ERK-mediated uterine artery contraction: role of thick and thin filament regulatory pathways

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Xiao, DaLiao, William J. Pearce, Lawrence D. Longo, and Lubo Zhang. ERK-mediated uterine artery contraction: role of thick and thin filament regulatory pathways. Am J Physiol Heart Circ Physiol 286: H1615–H1622, 2004; 10.1152/ajpheart.00981.2003.—We have demonstrated that extracellular signal-regulated kinase (ERK) plays an important role in the regulation of uterine artery contraction. The present study tested the hypothesis that ERK regulates thick and thin filament regulatory pathways in the uterine artery. Isometric tension, intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), and 20-kDa myosin light chain (LC\(_{20}\)) phosphorylation were measured simultaneously in uterine arteries isolated from near-term (140 days gestation) pregnant sheep. Phenylephrine produced time-dependent increases in [Ca\(^{2+}\)]\(_i\) and LC\(_{20}\) phosphorylation that preceded the contraction, which were inhibited by the MEK (ERK) inhibitor PD-098059. In addition, PD-098059 decreased the intercept of the regression line of LC\(_{20}\) phosphorylation vs. [Ca\(^{2+}\)]\(_i\) but increased the rate of tension development vs. LC\(_{20}\) phosphorylation. In contrast to phenylephrine, phorbol 12,13-dibutyrate (PDBu) produced contractions without changing [Ca\(^{2+}\)]\(_i\) or LC\(_{20}\) phosphorylation. PD-098059 potentiated PDBu-induced contractions without affecting [Ca\(^{2+}\)]\(_i\) and LC\(_{20}\) phosphorylation. PDBu produced time-dependent increases in phosphorylation of p42 and p44 ERK and ERK-dependent phosphorylation of caldesmon at Ser\(^{789}\) in the uterine artery. PD-098059 blocked PDBu-mediated phosphorylation of p42 and p44 ERK and caldesmon. The results indicate that ERK may regulate force by a dual regulation of thick and thin filaments in uterine artery smooth muscle. ERK potentiates the thick filament regulatory pathway by enhancing LC\(_{20}\) phosphorylation via increases in [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) sensitivity of LC\(_{20}\) phosphorylation. In contrast, ERK attenuates the thin filament regulatory pathway and suppresses contractions independent of changes in LC\(_{20}\) phosphorylation in the uterine artery.

isometric tension; intracellular free calcium concentration; myosin light chain phosphorylation; PD-098059; phenylephrine; phorbol 12,13-dibutyrate

SMOOTH MUSCLE CONTRACTION is regulated by phosphorylation and dephosphorylation of the 20-kDa regulatory light chain of myosin (LC\(_{20}\)) (21, 25, 39). Phosphorylation of LC\(_{20}\) is regulated primarily by an increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), resulting in activation of myosin light chain kinase (MLCK) and subsequent phosphorylation of LC\(_{20}\). Dephosphorylation of LC\(_{20}\) by myosin light chain phosphatase (MLCP) results in smooth muscle relaxation. The extent of LC\(_{20}\) phosphorylation and, hence, of the amplitude of force production depends on the balance of the activities of MLCK and MLCP. In addition to thick filament regulation, many studies have demonstrated dissociation between LC\(_{20}\) phosphorylation and cross-bridge cycling rates/tension development (6, 12, 18, 20, 32), suggesting an additional thin filament regulation in smooth muscle contraction (21, 33).

Extracellular signal-regulated kinase (ERK) has been proposed to regulate smooth muscle contraction (2, 11, 16, 17, 26, 52, 55, 57). Different signaling transduction mechanisms have been reported in ERK-mediated regulation of smooth muscle contraction. Previous studies showed that the MEK (ERK) inhibitor PD-098059 did not affect agonist-induced [Ca\(^{2+}\)]\(_i\), in isolated smooth muscle cells but inhibited agonist-induced smooth muscle contraction by decreasing Ca\(^{2+}\) sensitivity of contractile proteins (1, 2, 11, 17, 21, 38, 49). It has been proposed that ERK mediates smooth muscle contraction through the thin filament regulatory pathway by phosphorylation of caldesmon (CaD) (2, 21, 33). However, by measuring [Ca\(^{2+}\)]\(_i\) and tension simultaneously in the intact tissue, our recent studies clearly demonstrated that PD-098059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) sensitivity in sheep uterine artery smooth muscle (55). This suggests that, in addition to Ca\(^{2+}\)-independent pathways, as proposed previously, the ERK signaling pathway also involves Ca\(^{2+}\)-dependent components of vascular contractions. Similarly, recent studies in porcine carotid artery suggest that ERK might regulate force in arterial smooth muscle by inhibiting LC\(_{20}\) phosphorylation (8).

