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Regulatory Mechanisms of Uterine Artery Contractility : Effect of Pregnancy

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Regulatory Mechanisms of Uterine Artery Contractility: Effect of Pregnancy

by

DaLiao Xiao

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Pharmacology

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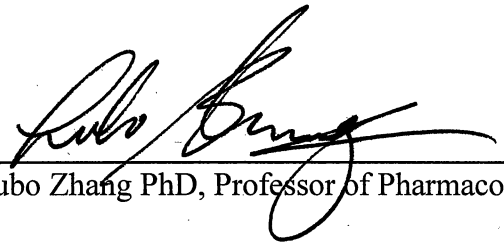
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.



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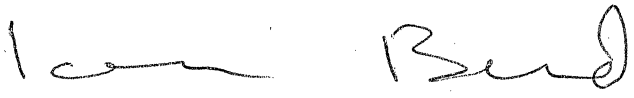

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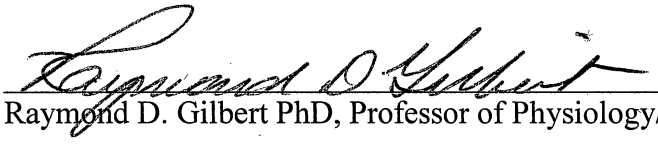

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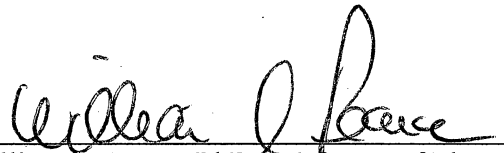

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LIST OF ABBREVIATIONS

5-HT---------- 5-hydroxytryptamine (serotonin)
$[Ca^{2+}]_i$---------- Intracellular calcium concentration
CaD---------- Caldesmon
CaP---------- Calponin
DAG---------- Diacylglycerol
ERK---------- Extracellular signal-regulated kinase
IP_3---------- Inositol 1, 4, 5-trisphosphate
MAP kinase---------- Mitogen-activated protein kinase
MEK---------- MAP kinase kinase
(M)LC20---------- 20-kDa light chain of myosin
(M)LC20-P---------- (M)LC20 phosphorylation
MLCK---------- Myosin light chain kinase
MLCP----------Myosin light chain phosphatase
MYPT---------- Myosin phosphatase regulatory targeting subunit
NE---------- Norepinephrine
NO---------- Nitric oxide
p-CaD---------- Phosphorylation of Caldesmon
p-CPI-17---------- Phosphorylation of CPI-17
PD098059---------- (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)
PDBu ---------- Phorbol 12,13-dibutyrate
PE ---------- Phenylephrine
p-ERK ----------Phosphorylation of ERK
PKC ----------Protein kinase C
p-MYPT1 ---------- Phosphorylation of MYPT1
PP1c ---------- MLCP catalytic subunit
$R_{f340/380}$ ---------- Ratio of fluorescence intensity at wavelength 340 and 380 nm
ROK ---------- Rho-associated kinase
TCA ---------- Trichloroacetic acid

ABSTRACT OF THE DISSERTATION

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Effect of Pregnancy

by

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Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, June 2004
Dr. Lubo Zhang, Chairperson

Regulation of uterine blood flow during pregnancy is important not only for the growth and survival of the fetus but also for cardiovascular well-being of the mother. Pregnancy is associated with a significant decrease in uterine artery vascular tone and a dramatic increase in uterine blood flow. Previous studies have been focused on adaptation of the endothelium to pregnancy and indicated that increased NO sythesis/release plays an important role in vasodilation of the uterine artery in pregnancy. However, little is known about the adaptation of contractile mechanisms of the uterine artery to pregnancy. The central hypothesis of the proposed studies is that extracellular signal-regulated kinase (ERK) and protein kinase C (PKC) pathways interact in the regulation of uterine artery contractility and play an important role in the adaptation of uterine artery contractile mechanisms to pregnancy. To test this hypothesis, we propose a series of experiments in the uterine arteries obtained from nonpregnant and near-term (~140 Days) pregnant sheep. We found that ERK plays an important role in the regulation of uterine artery contractility, and its effect is agonist-dependent. More importantly, pregnancy selectively enhances the role of ERK in α_1-adrenoceptor-mediated contractions and its effect in suppressing protein kinase C-mediated contraction

in the uterine artery. Our data indicate that 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. We also found that pregnancy upregulates α_1-adrenoceptor-mediated Ca^{2+} mobilization and LC_{20} phosphorylation. In contrast, pregnancy downregulates the Ca^{2+} sensitivity of myofilaments, which is mediated by both thick and thin filament pathways. ERK inhibitor, PD098059 significantly inhibited $ERK_{44/42}$, MYPT1/Thr^{850} phosphorylation but not MYPT1/Thr^{696} and CPI-17/Thr^{38}. It suggests that ERK mediated Ca^{2+} sensitivity is due to, at least, partly through inhibition of MLCP activity. On the other hand, activation of PKC by PDBu results increase in CPI-17/Thr^{38} phosphorylation and contraction without changes in LC_{20} phosphorylation. These results imply that PKC-CPI-17-mediated Ca^{2+} sensitivity of contraction might be through thin filament pathway in the uterine artery.

CHAPTER ONE

General Introduction

Introduction

A. Physiological Changes of Uterine Artery during Pregnancy

Pregnancy is associated with physiological changes in the maternal cardiovascular system that ensure normal fetal development. The dramatic alterations in arterial size and function during pregnancy are unique to the uterine artery. As pregnancy progresses, the uterine blood flow increases from 23 to >1,250 ml/min (80, 84). In late pregnancy, the uterine arteries carry about 20% of the maternal cardiac output, with approximately 80% of the uterine blood flow going to the placenta. Regulation of uterine blood flow during pregnancy is important not only for the growth and survival of the fetus but also for cardiovascular well-being of the mother. Several factors account for the striking increase of uterine blood flow during pregnancy, including growth of new vessels and remodeling of existing vessels in early pregnancy, and decreased vascular tone in these vessels.

The structure of the uterine artery changes markedly during pregnancy to accommodate the substantial increase in uterine blood flow. Uterine artery diameter markedly increased during pregnancy in the human (5, 80), pig (36) and sheep (30). The diameter of the uterine artery increases, as measured in situ, from 2.8 mm in nonpregnant ewes to 5.0 mm or greater during the late pregnancy (30). The increase in diameter of the uterine artery with pregnancy is not merely the result of passive dilation, since wall thickness is unchanged (35). This finding suggests that there is active growth of the uterine arterial wall. Griendling at al. (35) suggested that this

arterial growth is accomplished by a 2.5-fold increase in smooth muscle mass per unit length of vessel, despite no change in wall thickness. During pregnancy the number of cells per unit area and the diameter of the cells are unchanged; however, the numbers of nuclei per unit area decrease in the uterine artery (5). This could be explained in the case in which cells were elongated, thus reducing the probability of sectioning through the nucleus. Growth in cell length could explain the increase in uterine arterial diameter seen in pregnancy. Although it does not exclude a component of hyperplasia, it does favor hypertrophy as a major factor in accounting for vessel growth. Such hypertrophy might be more readily reversible after pregnancy than hyperplasia and may account for the rapid loss of those characteristics acquired during pregnancy (5). The biochemical changes in the uterine artery during pregnancy have also been observed. It has been found that there was a significant increase in protein contents of the uterine arteries from pregnant ewes than those from nonpregnant ewes but no difference in the fractional cellular composition of the muscularis (5). The increased protein content resulted in an increase in contractile protein contents and the total amount of myosin light chain phosphorylated in response to stimulation with phenylephrine. Thus significant biochemical alterations occur that can, in part, explain on a cellular level the observed increases in stress of uterine arterial smooth muscle associated with pregnancy.

Growth of new vessels as well as remodeling of existing vessels during early pregnancy contributes to the increased uterine blood flow. However, the fact that the period of greatest increase in uterine blood flow occurs after the completion of new vessel growth indicates that, in addition to those structural changes, functional

alterations and maintenance of vasodilation in existing or newly developed vessels are crucial. Normal pregnancy is associated with reductions in total vascular resistance and arterial pressure possible due to enhanced endothelium-dependent vascular relaxation and decreased vascular reactivity to vasoconstrictor agonists. Previous studies have been focused on adaptation of the endothelium to pregnancy and demonstrated that increased nitride oxide (NO) synthesis/release plays an important role in the maintenance of vasodilation in the uterine artery in pregnancy (9, 65, 74, 89, 98, 100, 101, 102). However, little is known about the adaptation of contractile mechanisms of uterine artery smooth muscle to pregnancy. Sympathetic activation of α_1-adrenoceptors plays an important role in moment-to-moment regulation of vascular contractility. Although pregnancy decreases uterine artery vascular tone, it paradoxically increases acute contractile response of the uterine artery to nonsynaptic α_1-adrenergic stimulation (4, 5, 18, 19, 77), due probably in part to the transient and reversible sympathetic denervation of uterine artery during pregnancy (56, 73). The sympathetic neurotransmitter norepinephrine, acting postsynaptically at the smooth muscle α_1-adrenergic receptors, is a principal determinant of vascular contractility of the uterine artery. Alpha$_1$-adrenergic receptors play a particularly important role in control of blood pressure via induction of vascular smooth muscle contraction. Alpha$_1$-adrenergic receptor signaling mechanism generally involve coupling to the G proteins; these G proteins activate phospholipase C, leading to increased hydrolysis of phospharidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and 1,2-diacylglycerol, which increase intracellular Ca^{2+} concentration and activate protein kinase C (PKC), respectively.

Although this predominant view of α_1-receptor signaling provides substantial insight into α_1-receptor-mediated responses in various cells, there are clear indications that these mechanisms may not explain all aspects of α_1-receptor signaling. Recent evidence demonstrates that α_1-receptor-stimulated mitogenic responses in myocytes may be due to activation of tyrosine protein kinases and MAP kinases, suggesting that α_1-adrenergic receptors may share common signal pathways with tyrosine kinase receptors in the stimulation of mitogenesis. Moreover, recent studies have demonstrated a direct relationship between MAP kinase activation and smooth muscle contraction (3, 20, 49). However, signaling pathways utilized by α_1-receptors in promoting mitogenic effects, such as growth-related gene expression and DNA synthesis, and the cause-and-effects relationship between MAP kinase activation and smooth muscle contraction are still not fully understood. On the other hand, it has been demonstrated that PKC is also important in the regulation of basal vascular tone and arterial caliber, which is a major determinant of blood flow (7, 16, 59, 67, 78). Although a growing body of evidence suggests a role for PKC in smooth muscle contraction, little information is available on the changes in vascular PKC activity and the regulation of PKC activity during pregnancy. It has reported that PKC activity (64) and PKC-mediated contractile pathway (46) in vascular smooth muscle is reduced during pregnancy. The results suggest that one possible mechanism of the pregnancy-associated changes in vascular reactivity may involve changes in the amount and/or activity of the PKC isoforms and the down stream signal pathways.

B. Hormonal regulation of the uterine artery contractility

Changes in uterine blood flow throughout pregnancy appear to be due to steroid

inducded alterations in uterine arterial tone and contractility. In pregnancy, tone is markedly depressed as estrogen concentrations rise, and the vessel is distended and flaccid. Estrogens are vasoactive substances, influencing the vascular wall mainly through gene modulation by the estrogen receptor(s), identified on endothelial and vascular smooth-muscle cells. A direct effect of estrogens was also postulated, which may be the result of post-translational modification of enzymes. Whereas gens modulation typically results in long-term effects, direct steroid actions can rapidly influence vascular function. Estrogens respectively augment and restore endothelium-dependent vasodilation in atherosclerotic blood vessels by increasing local NO concentraction. This is caused either by enhancing endothelial NOS activity or by inhibiting NO degradation, as estrogens are potent antioxidants. Besides their influences on the NO system, estrogens stimulate the activity of PGI_2 and EDHF while attenuating vasoconstrictor effects mediated by TXA_2 and ET-1. Direct effects of estrogens on proliferation and migration of vascular smooth-muscle cells and calcium channel blocker-like actions have been docemented (27). The progressive decrease in uterine arterial tone during gestation seems to result from the metabolism of placental estrogens to vasoactive catechol forms (27, 28). It has been demonstrated that peroxidases catalyze the conversion of estrogens to catechol estrogens. Extremely high concentrations of estrogens and peroxidase are detected in uterine lymphatic fluid during pregnancy (28). These lymphatics are in close contact with the uterine arterial vasculature and thus may serve to transport catechol estrogens. The catechol estrogens specifically inhibit calcium uptake through the potential-sensitive calcium channels and terminate the PKC-maintained tone of the

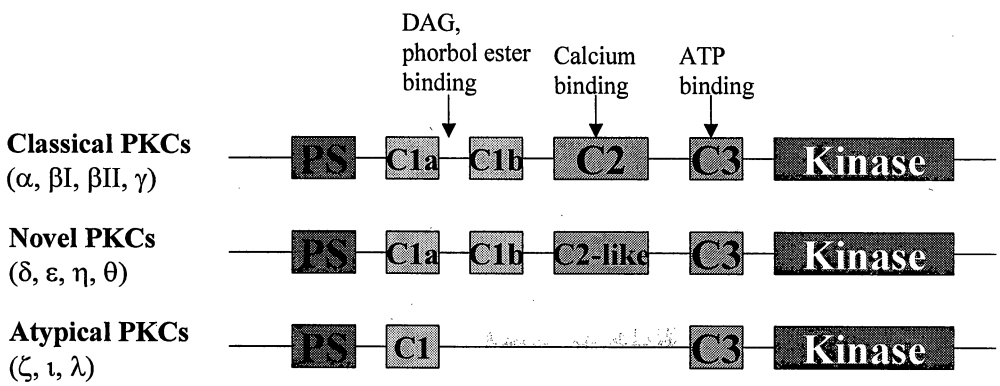
uterine arteries. In conjunction with the decreased calcium uptake through uterine arterial potential sensitive calcium channel, cellular PKC activity also progressively decreases throughout gestation in pigs (25). It is suggested that the decreasing PKC activity of uterine arterial smooth muscle may be a direct result of the catechol estrogen-induced inhibition of calcium uptake through the potential-sensitive calcium channel. Progesterone, in contrast to estrogen, augments the responsiveness of vascular smooth muscle to norepinephrine. During the estrous cycle of ewes, the higher the estrogen:progesterone ratio in systemic blood, the greater is the quantity of blood flowing through the uterine vascular bed. After the initial increase in blood flow to the uterus during early pregnancy, changes in the estrogen:progesterone ratio in fetal and maternal fluids appear to be unrelated to changes in uterine blood flow. This lack of association between the estrogen:progesterone ratio and uterine blood flow may result from a progressive reduction in number of adrenergic nerces observed in the uterus throughout pregnancy. If one accepts that the estrogen:progesterone ratio controls uterine blood flow throgh alterations in the function of periaterial adrenergic nerves, the impaired function of the nerves during mid and late gestation may preclude the effects of these steroids.

