AM, Moran AL, Warren GL, Lowe DA.** Estradiol's beneficial effect on murine muscle function is independent of muscle activity. *J Appl Physiol* 110: 109–115, 2011.
17. **Greising SM, Baltgalvis KA, Lowe DA, Warren GL.** Hormone therapy and skeletal muscle strength: a meta-analysis. *J Gerontol A Biol Sci Med Sci* 64: 1071–1081, 2009.
18. **Greising SM, Carey RS, Blackford JE, Dalton LE, Kosir AM, Lowe DA.** Estradiol treatment, physical activity, and muscle function in ovarian-senescent mice. *Exp Gerontol* 46: 685–693, 2011.
19. **Gros R, Ding Q, Davis M, Shaikh R, Liu B, Chorazyczewski J, Pickering JG, Feldman RD.** Delineating the receptor mechanisms underlying the rapid vascular contractile effects of aldosterone and estradiol. *Can J Physiol Pharmacol* 89: 655–663, 2011.
20. **Hamdi MM, Mutungi G.** Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. *J Physiol* 588: 511–525, 2010.
21. **Jin N, Siddiqui RA, English D, Rhoades RA.** Communication between tyrosine kinase pathway and myosin light chain kinase pathway in smooth muscle. *Am J Physiol Heart Circ Physiol* 271: H1348–H1355, 1996.
22. **Kadi F, Karlsson C, Larsson B, Eriksson J, Larval M, Billig H, Jonsdottir IH.** The effects of physical activity and estrogen treatment on rat fast and slow skeletal muscles following ovariectomy. *J Muscle Res Cell Motil* 23: 335–339, 2002.
23. **Kamm KE, Stull JT.** Signaling to myosin regulatory light chain in sarcomeres. *J Biol Chem* 286: 9941–9947, 2011.
24. **Kararigas G, Bito V, Sipido KR, Regitz-Zagrosek V.** Estrogen induces cardiomyocyte contractility via an MLCK/MRLC-dependent mechanism. *FASEB J* 26: 1054, 2012.
25. **Kararigas G, Bito V, Tinel H, Becher E, Baczko I, Knosalla C, Albrecht-Kupper B, Sipido KR, Regitz-Zagrosek V.** Transcriptome characterization of estrogen-treated human myocardium identifies myosin regulatory light chain interacting protein as a sex-specific element influencing contractile function. *J Am Coll Cardiol* 59: 410–417, 2012.
26. **Kararigas G, Nguyen BT, Zelarayan LC, Hassenpflug M, Toischer K, Sanchez-Ruderisch H, Hasenfuss G, Bergmann MW, Jarry H, Regitz-Zagrosek V.** Genetic background defines the regulation of postnatal cardiac growth by 17beta-estradiol through a beta-catenin mechanism. *Endocrinology* 155: 2667–2676, 2014.

27. **Kumar N, Liang Y, Parslow TG.** Receptor tyrosine kinase inhibitors block multiple steps of influenza a virus replication. *J Virol* 85: 2818–2827, 2011.
28. **Levine RJ, Kensler RW, Yang Z, Stull JT, Sweeney HL.** Myosin light chain phosphorylation affects the structure of rabbit skeletal muscle thick filaments. *Biophys J* 71: 898–907, 1996.
29. **Liu YH, Jia SS, Hou YX.** Effects of ovariectomy on rat genioglossal muscle contractile properties and fiber-type distribution. *Angle Orthod* 79: 509–514, 2009.
30. **Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, Wehling M.** Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* 83: 965–1016, 2003.
31. **Maggiolini M, Picard D.** The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J Endocrinol* 204: 105–114, 2010.
32. **Mangan G, Bombardier E, Mitchell AS, Quadrilatero J, Tiidus PM.** Oestrogen-dependent satellite cell activation and proliferation following a running exercise occurs via the PI3K signalling pathway and not IGF-1. *Acta Physiol* 212: 75–85, 2014.
33. **Margossian SS, Lowey S.** Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol* 85 Pt B: 55–71, 1982.
34. **Means AR.** Regulatory cascades involving calmodulin-dependent protein kinases. *Mol Endocrinol* 14: 4–13, 2000.
35. **Meeuwsen IB, Samson MM, Verhaar HJ.** Evaluation of the applicability of HRT as a preservative of muscle strength in women. *Maturitas* 36: 49–61, 2000.