Recently, we demonstrated that ERK plays an important role in the regulation of uterine artery contraction and that the effect of ERK on the uterine artery is altered during pregnancy (55). In the present study, we tested the hypothesis that ERK regulates uterine artery contraction through thick and thin filament regulatory pathways. We examined the effects of the MEK (ERK) inhibitor PD-098059 on phenylephrine- and phorbol 12,13-dibutyrate (PDBu)-induced increases in [Ca\(^{2+}\)]\(_i\), LC\(_{20}\) phosphorylation, and contractile tension in uterine arteries obtained from pregnant sheep. We also examined phosphorylation of ERK and ERK-dependent phosphorylation of the thin filament regulatory protein CaD. The present study provides evidence that ERK potentiates the thick filament regulatory pathway by enhancing LC\(_{20}\) phosphorylation but attenuates the thin filament regulatory pathway by suppressing contractions independent of changes in LC\(_{20}\) phosphorylation in the uterine artery.

METHODS

Tissue preparation. Pregnant (140 days gestation) sheep were anesthetized with thiamylal (10 mg/kg) administered via the external left jugular vein. The ewes were then intubated, and anesthesia was
maintained on 1.5–2.0% halothane in O2 throughout the surgery. An incision in the abdomen was made, and the uterus was exposed. The ureter and arteries were isolated, removed without stretching, and placed into modified Krebs solution (pH 7.4) of the following composition (in mM): 115.21 NaCl, 4.7 KCl, 1.80 CaCl2, 1.16 MgSO4, 1.18 KH2PO4, 22.14 NaHCO3, 0.03 EDTA, and 7.88 dextrose. The Krebs solution was oxygenated with 95% O2–5% CO2. After the tissues were removed, the animals were killed with euthanasia solution (T-61, Hoechst-Roussel, Somerville, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the National Institutes of Health guidelines for the care and use of laboratory animals.

**Contraction studies.** The fourth branches of the main uterine arteries were separated from the surrounding tissue and cut into 2-mm ring segments. The small branches of the main uterine artery were chosen, because they are much closer in characteristics to arterioles and play a substantial role in vascular resistance. Isometric tension was measured in Krebs solution in a tissue bath at 37°C, as described previously (55). After 60 min of equilibration, each ring was stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Tissues were pretreated with 30 μM PD-098059 or vehicle (DMSO) for 30 min and then stimulated with phenylephrine or PDBu. Tension was recorded with a chart recorder. To measure LC20 phosphorylation simultaneously in the same tissues, arterial rings were snap frozen with liquid N2-cooled clamps at the indicated times and immersed in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM DTT. The rings were stored at −80°C until they were used.

**Measurement of LC20 phosphorylation.** Tissues were brought to room temperature in the dry ice-acetone-TCA-DTT mixture and then washed three times with ether to remove the TCA. Tissues were then extracted in 100 μl of sample buffer containing 20 mM Tris base and 23 mM glycine (pH 8.6), 8.0 M urea, 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue, as previously described (8, 28). Samples (20 μl) were electrophoresed at 12 mA for 2.5 h after a 30-min prerun in 1.0 mm mini-polyacrylamide gels containing 10% acrylamide, 0.27% bisacrylamide, 40% glycerol, and 20 mM Tris base (pH 8.8). Proteins were transferred to nitrocellulose membranes and subjected to immunoblot with a specific LC20 antibody (1:500; Sigma, St. Louis, MO). Goat anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody (1:2,000; Calbiochem). Bands were detected with enhanced chemiluminescence, visualized on films, and analyzed with the Kodak electrophoresis documentation and analysis system and Kodak 1D image analysis software. Moles of phosphate per mole of light chain were calculated by dividing the density of the phosphorylated band by the sum of the densities of the phosphorylated plus the unphosphorylated bands.