C. The role of PKC in the regulation of smooth muscle contraction

The role of PKC in the regulation of many biological functions is widely accepted (40). There are at least 11 PKC isoenzymes divided into three major groups: 1) classical group A PKCs: α, βI, βII, and γ; 2) novel group B PKCs: δ, ε, η, and θ; 3) atypical group C PKCs: ζ, ι, and λ (49, 96). The molecular structures of PKC isozymes are shown in Fig. 1. The multiplicity of PKC isozymes creates a challenge

in determining the involvement of each in specific signaling events. Group A and group B PKCs translocate, in general, from the cytosol to membranous sites upon activation with diacylglycerol (DAG) and certain phorbol esters. However, they differ markedly in their sensitivity to Ca^{2+}. Group A PKCs are Ca^{2+}-dependent *via* their C2 domains and are activated by phosphatidylserine (PS), DAG and phorbol esters through their cysteine-rich C1 domains. Group B PKCs are Ca^{2+} independent but are also regulated by PS, DAG and phorbol esters. Group C PKCs are neither Ca^{2+}-dependent nor require PS, DAG or phorbol esters for their activation (75, 83). All three PKC groups contain a pseudo-substrate or autoinhibitory domain that binds to the substrate-binding site in the catalytic domain preventing their activation in the absence of cofactors or activators. PKC activation is also regulated by phosphorylation, and both PKC kinase(s) and PKC autophosphorylation are required for full activity. Phosphorylation also regulates PKC subcellular localization and down-regulation. The α, β, δ, ε, and ζ isoenzymes of PKC have been detected in vascular smooth muscle (96). Although not all of these appear to be in all vascular smooth muscle tissues, our preliminary data suggested that all of these isoenzymes of PKC were present in the uterine artery. The involvement of PKC activation in

Fig. 1. PKC kinase family members and their structure



Schematic representaion of PKC kinase family members and their structure, **CR,** cysteine rich-lipid binding domain in C1; **PS,**pseudosubstrate; **C2,** calcium binding domain; **C3,** ATP binding domain.

smooth muscle contraction has been well demonstrated from the studies showing that phorbol esters, known to activate PKC, induce slow sustained contractions in many types of vascular smooth muscle (44, 75, 88). Whether and to what extent PKC isoenzymes expression and activities in uterine artery smooth muscle adapt to pregnancy is unknown.

D. The role of ERK in the regulation of smooth muscle contraction

Extracellular signal-regulated kinases (ERK1/2) are involved in the regulation of essential cellular processes, including gene expression and cell proliferation (11, 86). Recent studies have demonstrated that ERK plays a role in the regulation of smooth muscle contraction (3, 20, 31, 32, 47, 97). Different signaling transduction mechanisms have been reported in the ERK-mediated regulation of smooth muscle contractions. In vascular smooth muscle, ERK1/2 activation in response to angiotensin (ANG II), thrombin, or ATP appears to involve both Ca^{2+} and PKC-dependent mechanisms (2, 10, 12). Other studies have demonstrated that Ca^{2+}-independent contractions are dramatically reduced with the MEK inhibitor PD098059, in contrast to the Ca^{2+}-dependent component of the agonists-evoked contractions that are not affected by ERK (20, 23). D'Angelo et al. (17) reported that pretreating carotid arterial strips for 1 h with the MEK inhibitor PD098059 attenuated ET-1-stimulated isometric force and the decrease in the rate of force development caused by PD-098059 was associated with a reduction in myosin light chain (LC20) phosphorylation. Pregnancy is associated with a growth of uterine vasculature and a dramatic increase in uterine artery blood flow. It has been shown recently that the pregnancy-induced increase in uterine artery endothelial vasodilator production is

mediated in part by a marked alteration in signaling pathway including activation of the ERK pathway (8, 21). It is unknown whether ERK plays a role in the regulation of uterine artery contractility, and more importantly, whether pregnancy affects the potential role of ERK in the uterine artery.

E. Interaction between PKC and ERK in the regulation of smooth muscle contraction.

Regulation and activation of PKC and ERK are interrelated in vascular smooth muscle. Adrenergic stimulation of vascular smooth muscle induces cellular redistribution of both PKC and ERK (50, 51, 52). Studies have shown a transient, Ca^{2+} independent, PKC-dependent redistribution of cytosolic ERK to the plasma membrane before cell contraction, followed by a second redistribution away from the plasmalemma to the contractile filaments during cell contraction. Further studies revealed that the initial association of ERK with the plasmalemma occurred in a tyrosine phosphorylation-independent manner, whereas the delayed redistribution of ERK, co-localizing with caldesmon, occurred in a tyrosine phosphorylation-dependent manner (49). These observations suggest a role for ERK as a link between membrane-bound PKC and caldesmon during cell contraction (Fig. 2).

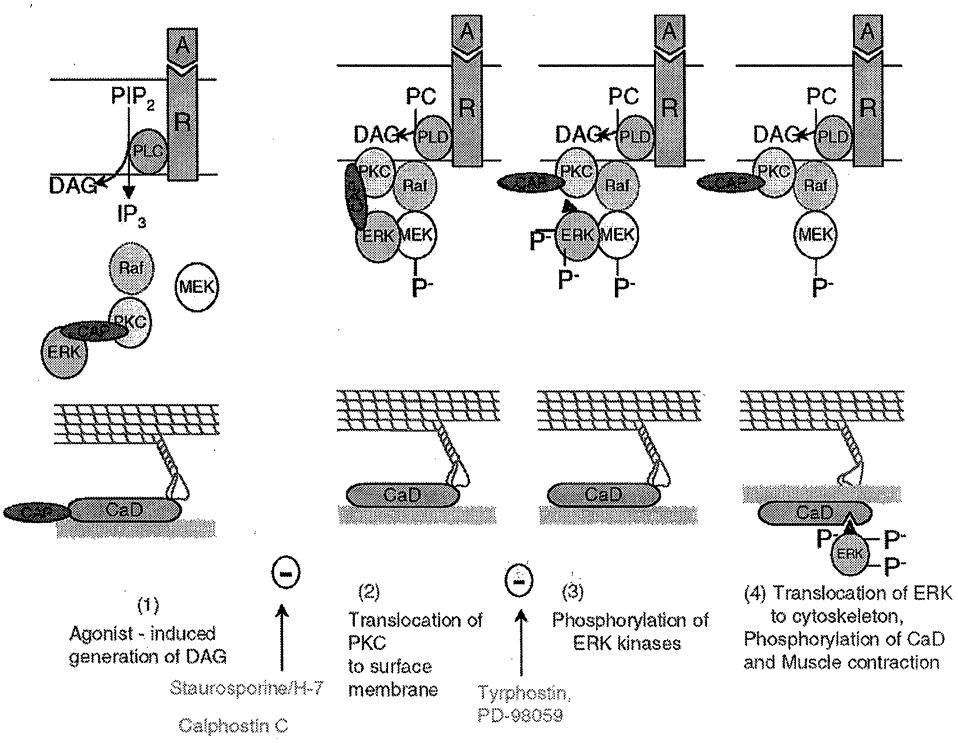


Fig. 2. Model of thin filament regulation, modified from Morgan (J Appl Physiol 91:953-962, 2001)

It has been proposed in several studies that ERK is a downstream signal of PKC in vascular smooth muscle (14, 48, 52). It has also been demonstrated that different agents, which produce contractions of smooth muscle, activate ERK at the same time (20, 31, 47). Although it has been suggested that PKC may be an upstream signal of ERK in agonist-stimulated contractions of vascular smooth muscle, several studies failed to see the inhibitory effect of PD-098059 on PDBu-induced contractions (1, 34, 69), suggesting that an alternative pathway may be involved. The finding that the PDBu-induced contraction was significantly attenuated in rat thoracic aorta during pregnancy (46), suggesting that the role of PKC in the regulation of vascular tone is down-regulated during pregnancy. However, the cellular and molecular mechanisms are unknown. One of the major goals of this project is to explore the cellular/molecular mechanisms underlying ERK suppressing PKC-mediated contraction during pregnancy, and how they affect uterine vascular resistance and regulate uterine blood flow in pregnant sheep.

F. Contractile mechanisms in vascular smooth muscle

Vascular smooth muscle contraction is regulated predominantly by phosphorylation and dephosphorylation of the 20-kD light chain of myosin (LC20) (38, 40, 45, 81). Phosphorylation of LC20 is affected primarily by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). An increase in $[Ca^{2+}]_i$ results in the activation of myosin light chain kinase (MLCK), which catalyzes phosphorylation of LC20. Phosphorylation of LC20 results in actin activation of myosin ATPase activity, the development of force, and contraction of the muscle. Dephosphorylation

in LC20 phosphorylation levels, perhaps by thin filament-associated signaling pathway (6, 71). Taken together, smooth muscle contraction is regulated by thick and thin filament regulatory pathways. Thick filament regulation is mediated by Ca^{2+}-dependent mechanisms that lead to activation of MLCK and LC20 phosphorylation and by Ca^{2+}-independent mechanisms that involve inactivation of MLCP and a decrease in LC20 dephosphorylation. On the other hand, thin filament signaling transduction pathway is regulated through both Ca^{2+}- and LC20 phosphorylation-independent mechanisms. An outline of some of the mechanisms that may regulate the thick and thin filaments in smooth muscle is shown in Fig. 3. The biochemical and physiological adaptations of these cascades that affect uterine arterial smooth muscle contractility during pregnancy are undefined.

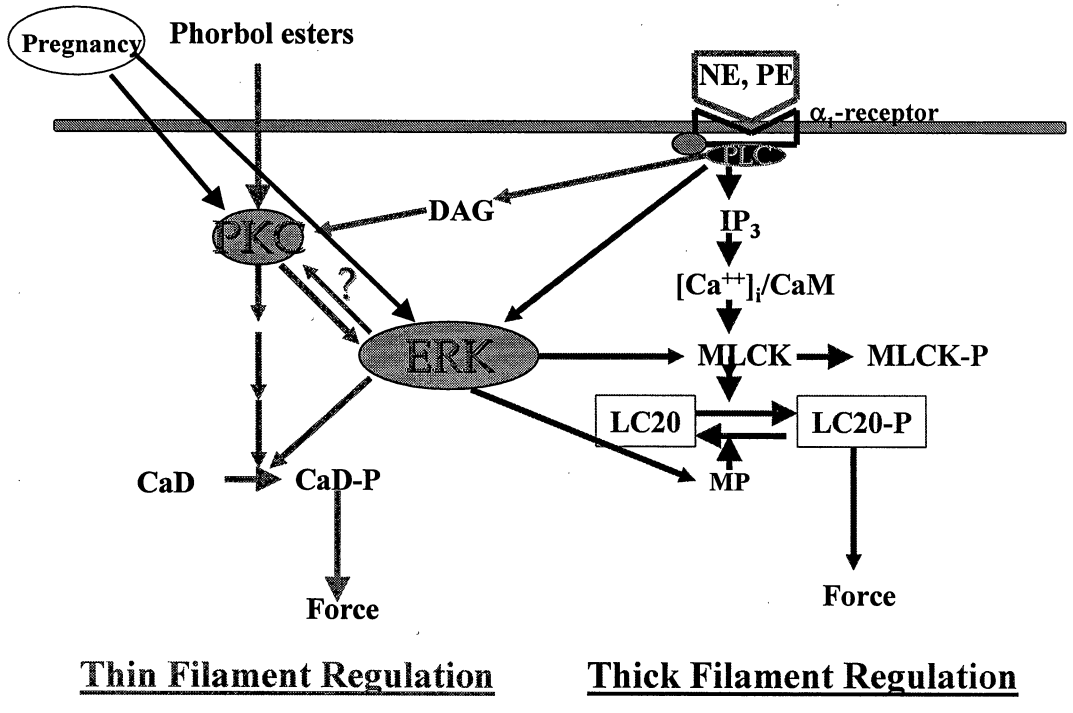


Fig. 3. Possible signaling pathways in uterine arterial smooth muscle

G. The role of MLCP in the regulation of smooth muscle contraction

A key event in agonist-induced Ca^{2+} sensitization is G protein-dependent inhibition of MLCP (55). MLCP consists of three subunits: a 110 to 130 kDa regulatory subunit (MYPT1), a ~37 kDa catalytic subunit (PP-1C), and a 20 kDa subunit of unknown function (39). It has been reported that inhibition of MLCP is, at least, regulated by following three independent mechanisms (Fig. 4). 1) Phosphorylation of the MYPT1 leads to inhibition of the phosphatase (41). This may occur *via* activation of Rho-associated kinase (ROK) (53), a kinase that is activated by the small GTPase RhoA. 2) The second messenger arachidonic acid inhibits MLCP activity by dissociation of the holoenzyme, as well as activation of PKC and Rho kinase (33). 3) Activation of PKC leads to inhibition of MLCP (68). This may be mediated by an endogenous, smooth muscle-specific phosphopeptide, CPI-17, which becomes a very potent inhibitor of PP-1C upon phosphorylation by PKC (62). Phosphorylation of MYPT1 by ROK leads to inhibition of MLCP. Other substrates of ROK include CPI-17 and calponin. However, there are concerns as to the access of ROK to its substrate MYPT1 because both Rho and ROK have to be recruited from a cytosolic pool to the cell membrane for activation. Perhaps, a kinase, such as ZIP kinase or ERK, could be a link between activated ROK and MYPT1, which leads to a Ca^{2+} independent increase in force. Recent studies from Morgan's laboratory (87) have provided evidence for another mechanism for Ca^{2+} sensitization. Their data show that there is an agonist-specific translocation of MLCP to smooth muscle cell membrane. $PGF_{2\alpha}$ stimulation of smooth muscle cells results in phosphorylation of