36. **Metzger JM, Greaser ML, Moss RL.** Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. Implications for twitch potentiation in intact muscle. *J Gen Physiol* 93: 855–883, 1989.
37. **Moore RL, Stull JT.** Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. *Am J Physiol Cell Physiol* 247: C462–C471, 1984.
38. **Moran AL, Nelson SA, Landisch RM, Warren GL, Lowe DA.** Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice. *J Appl Physiol* 102: 1387–1393, 2007.
39. **Moran AL, Warren GL, Lowe DA.** Removal of ovarian hormones from mature mice detrimentally affects muscle contractile function and myosin structural distribution. *J Appl Physiol* 100: 548–559, 2006.
40. **Muthu P, Kazmierczak K, Jones M, Szczesna-Cordary D.** The effect of myosin RLC phosphorylation in normal and cardiomyopathic mouse hearts. *J Cell Mol Med* 16: 911–919, 2012.
41. **Phillips SK, Rook KM, Siddle NC, Bruce SA, Woledge RC.** Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy. *Clin Sci (Lond)* 84: 95–98, 1993.
42. **Piccone CM, Brazeau GA, McCormick KM.** Effect of oestrogen on myofibre size and myosin expression in growing rats. *Exp Physiol* 90: 87–93, 2005.
43. **Qaisar R, Renaud G, Hedstrom Y, Pollanen E, Ronkainen P, Kaprio J, Alen M, Sipila S, Artemenko K, Bergquist J, Kovanen V, Larsson L.** Hormone replacement therapy improves contractile function and myonuclear organization of single fibres from postmenopausal monozygotic female twin pairs. *J Physiol* 591: 2333–2344, 2013.
44. **Rayment I, Holden HM, Whittaker M, Yohn CB, Lorenz M, Holmes KC, Milligan RA.** Structure of the actin-myosin complex and its implications for muscle contraction. *Science* 261: 58–65, 1993.
45. **Rogers NH, Perfield JW, 2nd Strissel KJ, Obin MS, Greenberg AS.** Loss of ovarian function in mice results in abrogated skeletal muscle PPARdelta and FoxO1-mediated gene expression. *Biochem Biophys Res Commun* 392: 1–3, 2010.
46. **Rogers NH, Witczak CA, Hirshman MF, Goodyear LJ, Greenberg AS.** Estradiol stimulates Akt, AMP-activated protein kinase (AMPK) and TBC1D1/4, but not glucose uptake in rat soleus. *Biochem Biophys Res Commun* 382: 646–650, 2009.
47. **Romani WA, Russ DW.** Acute effects of sex-specific sex hormones on heat shock proteins in fast muscle of male and female rats. *Eur J Appl Physiol* 113: 2503–2510, 2013.
48. **Ronda AC, Buitrago C, Boland R.** Role of estrogen receptors, PKC and Src in ERK2 and p38 MAPK signaling triggered by 17beta-estradiol in skeletal muscle cells. *J Steroid Biochem Mol Biol* 122: 287–294, 2010.
49. **Ronkainen PH, Kovanen V, Alen M, Pollanen E, Palonen EM, Ankarberg-Lindgren C, Hamalainen E, Turpeinen U, Kujala UM, Puolakka J, Kaprio J, Sipila S.** Postmenopausal hormone replacement therapy modifies skeletal muscle composition and function: a study with monozygotic twin pairs. *J Appl Physiol* 107: 25–33, 2009.
50. **Ryder JW, Lau KS, Kamm KE, Stull JT.** Enhanced skeletal muscle contraction with myosin light chain phosphorylation by a calmodulin-sensing kinase. *J Biol Chem* 282: 20447–20454, 2007.
51. **Samson MM, Meeuwsen IB, Crowe A, Dessens JA, Duursma SA, Verhaar HJ.** Relationships between physical performance measures, age, height and body weight in healthy adults. *Age Ageing* 29: 235–242, 2000.
52. **Schiaffino S, Reggiani C.** Fiber types in mammalian skeletal muscles. *Physiol Rev* 91: 1447–1531, 2011.
53. **Schneider BS, Fine JP, Nadolski T, Tiidus PM.** The effects of estradiol and progesterone on plantarflexor muscle fatigue in ovariectomized mice. *Biol Res Nurs* 5: 265–275, 2004.
54. **Schwab K, Stein R, Scheler C, Theuring F.** Dietary genistein enhances phosphorylation of regulatory myosin light chain in the myocardium of ovariectomized mice. *Electrophoresis* 33: 1795–1803, 2012.