**Simultaneous measurement of [Ca2+]i, and tension.** Simultaneous recordings of contraction and [Ca2+]i, in the same tissue were conducted as described previously (56). Briefly, each arterial ring was attached to an isotonic force transducer in a 5-ml tissue bath mounted on an intracellular Ca2+ analyzer (model CAF-110, Jasco, Tokyo, Japan). The tissue was equilibrated in Krebs buffer under a resting tension of 0.5 g for 40 min. The ring was then loaded with 5 μM fura 2-AM for 3 h in the presence of 0.02% Cremophor EL at room temperature (25°C). After loading, the tissue was washed with Krebs solution at 37°C for 30 min to allow for hydrolysis of fura 2 ester groups by endogenous esterase. Contractile tension and fura 2 fluorescence were measured simultaneously at 37°C in the same tissue. The tissue was illuminated alternately (125 Hz) at excitation wavelengths of 340 and 380 nm, respectively, by means of two monochromators in the light path of a 75-W xenon lamp. Fluorescence emission from the tissue was measured at 510 nm by photographing tissue fluorescence on photographic film. The fluorescence intensity was measured at each excitation wavelength and the ratio of the two fluorescence values were recorded with a time constant of 250 ms and stored with the force signal on a computer.

**Western immunoblotting analysis.** Arterial rings were equilibrated in the tissue bath, and the optimal tensions were obtained as described above. The tissues were then incubated for 30 min with 30 μM PD-098059 or vehicle alone in the organ bath (37°C). After incubation, they were stimulated with 5 μM PDBu. The reaction was stopped at various times by snap freezing the tissues in liquid N2, and the tissues were stored at −80°C until they were used. Protein levels of phosphorylated p42 and p44 ERK and CaD at Ser789 were determined by Western blot analysis, as previously described (55). Briefly, samples with equal protein were loaded on 10% (p42 and p44 ERK) and 7.5% (CaD) SDS-polyacrylamide gels, separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were incubated with the antibodies against phosphorylated p42 and p44 ERK (Thr202/Tyr204) and phosphorylated CaD (Ser789) and then with secondary antibodies of horseradish peroxidase-conjugated goat anti-rabbit. Proteins were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak 1D image analysis software. The data were normalized by actin.

**Materials.** Phenylenephrine, PDBu, PD-098059, and monoclonal antityrosine (20-kDa light chains) were obtained from Sigma; phosphorylated p42 and p44 ERK (Thr202/Tyr204) antibodies from Cell Signaling Technology (Beverly, MA); phosphorylated CaD (Ser789) antibody from Santa Cruz Biotechnology (Santa Cruz, CA); all electrophoretic and immunoblot reagents from Bio-Rad; and fura 2-AM from Molecular Probes (Eugene, OR). Other reagents were of analytic grade or better and were purchased from Sigma or Fisher Scientific. All chemicals were prepared fresh each day and kept on ice throughout the experiment.

**Data analysis.** Data were analyzed by computer-assisted linear or nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA). Values are means ± SE. Differences were evaluated for statistical significance (P < 0.05) by one-way ANOVA and Student’s t-test.

**RESULTS**

**Effect of PD-098059 on phenylephrine-induced increases in [Ca2+]i, LC20 phosphorylation, and tension.** Phenylephrine produced time-dependent increases in [Ca2+]i, LC20 phosphorylation, and tension development in the uterine artery (Fig. 1). The tension development was preceded by [Ca2+]i, and LC20 phosphorylation. Although the agonist-induced tension was sustained for up to 5 min, LC20 phosphorylation and [Ca2+]i, progressively declined to a steady state of 50% and 65%, respectively, of their peak values. PD-098059 significantly inhibited the phenylephrine-induced increase in [Ca2+]i, in the uterine artery and decreased the peak level to 56% of control (Fig. 1). Likewise, PD-098059 significantly decreased the rate of phenylephrine-mediated LC20 phosphorylation and the maximum phosphorylation in the uterine artery. The peak phosphorylation level was significantly decreased to 35.6 ± 8.7% of control, and the time to the peak was delayed by 20 s compared with control. Although PD-098059 did not change the time to the peak of phenylephrine-induced force development, it slowed the initial rate of tension development and significantly decreased the contractile tension compared with control.