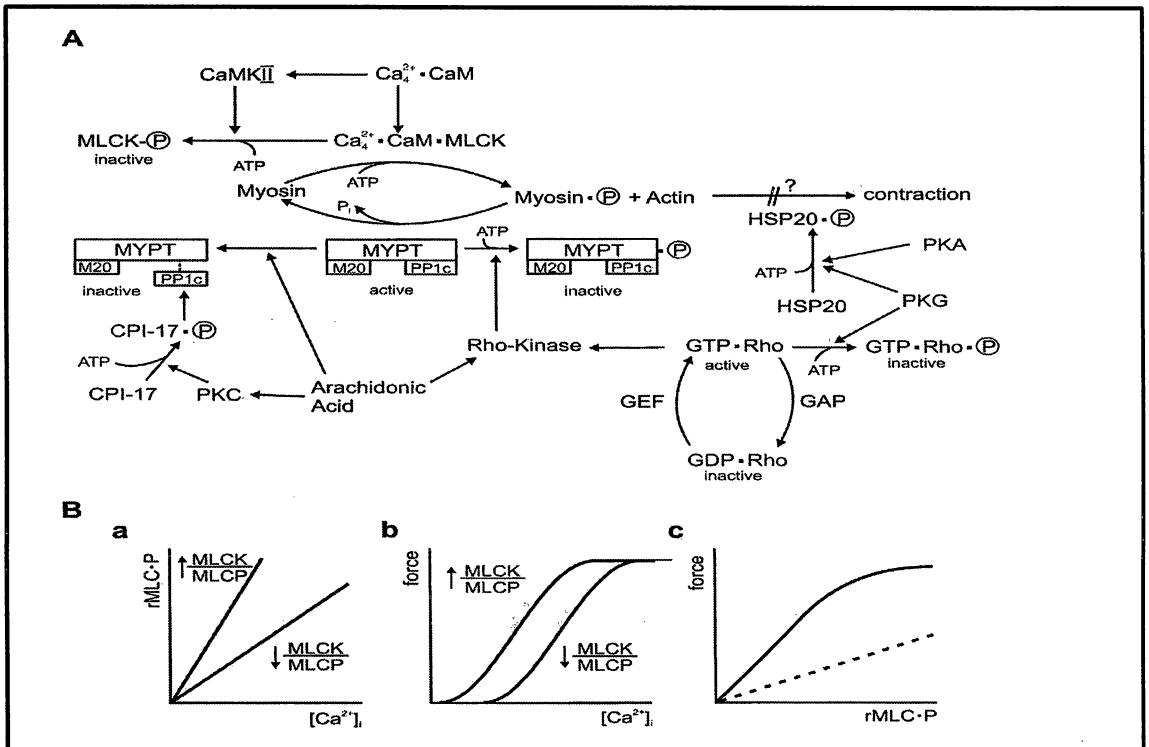


Fig. 4. A: signaling cascades that modulate Ca2+ sensitivity of phosphorylation of regulatory light chains of myosin (rMLC) and contraction. B: increasing the ratio of active MLCK to active MLCP will lead to an increase in Ca2+ sensitivity of rMLC phosphorylation (a) and contraction (b). A decrease in this ratio will have the opposite effect. Modulating the MLCK-to-MLCP ratio will not affect the dependence of force on rMLC phosphorylation (c, solid line). (Pfitzer G. J Appl Physiol. 91: 497-503, 2001)

MYPT at Thr^{696} and a translocation of MLCP from cytosol to cell membrane. The catalytic and targeting subunits of the MLCP are dissociated from each other in an agonist-specific manner and that the dissociation is accompanied by a slower rate of myosin phosphorylation. The phosphorylation and membrane translocation of the MLCP targeting subunit are inhibited with a ROK inhibitor. Translocation of PKC, ERK, calponin, and Rho A have all been reported, but how these proteins interact with MLCP and lead to inhibition of MLCP activity and to produce a Ca^{2+} independent contractions remain to be determined in uterine arteries.

At least two phosphorylation sites of MYPT1 have been identified at Thr^{696} and Thr^{850} (91). Previous reports have shown that phosphorylation of MYPT1/Thr^{696} through the RhoA/ROK pathway has been detected in a variety cells, including smooth muscle (43, 81, 94). On the other hand, MYPT1/Thr^{696} is constitutively phosphorylated, by kinases other than Rho-kinase, and does not respond to agonists (54, 76, 91). This implies that there is no agonist mediated G-protein-coupled signaling pathway leading to Thr^{696} phosphorylation in the smooth muscle. In contrast to Thr^{696}, MYPT1/Thr^{850} is significantly phosphorylated in response to agonist. These findings suggest an exciting hypothesis that the different phosphorylation sites may regulate MLCP activities differently at basal and agonist-stimulated states. MYPT1/Thr^{850} is not an inhibitory site in vitro (26), but its phosphorylation state could affect localization of MLCP and its catalytic subunit on myosin (95), and thereby, in conjunction with Thr^{696} phosphorylation, may be responsible for Ca^{2+}-sensitizing inhibition of MLCP when Thr^{696} is constitutively phosphorylated. It remains to be determined whether the difficulty in detecting

phosphorylation of MYPT1/Thr^{696} reflects its rapid dephosphorylation or operation of another RhoA/ROK-mediated mechanism that does not require Thr^{696} phosphorylation or is targeted to a different inhibitory residue not detected by the Thr^{696}-specific antibody.

Recent reports have indicated that CPI-17 plays a very important role in the G-protein- and PKC-mediated inhibition of MLCP and Ca^{2+} sensitization. CPI-17 was initially recognized as a PKC substrate and phosphorylated at the site of Thr^{38} by PKC (24) and it has been shown that Rho Kinase does also phosphoryalte CPI-17 in vitro (57). Thus, both PKC and ROK signaling pathways may contribute to regulation of MLCP activity through an increase in CPI-17 phosphorylation (54, 76, 79). Protein kinases other than PKC can also phosphorylate the inhibitory (Thr^{38}) site of CPI-17: ZIP-like kinase, PKN, integrin-linked kinase, PAK, and, most importantly, ROK (91). It remains to be shown which of these kinases, particularly ROK, is spatially and kinetically appropriate for phosphorylating CPI-17.

H. Thin filament regulation of smooth muscle contraction and the major thin filament-associated proteins

Thin filaments are defined as those filaments 6-8 nm in diameter and composed of filamentous actin. The thin filament signal transduction pathway is regulated through both Ca^{2+} independent and LC20 phosphorylation independent mechanisms. If Ca^{2+} sensitization were to be explained solely by inhibition of MLCP or activation of MLCK, sustained tension levels should always be paralleled by sustained LC20 phosphorylation levels, even if Ca^{2+} level falls. However, it is clear that during agonist-induced contractions of vascular smooth muscle, LC20 phosphorylation

levels do not parallel tension levels (22). This dissociation of LC20 phosphorylation levels (myosin or thick filament regulation) and tension levels has been used as evidence for the existence of thin filament regulation. In addition, there is also considerable direct evidence pointing to thin filament regulation in smooth muscle. Evidence includes: 1) the fact that crossbridge cycling rates can vary without detectable changes in LC20 phosphorylation (13, 37); and, most importantly, 2) the fact that contraction can occur in the absence of changes in LC20 phosphorylation (70). Thin filament regulatory proteins include caldesmon and calponin.

Caldesmon Recent studies on the smooth muscle actin-binding protein, caldesmon (CaD), have strongly suggested an important, and physiologically relevant, role for this protein in thin filament regulation. CaD is a thin filament-associated, actin, tropomyosin, myosin and calmodulin (CaM) binding protein. Its properties have been reviewed previously (40, 71). The molecular structure of CaD is shown in Fig. 5). Carboxy terminal domains are responsible for actin-binding and inhibition of myosin ATPase activity. Either binding of CaM or phosphorylation of sites in C-terminal half of the molecule can reverse the myosin inhibition. The N-terminal half of the molecule has been shown to bind myosin. CaD can acts as a cross-linker of actin and myosin. The consistency between the peptide studies with GS17C and the antisense studies strongly suggests that CaD plays a physiologically important role in suppressing smooth muscle tone. Studies have suggested two possible mechanisms by which inhibitory actions of CaD can be reversed: by the binding of Ca^{2+}/CaM and by phosphorylation (71). It is known to be phosphorylated in vitro by a number of protein kinases including CaMKII, casein kinase II, PKA, cdc2 kinase, ERK, and

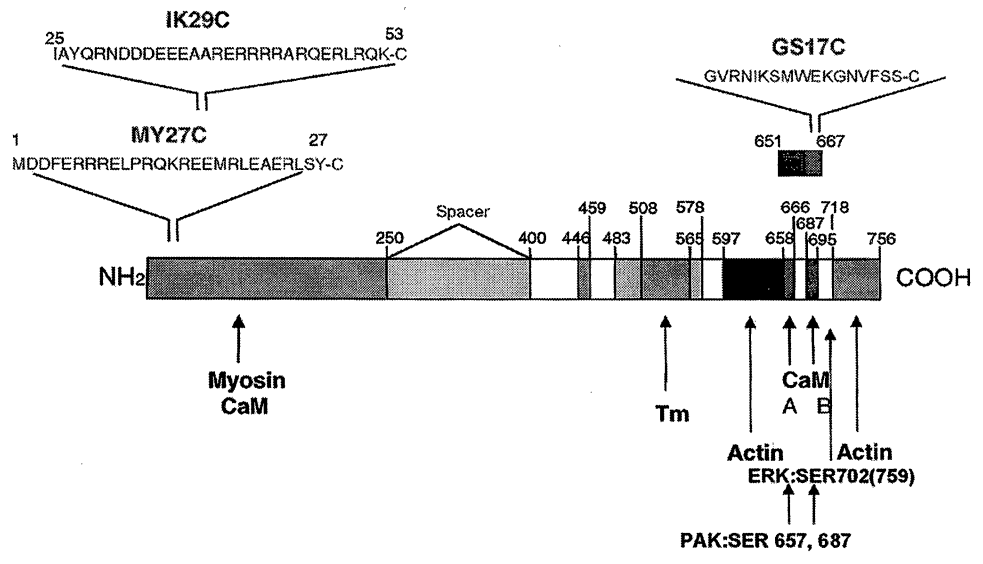


Fig. 5. Domain structure of caldesmon, modified from Lee et al. (J Biol Chem 275: 3213-20,2000). Numbering is according to the chicken gizzard sequence, except that the analogous mammalian extracellular regulated kinase (ERK) phosphorylation site (759) is given after the ERK gizzard phosphorylation site and the peptide IK29C is from the human sequence. CaM, calmodulin; Tm, tropomyosin; PAK, p21-activated kinase.

PKC. In vitro experiments have shown that phosphorylation of CaD by PKC or PAK can reverse the inhibitory activity of CaD on myosin ATPase activity and can also decrease the actin binding affinity of CaD. The function of CaD and the importance of CaD phosphorylation at ERK sites are controversial issues. Childs et al. (15) reported a significant effect of ERK phosphorylation on actin co-sedimentation of CaD. In contrast, Krymsky et al. (58) have shown that ERK phosphorylation of gizzaed CaD did not reverse its effects on myosin ATPase. Li et al. (63) have recently shown that ERK phosphorylation at both S759 and S789 of a C-terminal fragments actin binding. Similar results were presented by Marston et al. (66). The role of PKC and ERK in phosphorylation of CaD and its role in the regulation of contraction in the uterine artery are unknown.

Calponin Calponin (CaP) is another actin-binding protein that inhibits myosin ATPase activity. CaP has three isoforms that are separate gene products: 1) a smooth muscle-specific basic CaP, h1, 2) a neutral CaP, h2, that has a very high sequence homology with h1 CaP but is less basic and is localized primarily in cardiac muscle, and 3) acidic variant, which is not tissue specific, identified from rat brain and fibroblasts. Regarding molecular structure of CaP, the CaP homology (CH) domain, which has very high homology across isoforms, is followed by a variable region that contains an actin binding region with homology to troponin I (TnI), referred to as the TnI-like sequence. This is followed by three C-terminal repeats that are also involved in actin binding. CaP activity is regulated by phosphorylation by PKC or CaMKII, both of which reverse CaP's inhibitory activity. The major phosphorylation site in

vitro was identified as either Ser175 (99) or Thr184 (72). It was also identified that the CH domain of CaP is ERK binding domain (60).

The role of CaP in the regulation of smooth muscle contraction is controversial. Two mechanisms have been proposed for its function. On one hand, it has been suggested that CaP may directly inhibit actin-activated Mg-ATPase activity of myosin. On the other hand, it has also been proposed that CaP may facilitate agonist-induced signal transduction (71). This hypothesis is based on the observations that, in ferret vascular smooth muscle, in the early time points after agonist activation, ERK and PKC co-immunoprecipitate and co-translocate with CaP to the vicinity of the plasmalemma and the N-terminal 'CH domain' of CaP binds ERK, while the C-terminal half of CaP binds the regulatory domain of PKC and facilitates the activation of PKC *in vitro* (61). Based on these results, these investigators have speculated that CaP may function as an adaptor protein connecting the PKC cascade to the ERK cascade. After docking and being activated at the cell surface, ERK redistributes to the contractile filaments, phosphorylates CaD, and triggers contractions.

Aim of this project

The aim of the present project was to examine the main hypothesis that **regulatory mechanisms of ERK/PKC pathways in governing uterine artery contractility are altered during pregnancy.** To test this hypothesis, we propose a series of experiments in uterine arteries from nonpregnant and near-term (~140 days) pregnant sheep. Our specific aims are: 1) To test the hypothesis that pregnancy selectively enhances the role of ERK in the regulation of α_1-adrenoceptor-induced contractions and

its effect in suppressing PKC-mediated contraction in the uterine artery; 2) To test the hypothesis that ERK regulates smooth muscle contractility through both thick and thin filament regulatory pathways in the uterine artery; 3) To test the hypothesis that pregnancy up-regulates the thick filament regulatory pathway and down-regulates the thin filament regulatory pathway in the uterine artery; 4) To test the hypothesis that ERK inhibits myosin phosphatase activity by phosphorylating its regulatory/targeting subunit MYPT1, and CPI-17 which contributes to pregnancy-mediated up-regulation of the thick filament regulatory pathway in the uterine artery.

Significance

The proposed studies are of importance from both basic science and clinical perspectives. From the basic science point of view, the proposed studies of both thick and thin filament regulatory pathways are especially powerful means of evaluating mechanisms governing uterine artery contractility, and thereby enhance our basic understanding of uterine vasculature adaptation to pregnancy. They will provide exciting novel information from several standpoints. They will be the first to examine PKC isoenzymes expression and their functions in the uterine artery, and examine the effect of pregnancy. They will be the first to offer assessment of the dynamic interactions of ERK and PKC in the uterine artery, and to test the novel hypothesis that ERK is an upstream signal in suppressing PKC activity. They will be the first concerted effort to understand the mechanisms underlying both thick and thin filament regulatory pathways in the uterine artery. Most importantly, these novel assessments will extend to pregnant uterine artery in which such studies are extremely rare. Not only are these studies important to

understand basic mechanisms underlying adaptation of uterine artery contractile machinery to pregnancy, but they will also offer novel information in understanding signaling mechanisms for vascular contraction in general.

The proposed studies are relevant to the mechanisms involved in adjusting uteroplacental circulation to pregnancy. Given the importance of uterine blood flow in fetal development and maternal health, failure to make these adjustments is likely to contribute to many fetal abnormalities, including intrauterine growth restriction, as well as maternal cardiovascular disorders. The proposed studies will help provide a mechanistic basis for this functional adaptation, and thereby improve our understanding of problems associated with the maladaptation and abnormal pregnancy and permit us to address them in a more meaningful way.