55. **Sheikh F, Ouyang K, Campbell SG, Lyon RC, Chuang J, Fitzsimons D, Tangney J, Hidalgo CG, Chung CS, Cheng H, Dalton ND, Gu Y, Kasahara H, Ghassemian M, Omens JH, Peterson KL, Granzier HL, Moss RL, McCulloch AD, Chen J.** Mouse and computational models link Mlc2v dephosphorylation to altered myosin kinetics in early cardiac disease. *J Clin Invest* 122: 1209–1221, 2012.
56. **Sitnick M, Foley AM, Brown M, Spangenburg EE.** Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. *J Appl Physiol* 100: 286–293, 2006.
57. **Somlyo AP, Somlyo AV.** Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83: 1325–1358, 2003.
58. **Stewart MA, Franks-Skiba K, Chen S, Cooke R.** Myosin ATP turnover rate is a mechanism involved in thermogenesis in resting skeletal muscle fibers. *Proc Natl Acad Sci USA* 107: 430–435, 2010.
59. **Stull JT, Kamm KE, Vandenboom R.** Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. *Arch Biochem Biophys* 510: 120–128, 2011.
60. **Vandenboom R, Gittings W, Smith IC, Grange RW, Stull JT.** Myosin phosphorylation and force potentiation in skeletal muscle: evidence from animal models. *J Muscle Res Cell Motil* 34: 317–332, 2013.
61. **Velders M, Solzbacher M, Schleipen B, Laudenbach U, Fritzemeier KH, Diel P.** Estradiol and genistein antagonize the ovariectomy effects on skeletal muscle myosin heavy chain expression via ER-beta mediated pathways. *J Steroid Biochem Mol Biol* 120: 53–59, 2010.
62. **Wang L, Jia C, Yu Z, Liu X, Kang L, Cong Y, Shan Y, Zhao Z, Ma B.** Pennogenin tetraglycoside induces rat myometrial contraction and MLC20 phosphorylation via PLC-IP(3) and RhoA/Rho kinase signaling pathways. *PLoS One* 7: e51536, 2012.
63. **Warren GL, Ingalls CP, Armstrong RB.** Temperature dependency of force loss and Ca^{2+} homeostasis in mouse EDL muscle after eccentric contractions. *Am J Physiol Regul Integr Comp Physiol* 282: R1122–R1132, 2002.
64. **Warren GL, Lowe DA, Hayes DA, Karwoski CJ, Prior BM, Armstrong RB.** Excitation failure in eccentric contraction-induced injury of mouse soleus muscle. *J Physiol* 468: 487–499, 1993.
65. **Warren GL, Lowe DA, Inman CL, Orr OM, Hogan HA, Bloomfield SA, Armstrong RB.** Estradiol effect on anterior crural muscles-tibial bone relationship and susceptibility to injury. *J Appl Physiol* 80: 1660–1665, 1996.
66. **Wattanapermpool J, Reiser PJ.** Differential effects of ovariectomy on calcium activation of cardiac and soleus myofilaments. *Am J Physiol Heart Circ Physiol* 277: H467–H473, 1999.
67. **Wendt T, Taylor D, Messier T, Trybus KM, Taylor KA.** Visualization of head-head interactions in the inhibited state of smooth muscle myosin. *J Cell Biol* 147: 1385–1390, 1999.
68. **Wohlers LM, Sweeney SM, Ward CW, Lovering RM, Spangenburg EE.** Changes in contraction-induced phosphorylation of AMP-activated protein kinase and mitogen-activated protein kinases in skeletal muscle after ovariectomy. *J Cell Biochem* 107: 171–178, 2009.
69. **Xeni J, Gittings WB, Caterini D, Huang J, Houston ME, Grange RW, Vandenboom R.** Myosin light-chain phosphorylation and potentiation of dynamic function in mouse fast muscle. *Pflügers Arch* 462: 349–358, 2011.
70. **Zhi G, Ryder JW, Huang J, Ding P, Chen Y, Zhao Y, Kamm KE, Stull JT.** Myosin light chain kinase and myosin phosphorylation effect frequency-dependent potentiation of skeletal muscle contraction. *Proc Natl Acad Sci USA* 102: 17519–17524, 2005.
71. **Zoghbi ME, Woodhead JL, Moss RL, Craig R.** Three-dimensional structure of vertebrate cardiac muscle myosin filaments. *Proc Natl Acad Sci USA* 105: 2386–2390, 2008.