**Effect of PD-098059 on LC20 phosphorylation-[Ca2+]i, relation.** Contractions of smooth muscle can be regulated by altering the dependence of LC20 phosphorylation on [Ca2+]i, or the dependence of force on LC20 phosphorylation. To determine the potential role of ERK in the regulation of Ca2+ sensitivity of LC20 phosphorylation at fixed [Ca2+]i, we exam-
significantly decreased the intercept (from 0.164 ± 0.0097 to 0.008) significantly lower than the corresponding time point (from 3 to 30 s). Values are means ± SE from 4–9 animals. *All PD-098059 values at 5 s are significantly decreased compared with control (P < 0.05).

has the effect of showing a decreased LC20 phosphorylation at any given [Ca2+].

Effect of PD-098059 on force-LC20 phosphorylation relation. Although the majority of stimuli result in a unique relation between LC20 phosphorylation and force in smooth muscle, collateral regulation via thin filaments in modulating contraction can increase or decrease the force-to-LC20 phosphorylation ratio. We further examined the role of ERK in the regulation of contractions independent of changes in LC20 phosphorylation by evaluating the effect of PD-098059 on the relation between phenylephrine-stimulated increases in LC20 phosphorylation and force measured simultaneously in the same tissue. Phenylephrine-induced tensions were plotted against their corresponding phosphorylated LC20 levels of the initial rising phases in control and PD-098059-treated tissues, respectively (Fig. 3A). PD-098059 caused a leftward shift in the force-LC20 phosphorylation relation (Fig. 3A), suggesting that tension developed at a given level of LC20 phosphorylation in PD-098059-treated tissues was increased compared with that in control tissues. Because the relation between phenylephrine-stimulated LC20 phosphorylation and force development was nonlinear in control and PD-098059-treated tissues, analysis was performed to determine the levels of LC20 phosphorylation of control and PD-098059-treated tissues at corresponding tensions (from 0.4 to 2.8 g). There was a linear relation between the levels of LC20 phosphorylation of control and PD-098059-treated tissues at corresponding tensions (Fig. 3B), with the slope (0.456 ± 0.008) significantly lower than the line of identity, confirming that the amount of force produced per phosphorylated LC20 was enhanced by PD-098059.

Effect of PD-098059 on PDBu-stimulated [Ca2+]i, LC20 phosphorylation, and tension. Compared with phenylephrine, PDBu stimulated a much slower development of tension, which reached the maximum at 20 min. In contrast to phenylephrine, PDBu did not significantly increase [Ca2+]i (data not shown) or LC20 phosphorylation in the uterine artery (Fig. 4). Simultaneous measurement of [Ca2+]i and contractions showed that PD-098059 increased PDBu-induced contractions without changing [Ca2+]i (data not shown). The effect of PD-098059 on PDBu-induced contraction and LC20 phospho-

Fig. 1. Effect of PD-098059 on phenylephrine-induced intracellular Ca2+ concentration ([Ca2+]i; A), phosphorylation of 20-kDa myosin light chain (LC20; B), and contraction (C) in the uterine artery. Arterial rings were pretreated with 30 μM PD-098059 or the vehicle DMSO (control) for 30 min and then stimulated with 3 μM phenylephrine. [Ca2+]i, LC20 phosphorylation, and contraction were recorded simultaneously in the uterine artery. Representative Western immunoblot shows LC20 and phosphorylated LC20 (LC20-P) induced by phenylephrine at 0–300 s. Values are means ± SE from 4–9 animals. *All PD-098059 values at ≥5 s are significantly decreased compared with control (P < 0.05).

Fig. 2. Effect of PD-098059 on phenylephrine-mediated LC20 phosphorylation-[Ca2+]i, LC20 phosphorylation, and tension. Arterial rings were pretreated with 30 μM PD-098059 or DMSO for 30 min and then stimulated with 3 μM phenylephrine. Increases of LC20 phosphorylation stimulated by phenylephrine were plotted to show responses as a function of [Ca2+]i ([fura 2 signal, i.e., ratio of fluorescence at 340 nm to fluorescence at 380 nm (R340/380)]) at each corresponding time point (from 3 to 30 s). Values are means ± SE from 4–9 animals.
that preceded the contraction. We found that the decrease in phenylephrine-induced force development caused by PD-098059 was preceded by a reduction in LC20 phosphorylation, suggesting a role for ERK in thick filament regulation. Similar results were obtained in porcine carotid artery, in which PD-098059-mediated inhibition of endothelin-1-induced contraction was associated with a reduction in LC20 phosphorylation (8).