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CHAPTER TWO

ERK MAP Kinases Regulate Smooth Muscle Contraction in Ovine Uterine Artery: Effect of Pregnancy

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Abstract

The present study investigated the potential role of extracellular signal-regulated kinase (ERK) in uterine artery contraction, and tested the hypothesis that pregnancy up-regulated ERK-mediated function in the uterine artery. Isometric tension in response to phenylephrine (PE), serotonin (5-HT), PDBu and KCl was measured in the ring preparation of uterine arteries obtained from nonpregnant and near-term (140 d gestation) pregnant sheep. Inhibiting ERK activation with PD-98059 did not change the KCl-evoked contraction but significantly inhibited the contraction to 5-HT in both nonpregnant and pregnant uterine arteries. PD-98059 did not affect PE-induced contraction in nonpregnant but significantly decreased it in pregnant uterine arteries. In accordance, PE stimulated activation of ERK in pregnant uterine arteries, which was blocked by PD-98059. PD-98059-mediated inhibition of the PE-induced contraction was associated with a decrease in both intracellular Ca^{2+} concentration and Ca^{2+} sensitivity of contractile proteins in pregnant uterine arteries. PDBu-mediated contraction was significantly less in pregnant than nonpregnant uterine arteries. PD-98059 had no effect on PDBu-induced contraction in nonpregnant but significantly increased it in pregnant uterine arteries. In addition, PD-98059 significantly enhanced PDBu-stimulated protein kinase C activity. The results indicate that ERK plays an important role in the regulation of uterine artery contractility, and its effect is agonist-dependent. More importantly, pregnancy selectively enhances the role of ERK in α_1-adrenoceptor-mediated contractions and its effect in suppressing protein kinase C-mediated contraction in the uterine artery.

Introduction

Extracellular signal-regulated kinase (ERK) has been proposed to regulate smooth muscle contraction. Activation of ERK is dependent on a dual-phosphorylation on Tyr^{185} and Thr^{187} by mitogen-activated/extracellular-regulated kinase kinase or MEK (5,6,34). It has been demonstrated in many studies that different agents, which produce contractions of smooth muscle activate ERK at the same time (4,9,10,13,14,23,39). However, conflicting results were obtained regarding a role for ERK in smooth muscle contractile regulation. A cause-and-effect relationship between ERK activation and α_1-adrenoceptor-mediated contraction was demonstrated in ferret aorta (10). In addition, addition of activated ERK to permeabilized airway smooth muscle strips resulted in a contraction by increasing Ca^{2+} sensitivity of contractile proteins (13). In contrast, studies with permeabilized rabbit vascular smooth muscle preparations showed no effect of ERK on Ca^{2+} sensitivity (32). Furthermore, in swine carotid artery, inhibition of MEK with PD-98059 had no effect on histamine and phorbol 12,13-dibutyrate (PDBu)-mediated contractions (15).

It is unknown whether ERK plays a role in the regulation of uterine artery contractility, and more importantly, whether pregnancy effects the potential role of ERK in the uterine artery. Pregnancy is associated with a growth of uterine vasculature and a dramatic increase in uterine artery blood flow. It has been shown recently that the pregnancy-induced increase in uterine artery endothelial vasodilator production is mediated in part by a marked alteration in signaling pathway including activation of the ERK pathway (7). In the present study, we tested the hypothesis that the ERK pathway

played an important role in the regulation of uterine artery contractility in an agonist-dependent manner. Furthermore, we tested the hypothesis that pregnancy selectively enhanced the role of ERK in α_1-adrenoceptor-induced contractions and its effect in suppressing protein kinase C (PKC)-mediated contraction in the uterine artery. Specifically, we examined the effect of PD-98059 (a selective MEK inhibitor) on phenylephrine-, 5-HT-, PDBu- and KCl-induced contractions in both pregnant and nonpregnant uterine arteries. ERK activity was measured using a phosphospecific ERK 1/2 antibody. We also examined the role of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ca^{2+} sensitivity in the ERK-mediated response, and tested the hypothesis that both the Ca^{2+} dependent and independent components were involved in the ERK pathway in the uterine artery.

Methods

Tissue preparation. Nonpregnant and 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% to 2.0% halothane in oxygen throughout surgery. An incision in the abdomen was made and the uterus exposed. The uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution

(pH 7.4) of the following composition (in mM): 115.21 NaCl, 4.7 KCl, 1.80 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22.14 $NaHCO_3$, and 7.88 dextrose. EDTA (0.03 mM) was added to suppress oxidation of amines. The Krebs solution was oxygenated with a mixture of oxygen-carbon dioxide (95:5%). After removal of the tissues, animals were killed with T-61 (euthanasia solution, Hoechst-Roussel, Somervile, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines put forward in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Contraction studies. The third (nonpregnant) and fourth (pregnant) branches of the main uterine arteries were separated from the surrounding tissue, and special care was taken to avoid touching the luminal surface. The arteries were cut into 2-mm ring segments. In some rings the endothelium was removed by gentle rotation of the arterial rings on an approximately sized, rough-surfaced blunt hypodermic needle as described previously (20). Contractile responses were quantified in Krebs' solution in tissue baths at 37 °C as described previously (20). Isometric tensions were measured. After 60 min

of equilibration in the tissue bath, each ring was stretched to the optimal resting tension (1 g) as determined by the tension developed in response to potassium chloride (120 mM) added at each stretch level. Concentration-response curves were obtained by cumulative addition of the agonist in approximate one-half log increments. EC_{50} values for the agonist in each experiment were taken as the molar concentration at which the contraction-response curve intersected 50% of the maximum response, and were expressed as pD_2 ($-\log EC_{50}$) values. All responses are normalized to the maximal high KCl (120 mM) contraction.

Immunoblotting. Arterial rings were equilibrated in the tissue bath and the optimal tension was obtained as described above. The tissues were then incubated for 30 min with PD-98059 (30 μM) or vehicle alone in the organ bath (37 °C). After incubation, they were stimulated with phenylephrine (3 μM). The reaction was stopped by snap-freezing the tissues in liquid nitrogen and stored at –80 °C until used. Frozen samples were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, pH 7.4. Sample homogenates were then centrifuged at 4 °C for 5 min at 6,000 g and the supernatants were collected. Protein was quantified in the supernatant using protein assay kit from Bio-Rad. Samples with equal protein were loaded on 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS) and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred on to immobilon P membrane at 30 V for 50 min at room temperature using a semidry blotter (Bio-Rad). Nonspecific binding sites in the membranes were blocked by an overnight incubation at 4 °C in a Tris-buffered saline solution (TBS) containing 5%

dry milk. The membranes were washed in TBS (3x 15 min) and then incubated with rabbit phospho-p44/42 MAP kinase (Tyr-202/tyr-204) antibody (at 1:1,000 dilution; New England Biolabs, Beverly, MA). Total p44/42 MAP kinase (ERK-1/2) was determined using anti-ERK-1/2 antibody (New England Biolabs, Beverly, MA). Membranes were washed using TBS and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000) obtained from Amersham (Arlington Heights, IL). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL). The blots were exposed to hyperfilm. Results were quantified by scanning densitometer (model 670, Bio-Rad). The data were normalized by actin and presented as the percentage of the control protein levels within each group.

Measurement of PKC activity. PKC activity was determined as previously described (8,37). Briefly, after treatment, tissues were homogenized in buffer A containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, PMSF 1 mM, β-mercaptoethanol 10 mM, and benzamide 1 mM. The homogenate was centrifuged at 100,000g for 60 minutes at 4°C, and the supernatant was used as the cytosolic fraction. The pellet corresponding to the membrane particulate fraction was solubilized in buffer A containing Triton X-100 at a final concentration of 0.1% by stirring on ice for 45 minutes at 4°C, followed by centrifugation at 100,000g for 60 minutes at 4°C to remove insoluble membrane particles. Cytosolic and solubilized membrane fractions were applied to DEAE-cellulose columns, which had been pre-equilibrated with buffer A including 0.1% Triton X-100. The DEAE columns were washed with 5 mL of buffer A and 5 mL of buffer B containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 1 mM, EGTA 1 mM, PMSF 1 mM, β-mercaptoethanol 10 mM, and

benzamide 1 mM, and 0.1% Triton X-100. PKC was eluted with 2 mL of buffer B including 400 mM NaCl. Protein concentrations were determined with a protein assay kit (Bio-Rad). PKC activity was determined in the cytosol and solubilized membrane particulate fractions using a PKC ELISA assay that utilizes a synthetic peptide and a monoclonal antibody that recognizes the phosphorylated form of the peptide (Upstate Biotech).

Simultaneous measurement of $[Ca^{2+}]_i$ and tension. Simultaneous recordings of contraction and free intracellular calcium concentration ($[Ca^{2+}]_i$) in the same tissue were conducted as described previously (36,43). Briefly, the arterial ring was attached to an isometric force transducer in a 5-ml tissue bath mounted on a CAF-110 intracellular Ca^{2+} analyzer (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-acetoxymethyl ester (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 alternatively (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 a photomultiplier tube. The fluorescence intensity at each excitation wavelength (F_{340} and F_{380}, respectively) and the ratio of these two fluorescence values ($R_{340/380}$) were recorded with a time constant of 250 ms and stored with the force signal on a computer.

Materials. Phenylephrine, PDBu, serotonin, PD-98059, staurosporine, and calphostin C were obtained from Sigma Chemical (St. Louis, MO). All electrophoretic and immunoblot reagents were from Bio-Rad Laboratories (Richmond, CA). Fura 2-AM was obtained from Molecular Probes (Eugene, OR). PKC activity ELISA assay kit was obtained from Upstate Biotech (Lake Placid, NY). All drugs were prepared fresh each day and kept on ice throughout the experiment.

Data analysis. Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad software, San Diego, CA). Results were expressed as means ± S.E.M. obtained from the number (n) of experimental animals given. Differences were evaluated for statistical significance ($P < 0.05$) by one-way ANOVA and paired Student t-test.

Results

Effect of PD-98059 on agonist-mediated contractions. Fig. 1 shows the effect of PD-98059 on phenylephrine-induced contraction of the uterine artery. PD-98059 showed no effect on basal tension in both nonpregnant and pregnant uterine arteries. As depicted in Fig. 1, PD-98059 did not effect phenylephrine-induced contractions in nonpregnant uterine arteries (pD_2: 5.76 ± 0.12 *vs.* 5.50 ± 0.14, $P > 0.05$), but significantly shifted the phenylephrine concentration-response curve to the right in pregnant uterine arteries (pD_2: 6.25 ± 0.02 *vs.* 5.44 ± 0.24, $P < 0.05$). The maximal response was not effected (Fig. 1). Removal of the endothelium did not change the inhibition of PD-98059 on phenylephrine-induced contractions (data not shown).

Unlike its lack of effect on phenylephrine in nonpregnant uterine arteries, PD-98059 significantly decreased 5-HT-induced contractions in nonpregnant uterine arteries (pD_2: 6.87 ± 0.19 *vs.* 6.32 ± 0.06, $P < 0.05$) (Fig. 2, lower panel). Pregnancy did not change the PD-98059-mediated inhibition of 5-HT-induced contractions of the uterine artery (pD_2: 6.23 ± 0.03 *vs.* 5.61 ± 0.23, $P < 0.05$) (Fig. 2, upper panel).

Fig. 3 depicts the effect of PD-98059 on protein kinase C (PKC)-mediated contractions induced by PDBu in the uterine artery. PDBu produced a dose-dependent contraction in both nonpregnant and pregnant uterine arteries. However, PDBu was 10-time more potent in contracting nonpregnant than pregnant uterine arteries (pD_2: 6.64 ± 0.07 *vs.* 5.62 ± 0.17, $P < 0.05$), and produced significantly higher maximum response in nonpregnant (73.9 ± 14.3%) than pregnant (31.6 ± 4.0%) uterine arteries ($P < 0.05$). PD-98059 did not effect PDBu-mediated contractions in nonpregnant uterine arteries (pD_2:

6.64 ± 0.07 *vs.* 6.78 ± 0.06; maximum response: 73.9 ± 14.3% *vs.* 89.3 ± 22%, $P > 0.05$). In contrast, PD-98059 significantly increased contractile sensitivity of PDBu (pD_2: 6.32 ± 0.20 *vs.* 5.62 ± 0.17, $P < 0.05$) and its maximum response (60.3 ± 8.8% *vs.* 31.6 ± 4.0%, $P < 0.05$) in pregnant uterine artery. Thus, PD-98059 minimized the difference in PDBu-mediated contractions between nonpregnant and pregnant uterine arteries (Fig. 3). PKC activity was measured in tissue fractions of pregnant uterine artery smooth muscle. As shown in Fig. 4, PDBu significantly increased particulate/cytosolic PKC activity ratio, which was blocked by both staurosporine and calphostin C. PD-98059 had no effect on the resting P/C PKC activity ratio, but significantly enhanced PDBu-stimulated PKC activity (Fig. 4).

The effect of PD-98059 on KCl-induced contractions of uterine arteries is shown in Fig. 5. In contrast to its effect on agonist-mediated contractions, PD-98059 had no effect on KCl-induced contractions in both nonpregnant and pregnant uterine arteries. From the data presented in Fig. 5, the pD_2 values of KCl were 1.45 ± 0.01 and 1.48 ± 0.06 for the control and PD-98059-treated pregnant uterine arteries, and 1.47 ± 0.01 and 1.50 ± 0.08 for the control and PD-98059-treated non-pregnant uterine arteries.

Effect of α_1-adrenergic agonist on the ERK activation. Fig. 6 shows total ERK-1/2 protein levels in nonpregnant and pregnant uterine arteries, and demonstrates that pregnancy is associated with an increase in ERK-2 protein levels in the uterine artery. To demonstrate that the effect of PD-98059 observed in the contraction was associated with inhibition of ERK activation, we measured the phenylephrine-induced phosphorylation of ERK using an ERK1/2 phospho-MAP kinase antibody. Fig. 7 depicts data from experiments in which pregnant uterine artery rings in tissue baths were exposed to

phenylephrine (3 μM) following PD-98059 pre-treatment (30 min) or the vehicle (DMSO) alone. The tissues were treated with phenylephrine for 5 min followed by immediately frozen in liquid nitrogen to stop the reaction. As shown in Fig. 7, phenylephrine significantly increased tyrosyl-phosphorylation of two proteins with molecular weights of 44 kDa (207% above control) and 42 kDa (236% above control). PD-98059 alone did not affect basal p44/p42 MAP kinase phosphorylation, but significantly inhibited phenylephrine-induced tyrosyl-phosphorylation of both ERK1 and ERK2 by 50% and 53%, respectively (Fig. 7).