Previous studies in isolated smooth muscle cells suggested that ERK does not regulate [Ca2+]i (38, 49). However, our recent studies in intact arterial rings, in which [Ca2+]i and contractile tension were measured simultaneously in the same tissue, demonstrated clearly that PD-098059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in [Ca2+]i and Ca2+ sensitivity in the uterine artery (55). This suggests that, in addition to the Ca2+-independent pathway as previously proposed (1, 2, 11, 17, 21, 33), the ERK signaling pathway also involves the Ca2+-dependent components of vascular contractions. Consistent with our previous findings, the present study demonstrated that PD-098059 inhibited the phenylephrine-induced increase in [Ca2+]i. The mechanisms involved in ERK-mediated regulation of [Ca2+]i are not clear. Given the finding that PD-098059 did not affect force development elicited by KCl depolarization (8, 52, 55), it is likely that ERK may regulate the release of Ca2+ from intracellular pools but not Ca2+ entry via L-type Ca2+ channels. Because LC20 phosphorylation is primarily regulated by [Ca2+]i, our results suggest that ERK may regulate LC20 phosphorylation, in part, through modulating [Ca2+]i.

In addition, we found that PD-098059 significantly decreased Ca2+ sensitivity of LC20 phosphorylation in response to phenylephrine, i.e., less LC20 phosphorylation at the given

**DISCUSSION**

**ERK and thick filament regulation.** Smooth muscle contraction is regulated by thick and thin filament regulatory pathways. Thick filament regulation is mediated by Ca2+-dependent mechanisms that lead to activation of MLCK and LC20 phosphorylation and by Ca2+-independent mechanisms that involve inactivation of MLCP and a decrease in LC20 dephosphorylation. In the present study, we examined the temporal relations among [Ca2+]i, LC20 phosphorylation, and isometric force in the pregnant uterine artery and, consistent with previous findings, demonstrated that phenylephrine produced a time-dependent increase in [Ca2+]i and LC20 phosphorylation.
suggests that ERK may have a positive tonic effect on Ca$^{2+}$ through changes in pMLCK activity in COS-7 cells, which were reversed by pretreatment with PD-098059 (29).

**ERK and thin filament regulation.** The present findings that [Ca$^{2+}$]i and the degree of LC20 phosphorylation decline from their peak values to lower steady-state levels during phenylephrine-induced sustained uterine artery contraction are in agreement with previous results (8, 12, 34, 54). Previously, the dissociation of force and LC20 phosphorylation was postulated to be a consequence of the “latch” state (25, 35). However, recent studies suggested an additional thin filament regulation in smooth muscle contraction (21, 33). The present study demonstrated that PD-098059 increased force development at given levels of LC20 phosphorylation mediated by phenylephrine. This suggests that, in the uterine artery, α1-adrenoceptor-mediated contractions are regulated, in addition to the thick filament pathway, by thin filament pathways. More importantly, the present results suggest that ERK inhibits this α1-adrenoceptor-mediated thin filament regulation of contractions. Nevertheless, PD-098059 inhibited the phenylephrine-induced contraction, suggesting that the thick filament regulatory pathway, i.e., LC20 phosphorylation, predominates in α1-adrenoceptor-mediated uterine artery contractions.

The idea that ERK may inhibit thin filament regulatory pathways is further supported by the results of PKC-mediated contractions in the uterine artery. PKC activation of smooth muscle contraction has been well demonstrated from studies showing that phorbol esters, known to activate PKC, induce slow sustained contractions in many types of vascular smooth muscle (7, 10, 23, 24, 40, 44). In our previous studies in the uterine artery, we showed that PDBu increased PKC activity and induced contractions (55). Phosphorylation of LC20 has been reported in phorbol ester-induced contractions (22, 41–43). However, the significance of phorbol ester-induced LC20 phosphorylation in the contraction is controversial, and there is an inconsistency in the dependence of LC20 phosphorylation on [Ca$^{2+}$]i. For instance, it has been reported that PDBu induces LC20 phosphorylation in Ca$^{2+}$-depleted rat aortas (22,

Fig. 5. Effect of PD-098059 on PDBu-stimulated phosphorylation of p42 and p44 ERK in the uterine artery. Arterial rings were pretreated with 30 μM PD-098059 or DMSO for 30 min and then stimulated with 5 μM PDBu. PDBu-induced increase in phosphorylated p42 (A) and p44 (B) ERK (p-ERK42 and p-ERK44) was determined by Western immunoblot analysis. Values are means ± SE from 6 animals.