Effect of PD-98059 on phenylephrine-induced changes in $[Ca^{2+}]_i$ and Ca^{2+} sensitivity. To examine the potential effect of ERK on agonist-mediated Ca^{2+} concentration and Ca^{2+} sensitivity, contractile tension and $[Ca^{2+}]_i$ were measured simultaneously in the same tissue as described in the *Methods*. In the arterial rings loaded with fura-2, an increase in $[Ca^{2+}]_i$ resulted in an increase in F_{340}, a decrease in F_{380}, and an increase in $R_{340/380}$. Typical traces of simultaneous measurement of phenylephrine-stimulated increase in $[Ca^{2+}]_i$ and muscle tension development in the pregnant uterine artery are shown in Fig. 8. Because preliminary studies demonstrated that 10 μM and 30 μM PD-98059 completely blocked phenylephrine-induced contractions in this preparation, subsequent studies were performed using 3 μM PD-98059. PD-98059 significantly decreased basal $[Ca^{2+}]_i$ as evidenced of reducing fura-2 fluorescence ratio ($R_{340/380}$) from 0.198 ± 0.012 to 0.146 ± 0.008 ($P < 0.05$) in pregnant, but not nonpregnant (0.099 ± 0.01 *vs.* 0.092 ± 0.009, $P > 0.05$) uterine arteries. Fig. 8 shows the real time effect of PD-98059 on phenylephrine-induced $[Ca^{2+}]_i$ response and contractile tension in intact pregnant uterine arteries. As shown in Fig. 8, 10 μM

phenylephrine caused an increase in $[Ca^{2+}]_i$ and contractile tension simultaneously. After washing and recovery, the same tissue was pretreated with 3 μM PD-98059 for 30 min, and then challenged again with 10 μM phenylephrine. Both the phenylephrine-induced $[Ca^{2+}]_i$ and contractile tension were significantly reduced in the presence of PD-98059 (Fig. 8). The responses to phenylephrine were completely recovered after the removal of PD-98059 (Fig. 8). Quantitative analysis of the data revealed that PD-98059 decreased phenylephrine-induced contractile tension and $[Ca^{2+}]_i$ by 71% and 53%, respectively ($P < 0.05$, paired *t*-test) (Fig. 9). In addition, the simultaneous measurement of $[Ca^{2+}]_i$ with tension in the same intact tissue allowed us to determine directly the precise relationship between fura-2 $R_{340/380}$ and tension in the uterine artery and thus to estimate Ca^{2+} sensitivity of myofilaments. As shown in Fig. 9, the contraction of pregnant uterine artery at a given amount of increase in $[Ca^{2+}]_i$ mediated by phenylephrine was significantly decreased by PD-98059. In contrast, PD-98059 had no effect on $[Ca^{2+}]_i$ and Ca2+ sensitivity induced by phenylephrine in nonpregnant uterine arteries (data not shown).

Discussion

The present study has demonstrated that the ERK pathway plays a key role in the regulation of uterine artery contractility. More importantly, the effect of ERK on the uterine artery is regulated by pregnancy. There are several important observations in the present study. First, among the several agonists tested, PD-98059 selectively inhibited 5-HT-induced contractions in nonpregnant uterine artery. Second, pregnancy selectively augmented the inhibition of PD-98059 on α_1-adrenoceptor-, but not 5-HT-, induced contractions in uterine arteries. Third, in agreement with the previous finding in rat thoracic aorta (22), the PDBu-induced contraction was significantly attenuated in pregnant uterine artery. PD-98059 did not effect the PDBu-mediated contraction in nonpregnant uterine arteries, but significantly increased it in pregnant uterine arteries. In accordance, PD-98059 significantly enhanced PDBu-stimulated PKC activity in pregnant uterine artery. Fourth, PD-98059 had no effect on KCl-mediated contractions in both nonpregnant and pregnant uterine arteries. Fifth, activation of α_1-adrenoceptors increased tyrosyl-phosphorylation of ERK1/2, which was blocked by PD-98059. Sixth, PD-98059-mediated inhibition of the phenylephrine-induced contraction was associated with a decrease in both intracellular Ca^{2+} concentration and Ca^{2+} sensitivity of contractile proteins in the uterine artery.

The agonist-stimulated activation of ERK has been well documented in cultured smooth muscle cells (16,21,28,29), and intact smooth muscle (3,10,23). The lack of effect of PD-98059 on KCl-induced contractions in both nonpregnant and pregnant uterine arteries suggests that the function of Ca^{2+} channels may not be regulated by the

ERK pathway in uterine arteries. This is in agreement with several previous findings (10,12,30,39). On the other hand, it was also reported that tyrosine kinase inhibitors inhibited L-type voltage gated Ca^{2+} channels (4,23,40). Similarly, several previous studies have explored the role of ERK in the regulation of agonist-mediated arterial contractions in different animal models and arterial types, but the results are controversial (10,13,15,32). The present finding that PD-98059 inhibited 5-HT-induced contractions of the uterine artery is in agreement with the previous study in which 5-HT-mediated contractions were inhibited by PD-98059 in rat aorta, mesenteric artery, and tail artery (39). We have previously demonstrated that 5-HT-elicited contractions of the uterine artery are mediated by the increase of inositol 1,4,5-trisphosphate, leading to release of Ca^{2+} from intracellular stores (42,43). The present results suggest the involvement of the ERK pathway in 5-HT-induced contractions of the uterine artery. It has been demonstrated that 5-HT stimulates the activation of ERK 1/2 in arterial smooth muscle (12,39). The finding that the inhibitions of PD-98059 on 5-HT-induced contractions were not different in pregnant and nonpregnant uterine arteries suggest that the effect of ERK on the 5-HT-mediated contraction is not regulated by pregnancy.

Unlike its effect on the 5-HT-mediated contraction, PD-98059 showed no effect on the phenylephrine-induced contractions in nonpregnant uterine artery. This is contrary to the previous finding in ferret aorta in which PD-98059 inhibited phenylephrine-induced contractions (10). However, PD-98059 did not effect phenylephrine-induced contractions in rat mesenteric resistance arteries (30). These results suggest that the role of ERK in the α_1-adrenoceptor-mediated contraction show a considerable heterogeneity in vessel types. Nevertheless, PD-98059 did inhibit phenylephrine-mediated contractions

in pregnant uterine artery. In consistent with the contraction results, Western analysis indicated that phenylephrine increased protein tyrosyl-phosphorylation of both p42/p44 MAP kinase in pregnant uterine artery, which was blocked by PD-98059. These results suggest that pregnancy up-regulates the coupling of the ERK pathway to α_1-adrenoceptor-mediated contractions in the uterine artery. Given that both 5-HT and α_1-adrenoceptor-mediated contractions share a common downstream signal, *i.e.* inositol 1,4,5-trisphosphate, in the uterine artery (19,42,43,44), it is intriguing that PD-98059 differentially regulated 5-HT and phenylephrine-induced contractions in uterine arteries. This would suggest a specific coupling of the ERK pathway to individual receptor signaling pathways. Although the cellular mechanisms for this selectivity are not clear at present, it is postulated that scaffolding proteins may play an important role.

It is generally believed that during pregnancy the uterine vasculature acts as a low-resistance shunt to accommodate the large increase in uteroplacental blood flow required for normal fetal development. The mechanisms for the attenuated vascular tone may involve a decreased role of endogenous vasoconstrictors and/or an increased role of both endogenous vasodilator and placental angiogenic factors (31,35,41). Given that α_1-adrenoceptors play a key role in moment-to-moment regulation of uterine vascular tone, the finding that pregnancy selectively up-regulated the role of ERK in α_1-adrenoceptor (but not 5-HT)-mediated contractions in the uterine artery warrants a physiological significance of the ERK pathway in the regulation of uterine artery contractility during pregnancy. Nonetheless, it is not clear at present whether and to what extent the enhanced ERK pathway in α_1-adrenoceptor-mediated signaling affects the vascular tone of pregnant uterine artery. It has been shown recently that the pregnancy-induced

increase in uterine artery endothelial vasodilator production is mediated in part by a marked alteration in signaling pathway including activation of the ERK pathway (7). The present finding that the inhibition of PD-98059 on the phenylephrine-induced contraction was not changed with and without the endothelium in both pregnant and non-pregnant uterine arteries suggests a less important role of ERK on the uterine artery endothelium.

The finding that the PDBu-induced contraction was significantly attenuated in pregnant uterine artery is in agreement with previous results in rat thoracic aorta (22), suggesting that the role of PKC in the regulation of uterine vascular tone is down-regulated during pregnancy. It has been well documented that PKC plays an important role in the regulation of the sustained phase of contraction in vascular smooth muscle (18). In consistent with the previous findings (1,15,30,39), PD-98059 had no effect on the PDBu-induced contraction in nonpregnant uterine arteries. To our surprise, PD-98059 significantly increased PDBu-induced contractions in pregnant uterine arteries. To our knowledge, it has not been reported previously that PD-98059 increases contractions to any agonists examined. It has been proposed in several studies that PKC is an upstream signal of the activation of the ERK pathway in the vascular smooth muscle (24,25,26,27,29,30). In contrast, the present result that the PBDu-induced contraction was increased after the ERK inhibition by PD-98059 would suggest a role for ERK in the regulation of PKC as a downstream signal in the uterine artery of pregnant animals. This is supported by the finding that PD-98059 significantly enhanced PDBu-stimulated PKC activity in pregnant uterine arteries. The finding that PD-98059 increased PDBu-induced contractions of pregnant uterine arteries and eliminated its difference between nonpregnant and pregnant uterine arteries is likely to have physiological significance, and

suggests that ERK may play a very important role in increased uterine blood flow by suppressing the PKC-mediated contraction during pregnancy. Consistent with this notion, the present study demonstrated a significant increase in ERK-2 protein levels in pregnant, as compared with nonpregnant, uterine arteries. This is in agreement with our recent findings in uterine artery endothelial cells in which pregnancy is associated with an enhancement in ERK-2 signaling pathway (7,11).

Whereas the mechanisms underlying ERK-mediated inhibition of PKC-induced contractions are not clear at present, our study demonstrated that PD-98059 inhibited the phenylephrine-mediated contraction by decreasing both phenylephrine-induced intracellular Ca^{2+} concentration and Ca^{2+} sensitivity. To our knowledge, our results are the first to show a direct relation between PD-98059-mediated inhibitions of agonist-induced contraction and Ca^{2+} concentration in intact muscle. Previous studies have suggested that PD-98059 inhibits agonist-induced contraction by decreasing Ca^{2+} sensitivity of contractile proteins through the inhibition of ERK-mediated phosphorylation of caldesmon (2,10,12,17,18). Studies in isolated smooth muscle cells indicated that PD-98059 did not effect agonist-induced intracellular Ca^{2+} concentration (33,38). However, the effect of PD-98059 on Ca^{2+} concentration in intact smooth muscle was not examined. We have developed a method to measure contractile tension and intracellular Ca^{2+} concentration simultaneously in the same intact arterial ring (43). This allowed us to directly determine the precise relationship between fura-2 $R_{340/380}$ and tension in the uterine artery, and thus to estimate not only Ca^{2+} concentration but also Ca^{2+} sensitivity of myofilaments with unimpaired excitation-contraction coupling processes and retained regulatory targets for second messenger pathways. The present

study clearly demonstrated that the PD-98059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in both Ca^{2+} concentration and Ca^{2+} sensitivity in the uterine artery. Our findings suggest, therefore, that, in addition to the Ca^{2+}-independent pathway as proposed previously, the ERK signaling pathway also involves the Ca^{2+}-dependent component of vascular contractions. It is noteworthy that PD-98059 decreased not only the agonist-induced Ca^{2+} concentration but also the basal Ca^{2+} concentration. This raises the possibility that ERK may play a role in the regulation of basal tone of the uterine artery.

In summary, the results indicate that ERK plays an important role in the regulation of uterine artery contractility, and its effect is agonist-dependent. More importantly, pregnancy selectively enhances the role of ERK in α_1-adrenoceptor-mediated contractions and its effect in suppressing the protein kinase C-mediated contraction in the uterine artery. In addition, both the Ca^{2+} dependent and independent components are involved in the ERK pathway in the uterine artery. The physiological role of the ERK pathway and it mechanisms in the adaptation of uterine vascular reactivity to pregnancy are important avenues for future studies.

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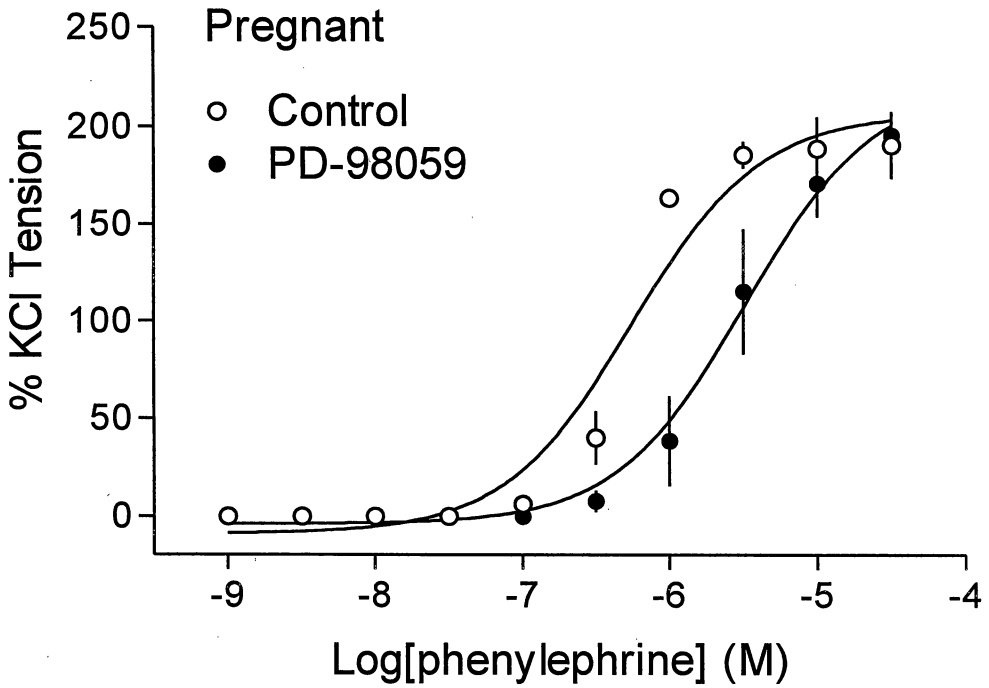
Figure 1. Effect of PD-98059 on phenylephrine-induced contraction in ovine uterine arteries. Arterial rings were pretreated with 30 μM PD-98059 or with the vehicle DMSO (Control) for 30 min and then subjected to the cumulative addition of phenylephrine in Krebs' solution. Data are expressed as percentages of contraction produced by 120 mM KCl, and each point represents mean ± SEM of four animals. The pD_2 (-log EC_{50}) values were presented in the text. Upper panel: pregnant uterine artery. Lower panel: nonpregnant uterine artery.