[Ca$^{2+}$]i, in the presence of PD-098059. Because alterations in the activities of MLCK or MLCP at fixed [Ca$^{2+}$]i, will affect the Ca$^{2+}$ sensitivity of LC20 phosphorylation, the results suggest that, in addition to the regulation of MLCK activity through changes in [Ca$^{2+}$]i, ERK may also regulate MLCK or/and MLCP activities independent of changes in [Ca$^{2+}$]i. The finding that PD-098059 decreased the intercept without affecting the slope of the LC20 phosphorylation-[Ca$^{2+}$]i relation suggests that MLCK and MLCP can be involved in the regulation of Ca$^{2+}$ sensitivity of LC20 phosphorylation but may not affect agonist-mediated Ca$^{2+}$ sensitivity. MLCK and MLCP can be involved in agonist-induced Ca$^{2+}$ sensitization (39). Although the present study cannot rule out effects on MLCP, we speculate that the tonic effect of ERK on Ca$^{2+}$ sensitivity observed in the present study may be mediated by an increase in MLCK activity. It has been demonstrated that purified, constitutively active ERK1/2 can phosphorylate chicken gizzard MLCK and increase its phosphotransferase activity in vitro (29). In addition, transient transfection of a mutationally activated MEK1 increased MLCK and LC20 phosphorylation in COS-7 cells, which were reversed by pretreatment with PD-098059 (29).

Fig. 6. Effect of PD-098059 on PDBu-stimulated phosphorylation of caldesmon (Ser$^{789}$) in the uterine artery. Arterial rings were pretreated with 30 μM PD-098059 or DMSO for 30 min and then stimulated with 5 μM PDBu. PDBu-induced increase in phosphorylated caldesmon at Ser$^{789}$ (p-CaD$^{789}$) was determined by Western immunoblot analysis. Values are means ± SE from 4–6 animals.
haviour of smooth muscle contractions (13, 27, 36). It has been proposed that ERK-mediated phosphorylation of CaD reverses the inhibitory effect of CaD on acto-activated myosin ATPase, which is mediated predominantly through thin filament regulatory pathways. Phorbol ester-induced contractions have been shown to involve regulatory components, including CaD and calponin, associated with thin filaments (33, 45, 46, 53). Phosphorylation of CaD by PKC reverses its inhibitory effect on myosin ATPase (48). The present finding that PD-098059 enhanced PKC-mediated contraction without changing \([Ca^{2+}]\), or LC20 phosphorylation levels reinforces the conclusion that ERK inhibits the thin filament regulatory pathway in the uterine artery.

In intact vascular smooth muscle, ERK has been demonstrated as a physiologically relevant CaD kinase mediating CaD phosphorylation (2–5). CaD functions as a thin filament regulatory protein and exerts an inhibitory effect on vascular smooth muscle contractions (13, 27, 36). It has been proposed that ERK-mediated phosphorylation of CaD reverses the inhibitory activity of CaD on actin-activated myosin ATPase, hence activating the thin filament pathway (15, 17, 21, 33). Nonetheless, the importance of CaD phosphorylation at ERK-specific sites (particularly at Ser789) in smooth muscle contraction remains controversial. Krymsky et al. (30) showed that ERK phosphorylation of gizzard smooth muscle CaD did not reverse its inhibitory effects on myosin ATPase. In addition, Nixon et al. (37) demonstrated that CaD phosphorylation by recombinant, activated ERK2 had no effect on the Ca\(^{2+}\) sensitivity of Triton-permeabilized vascular smooth muscle preparations and concluded that the phosphorylation of CaD by ERK is temporally associated with, but not involved in, force generation in smooth muscle. In porcine carotid artery stimulated with endothelin-1, D’Angelo and Adam (8) examined the interrelation among ERK activity, phosphorylation of CaD and LC20, and isometric force. They demonstrated that the inhibitory effect of PD-098059 on force could not be correlated with a corresponding effect on ERK-mediated CaD phosphorylation, because force in arterial strips stimulated with endothelin-1 in the absence or presence of PD-098059 tended to approximate each other over time, despite significant differences in the level of CaD phosphorylation.