Pregnant
Control
PD-98059
% KCl Tension
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
Log[phenylephrine] (M)

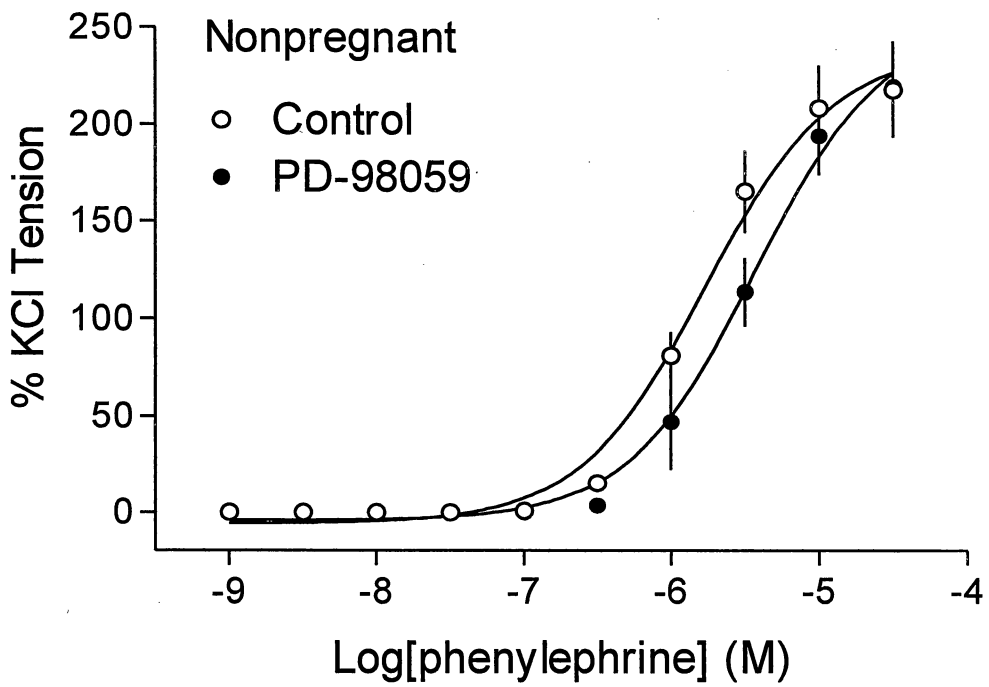
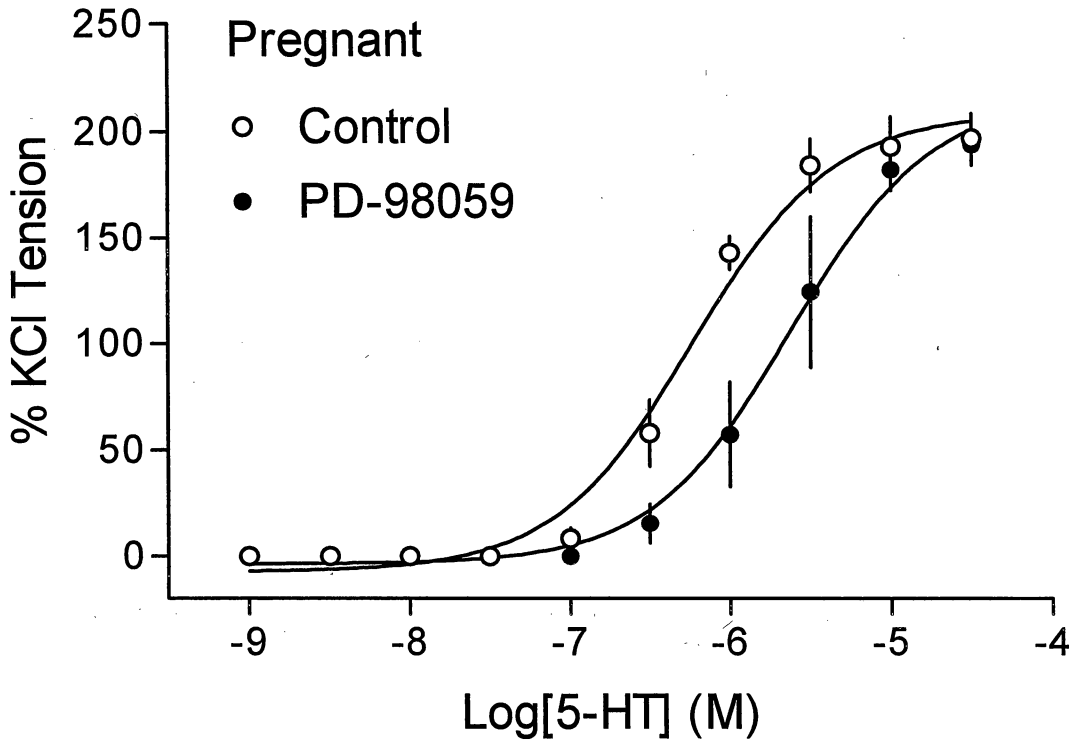

Nonpregnant
Control
PD-98059
% KCl Tension
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
Log[phenylephrine] (M)

Figure 2. Effect of PD-98059 on 5-HT-induced contraction in ovine uterine arteries. Arterial rings were pretreated with 30 μM PD-98059 or with the vehicle DMSO (Control) for 30 min and then subjected to the cumulative addition of 5-HT in Krebs' solution. Data are expressed as percentages of contraction produced by 120 mM KCl, and each point represents mean ± SEM of four animals. The pD_2 (-log EC_{50}) values were presented in the text. Upper panel: pregnant uterine artery. Lower panel: nonpregnant uterine artery.


Pregnant
Control
PD-98059
% KCl Tension
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
Log[5-HT] (M)

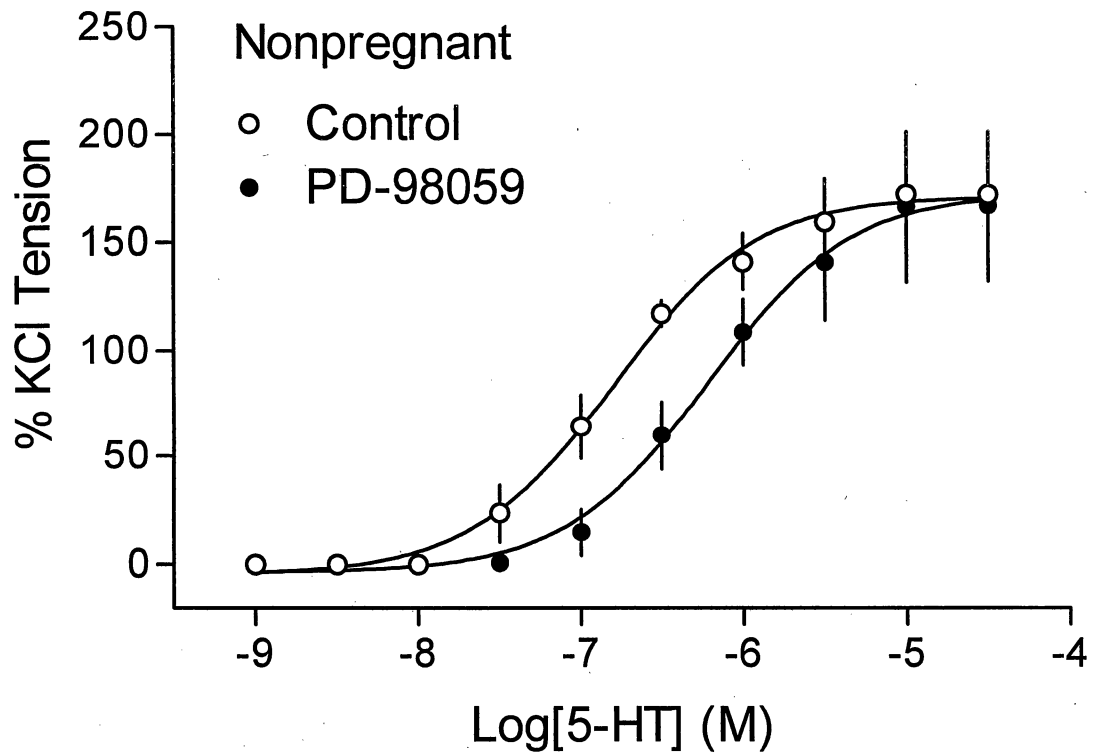
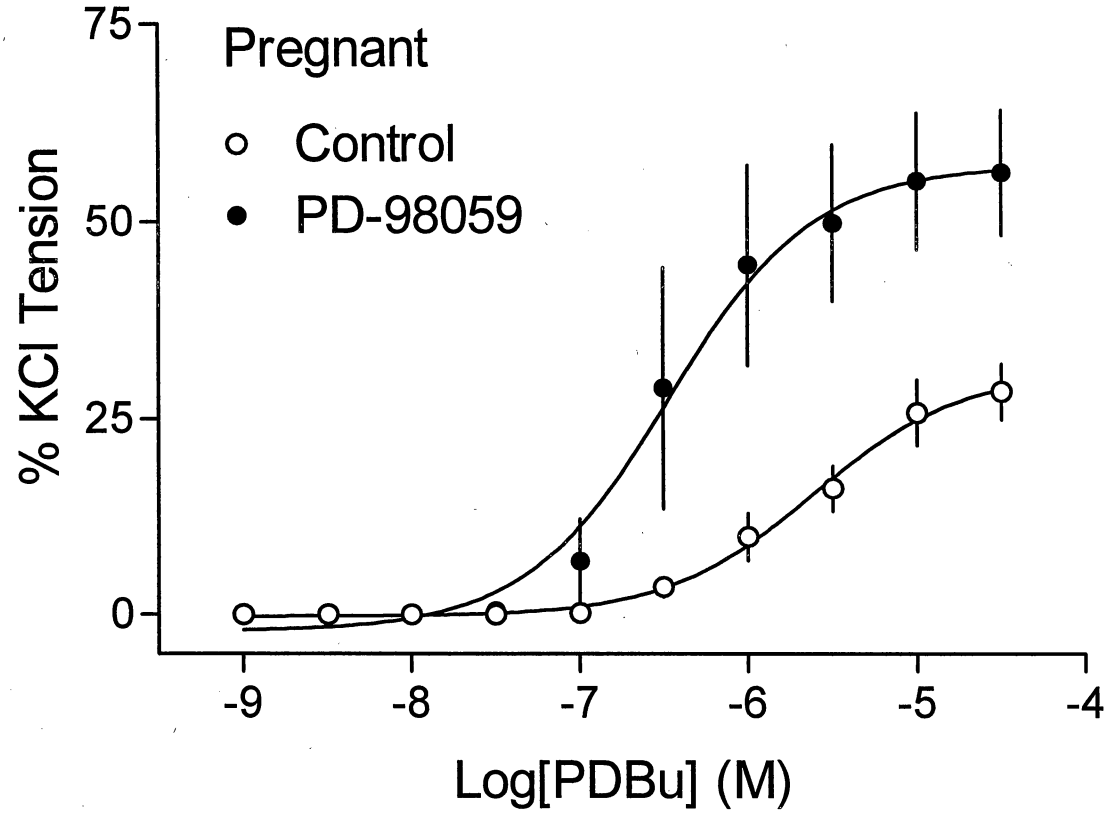

Nonpregnant
Control
PD-98059
% KCl Tension
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
Log[5-HT] (M)

Figure 3. Effect of PD-98059 on PDBu-induced contraction in ovine uterine arteries. Arterial rings were pretreated with 30 μM PD-98059 or with the vehicle DMSO (Control) for 30 min and then subjected to the cumulative addition of PDBu in Krebs' solution. Data are expressed as percentages of contraction produced by 120 mM KCl, and each point represents mean ± SEM of four animals. The pD_2 (-log EC_{50}) values were presented in the text. Upper panel: pregnant uterine artery. Lower panel: nonpregnant uterine artery.


Pregnant
Control
PD-98059
% KCl Tension
75
50
25
0
-9
-8
-7
-6
-5
-4
Log[PDBu] (M)

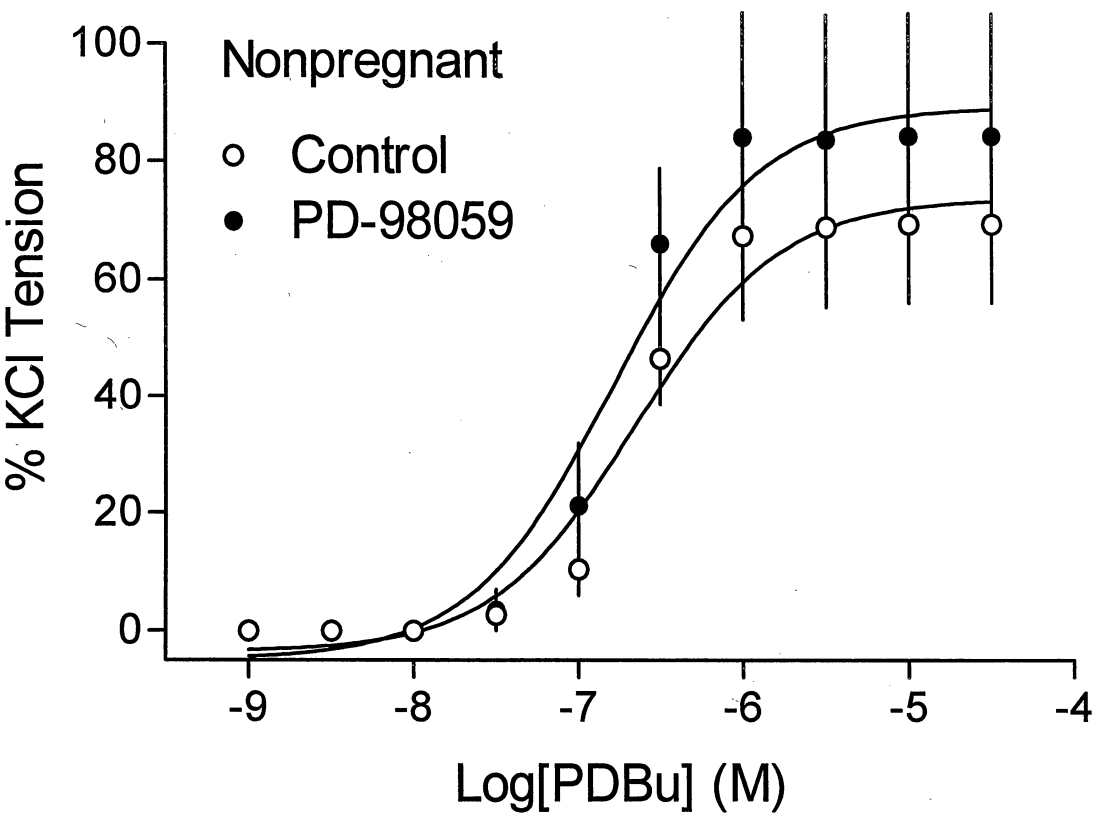

Nonpregnant
Control
PD-98059
% KCl Tension
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[PDBu] (M)

Figure 4. Effect of PD-98059 on PDBu-stimulated PKC activity in ovine uterine artery. Pregnant uterine arteries were pretreated with 30 μM PD-98059 (PD), 0.1 μM staurosporine (Stau), and 0.1 μM calphostin C (Calp) for 30 min before the addition of PDBu (3 μM, 30 min). PKC activity in the cytosolic and particulate fractions was determined as described in the Methods. Data are means ± SEM of four animals. **a**, $P < 0.05$, *vs.* rest; **b**, $P < 0.05$, *vs.* PDBu alone.

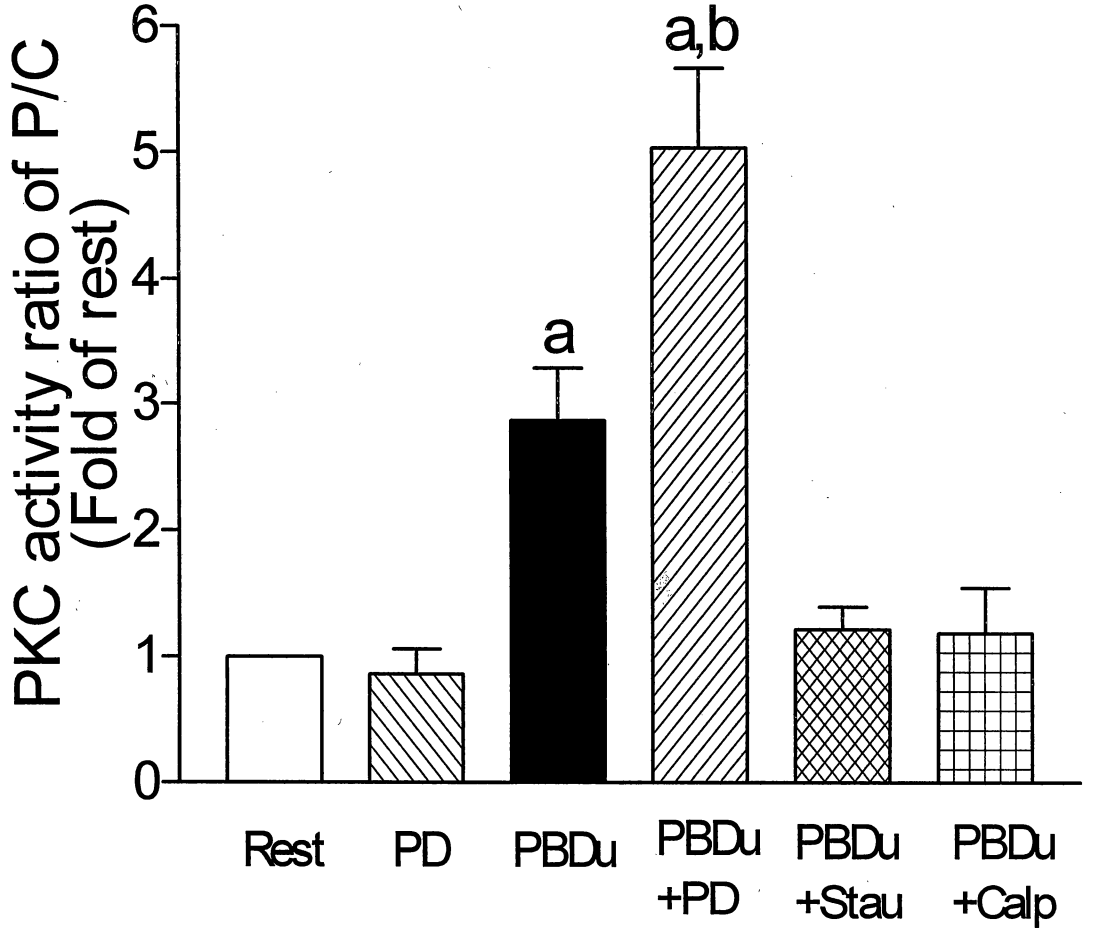
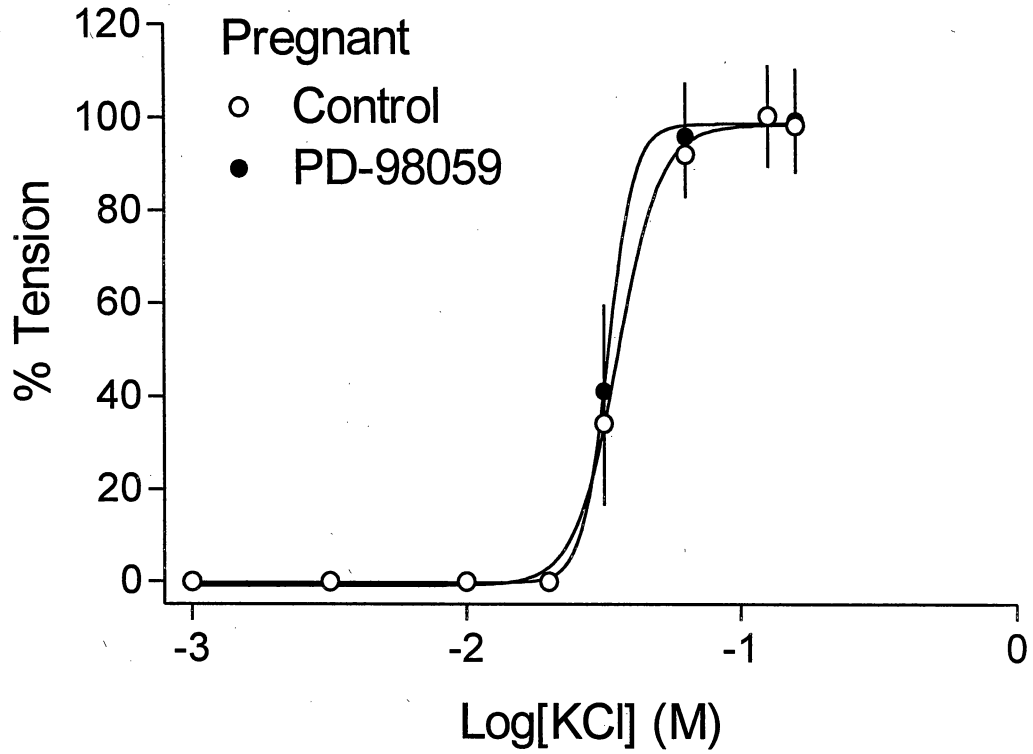

PKC activity ratio of P/C
(Fold of rest)
6
5
4
3
2
1
0
a,b
a
Rest
PD
PBDu
PBDu
+PD
PBDu
+Stau
PBDu
+Calp

Figure 5. Effect of PD-98059 on KCl-induced contraction in ovine uterine arteries. Arterial rings were pretreated with 30 μM PD-98059 or with the vehicle DMSO (Control) for 30 min and then subjected to the cumulative addition of KCl in Krebs' solution. Data are mean ± SEM of four to six animals. The pD_2 (-log EC_{50}) values were presented in the text. Upper panel: pregnant uterine artery. Lower panel: nonpregnant uterine artery.

A


Pregnant
Control
PD-98059
% Tension
120
100
80
60
40
20
0
-3
-2
-1
0
Log[KCl] (M)

B

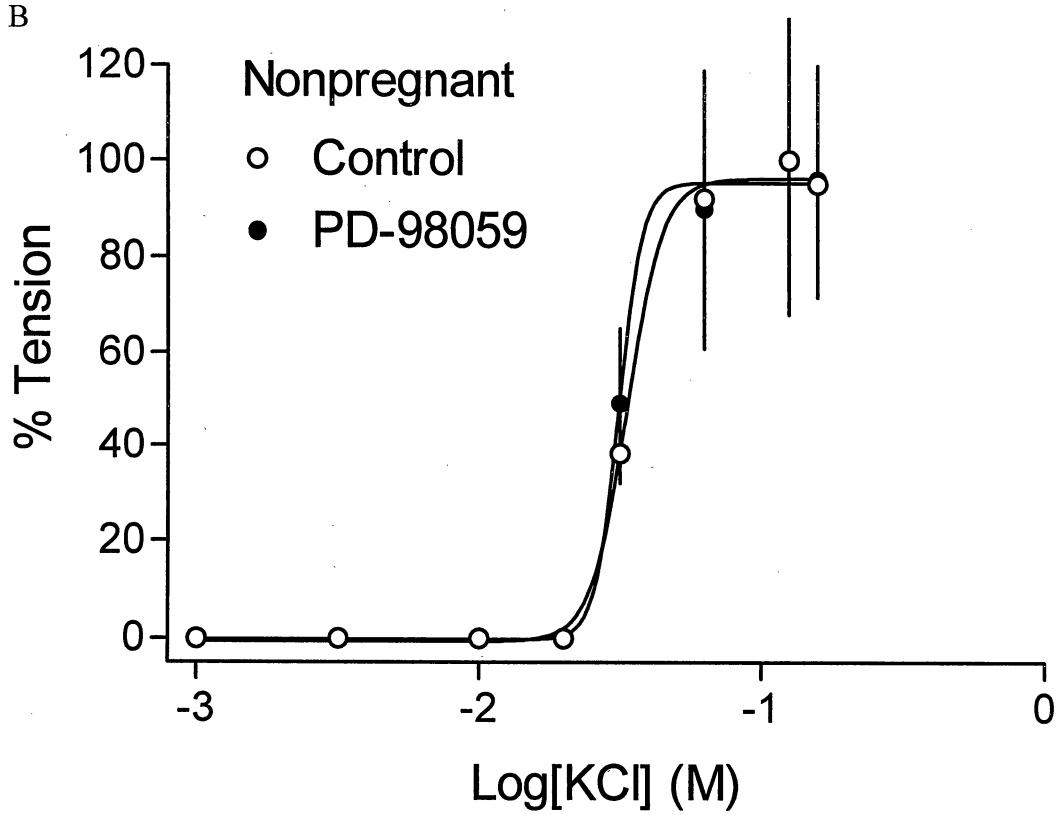
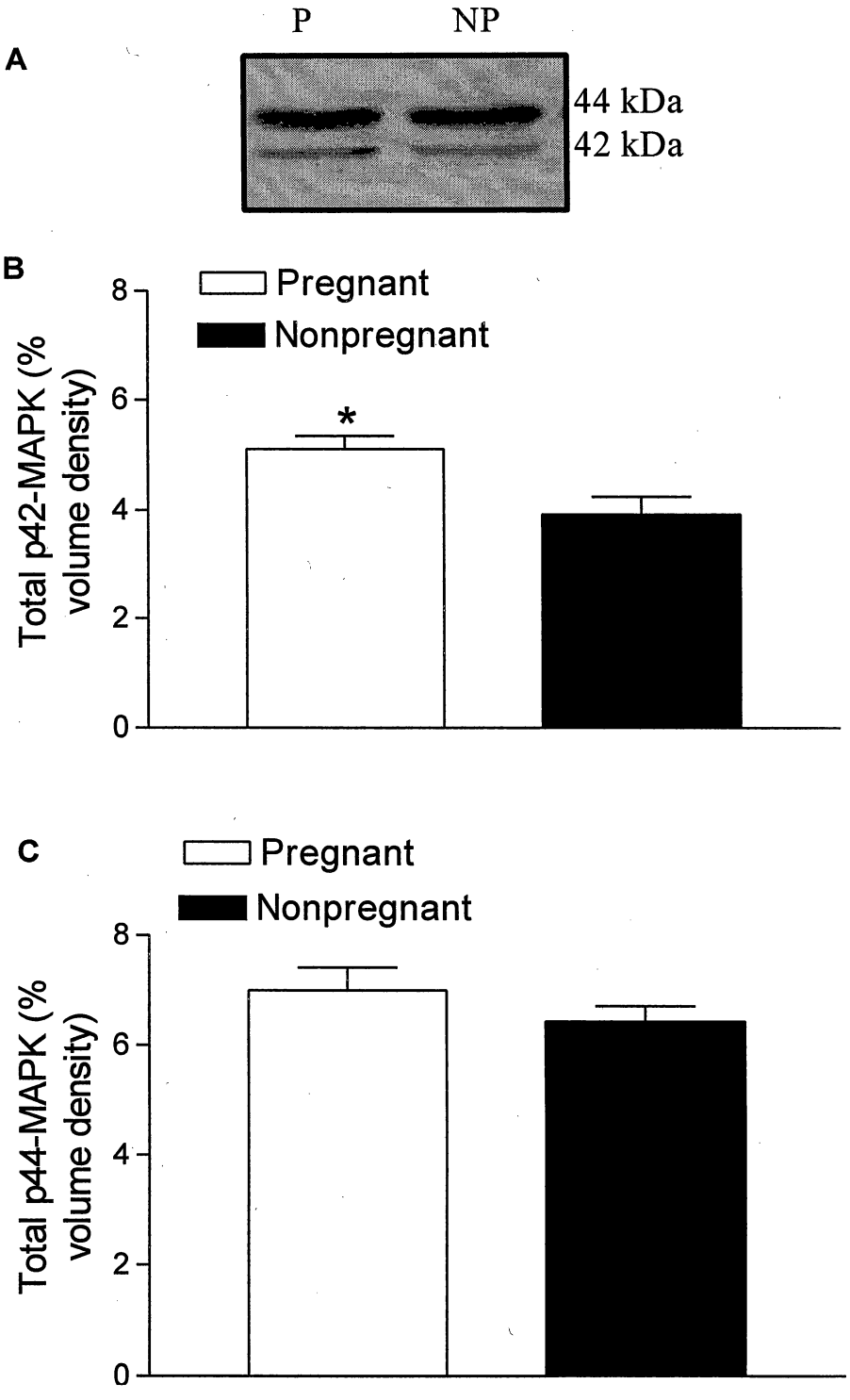

Nonpregnant
Control
PD-98059
% Tension
120
100
80
60
40
20
0
-3
-2
-1
0
Log[KCl] (M)

Figure 6. Effect of pregnancy on total ERK-1/2 protein levels in uterine arteries. Upper panel shows scanned images of representative anti-p44/42-MAP kinase immunoblot from pregnant (P) and nonpregnant (NP) uterine arteries. The two bands detected correspond to the 44-kDa isoform (ERK1) and 42-kDa isoform (ERK2). Middle panel shows ERK2 (42-kDa) determined by densitometry. Lower panel shows ERK1 (44-kDa) determined by densitometry. Data are means ± SEM of ten animals. *, $P < 0.05$, *vs.* nonpregnant.