In the present study, we demonstrated that PDBu produced parallel time courses in increasing phosphorylation of p42 and p44 ERK and CaD at Ser789 and that the phosphorylation was blocked by PD-098059, suggesting ERK-mediated phosphorylation of CaD at Ser789 in the uterine artery. These are in agreement with previous findings (9). The novel finding of the present study is that PD-098059 had opposite effects on PDBu-induced CaD phosphorylation at Ser789 and contractions. It blocked phosphorylation of CaD at Ser789 but potentiated the contractions. This suggests that ERK-dependent phosphorylation of CaD at Ser789 may not be involved in the PDBu-induced contraction but, rather, may inhibit it in the uterine artery. Although we were unable to determine phosphorylation of CaD at Ser759 because of the lack of appropriate antibody, a previous study demonstrated that the major site of ERK-dependent phosphorylation in CaD was Ser789. Although all the phosphate incorporated into CaD by the ERK is in Ser789 or Ser759, not all the phosphate in CaD is accounted for by these two sites (3, 9). In addition to ERK-dependent phosphorylation sites, phorbol esters and activation of PKC may increase phosphorylation of CaD at other sites, directly or indirectly, through unknown mechanisms (4, 9, 48, 50, 51). Although CaD phosphorylation in vitro by PKC was at sites that were different from those involved in PDBu-induced phosphorylation of CaD in intact canine aortas (3), endogenous CaD kinase activity in sheep aorta smooth muscle was purified and identified as a proteolytic fragment of PKC (51). PKC phosphorylated sheep aorta CaD in native thin filaments and in the isolated state at Ser27, Ser587, Ser610, Ser657, Ser686, and Ser726. PKC-mediated phosphorylation of intact CaD and of its COOH-terminal fragment containing 658–756 significantly decreased their ability to inhibit acto-heavy meromyosin ATPase (51). Taken together, we propose that, in sheep uterine artery, PKC induces phosphorylation of CaD at Ser789 through activation of ERK, as well as at other site(s) through unknown mechanisms (Fig. 7). It is the phosphorylation of CaD at site(s) other than Ser789 that may be important in reversing the inhibitory effect of CaD on myosin ATPase and leading to contractions. Phosphorylation of ERK-dependent Ser789 may not lead to the reversal of CaD inhibitory effects on myosin ATPase. Instead, phosphorylation of Ser789 may inhibit PKC-mediated phosphorylation of the other site(s).

In summary, we have shown in the pregnant uterine artery that \(\alpha_1\)-adrenoceptor-mediated contraction is regulated through thick and thin filament pathways, with the thick filament regulatory pathway, i.e., LC20 phosphorylation, predominating. However, PKC-mediated contraction is regulated predominantly through thin filament pathways, i.e., independent of changes in LC20 phosphorylation. ERK activation differentially regulates thick and thin filament pathways in the uterine artery. ERK potentiates the thick filament regulatory pathway.

Fig. 7. Proposed model: ERK-dependent phosphorylation of caldesmon at Ser789 inhibits PKC-mediated contractions. We propose that PKC induces phosphorylation of caldesmon at Ser789 through activation of ERK, as well as at other site(s) through unknown mechanisms in sheep uterine artery. It is the phosphorylation of caldesmon at site(s) other than Ser789 that may be important in reversing the inhibitory effect of caldesmon on myosin ATPase and leading to contractions. Phosphorylation of ERK-dependent Ser789 may not lead to the reversal of caldesmon inhibitory effects on myosin ATPase. Instead, phosphorylation of Ser789 may inhibit PKC-mediated phosphorylation of the other site(s).
by elevating LC20 phosphorylation via increases in [Ca2+] and 
Ca2+ sensitivity of LC20 phosphorylation. In contrast, the 
present study indicates that ERK attenuates thin filament regu-
laratory pathways and suppresses contractions independent of 
changes in LC20 phosphorylation in the uterine artery. Al-
though it is not clear whether the ERK-mediated inhibition of 
the thin filament regulatory pathway is unique to the pregnant 
uterine artery or is a more generalized mechanism, this novel 
finding suggests an intriguing hypothesis that, among ERK’s 
effects, CaD phosphorylation at ERK-specific sites may sta-
bilize its inhibitory effect on actin-activated myosin ATPase.

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