Figure 7. Effect of phenylephrine on ERK activation in uterine arteries. Pregnant uterine arteries were pretreated with 30 μM PD-98059 (PD) or with the vehicle DMSO (control, C) for 30 min before the addition of phenylephrine (PE). When the contraction reached its steady state (PE alone or PE +PD-98059 group) (5 min) the rings were snap frozen in liquid N_2. Upper panel shows scanned images of representative anti-phospho-MAP kinase immunoblot from uterine artery treated as above. The two bands detected correspond to the 44-kDa isoform (ERK1) and 42-kDa isoform (ERK2). Middle panel shows phosphorylation of ERK2 (42-kDa) determined by densitometry, and expressed relative to α-actin density. Lower panel shows phosphorylation of ERK1 (44-kDa) determined by densitometry, and expressed relative to α-actin density. Data are means ± SEM of four animals. **a**, $P < 0.05$, control *vs.* PE; **b**, $P < 0.05$, PE *vs.* PE+PD-98059.


A
P
NP
44 kDa
42 kDa
B
Pregnant
Nonpregnant
Total p42-MAPK (% volume density)
8
6
4
2
0
*
C
Pregnant
Nonpregnant
Total p44-MAPK (% volume density)
8
6
4
2
0

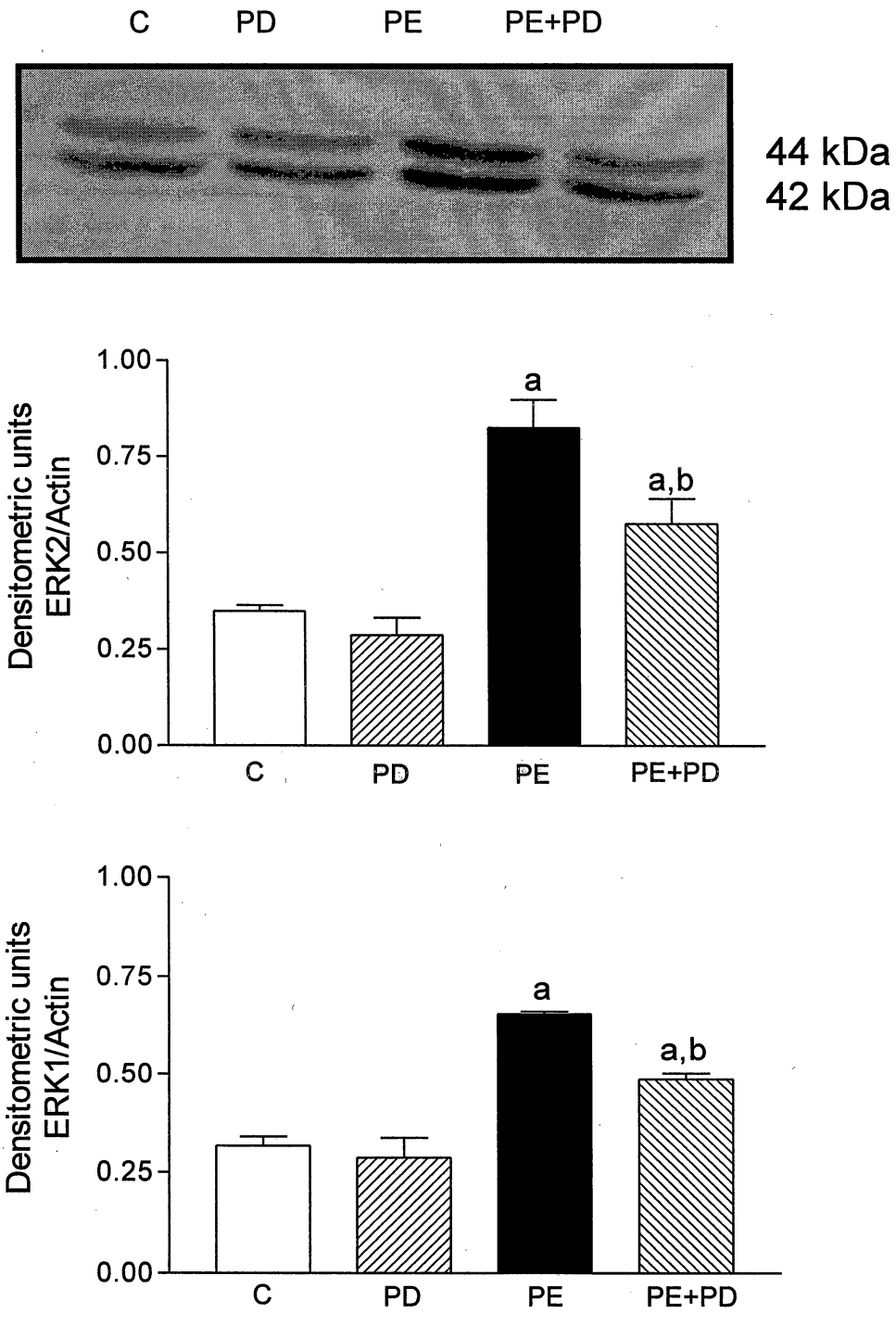

C
PD
PE
PE+PD
44 kDa
42 kDa
Densitometric units
ERK2/Actin
1.00
0.75
0.50
0.25
0.00
a
a,b
C
PD
PE
PE+PD
Densitometric units
ERK1/Actin
1.00
0.75
0.50
0.25
0.00
a
a,b
C
PD
PE
PE+PD

Figure 8. Effect of PD-98059 on phenylephrine-induced contraction and intracellular Ca^{2+} in the uterine artery. Representative traces show the effect of PD-98059 on phenylephrine (PE)-induced $[Ca^{2+}]_i$ (fura-2 signal $R_{f340/f380}$, upper panel) and contraction (lower panel) recorded simultaneously in the same tissue of pregnant uterine artery loaded with fura-2. The tissue was stimulated first with 10 μM phenylephrine. After washout, it was treated with 3 μM PD-98059 for 30 min, followed by the same concentration of phenylephrine. After washout, the same tissue was stimulated with phenylephrine again.

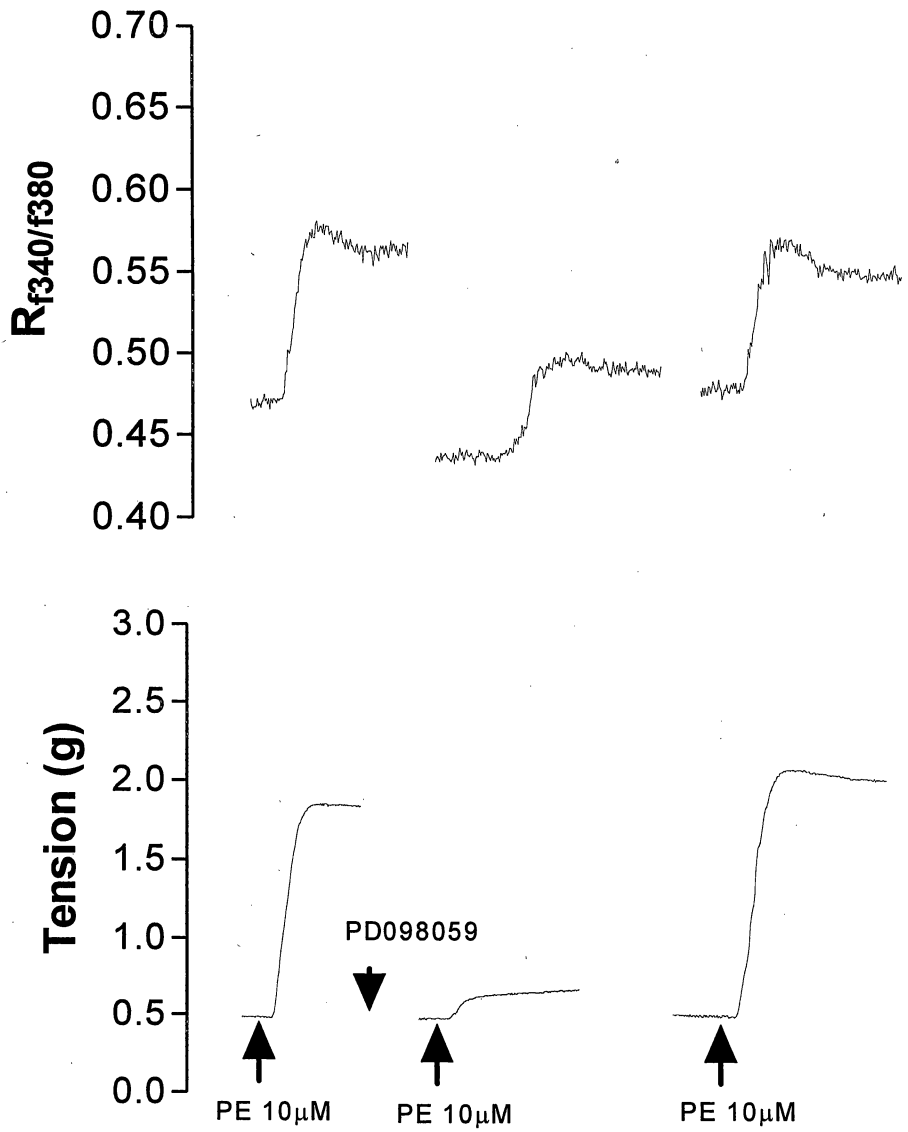
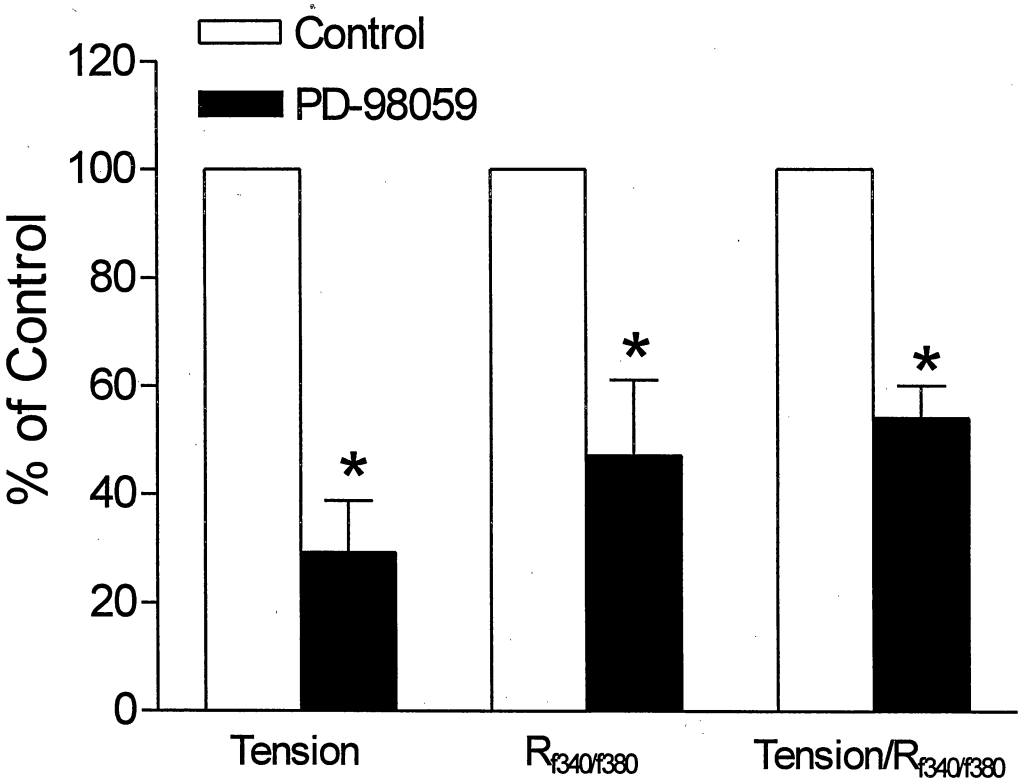

0.70
0.65
0.60
0.55
0.50
0.45
0.40
$R_{f340/f380}$
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tension (g)
PD098059
PE 10μM
PE 10μM
PE 10μM

Figure 9. Effect of PD-98059 on phenylephrine-induced intracellular Ca^{2+} concentration and Ca^{2+} sensitivity in the uterine artery. Phenylephrine (10 μM)-induced $[Ca^{2+}]_i$ (fura-2 signal $R_{f340/f380}$) and contraction were recorded simultaneously in the absence (control) or presence of PD-98059 (3 μM for 30 min) in the same tissue of pregnant uterine artery loaded with fura-2 as shown in Fig. 7. Data are means ± SEM of four animals. * $P < 0.05$.


Control
PD-98059
120
100
80
60
40
20
0
% of Control
*
*
*
Tension
$R_{f340/f380}$
Tension/$R_{f340/f380}$

CHAPTER THREE

ERK–Mediated Uterine Artery Contraction: Role of Thick and Thin Filament Regulatory Pathways

DaLiao Xiao, William J. Pearce, Lawrence D. Longo, and Lubo Zhang

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Abstract

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 both 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 PD098059. In addition, PD098059 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. Unlike phenylephrine, phorbol 12,13-bibutyrate (PDBu) produced contractions without changing $[Ca^{2+}]_i$ or LC_{20} phosphorylation. PD098059 potentiated PDBu-induced contractions without affecting $[Ca^{2+}]_i$ and LC_{20} phosphorylation. PDBu produced time-dependent increases in phosphorylation of $ERK_{42/44}$, and ERK-dependent phosphorylation of caldesmon at Ser^{789} in the uterine artery. PD098059 blocked PDBu-mediated phosphorylation of $ERK_{42/44}$ 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.

Introduction

Smooth muscle contraction is regulated by phosphorylation and dephosphorylation of the 20-kD 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, PD098059 did not affect agonist-induced intracellular Ca^{2+} concentration 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 (2, 21, 33). However, by measuring intracellular Ca^{2+} concentration and tension simultaneously in the intact tissue, our recent studies clearly

demonstrated that PD098059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in both intracellular Ca^{2+} concentration 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 suggested that ERK might regulate force in arterial smooth muscle by inhibiting LC_{20} phosphorylation (8).

Recently, we have demonstrated that ERK plays an important role in the regulation of uterine artery contraction, and 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 both thick and thin filament regulatory pathways. We examined the effects of the MEK (ERK) inhibitor PD098059 on both 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 caldesmon. 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% to 2.0% halothane in oxygen throughout the surgery. An incision in the abdomen was made and the uterus exposed. The uterine 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 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22.14 $NaHCO_3$, 0.03 EDTA, and 7.88 dextrose. The Krebs solution was oxygenated with a mixture of 95% oxygen-5% carbon dioxide. After the tissues were removed, 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 guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Contraction studies. The fourth branches of 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 potassium chloride (120 mM) added at each stretch level. Tissues were pretreated with either PD098059 (30 μM) or

vehicle (DMSO) for 30 min, followed by stimulation with phenylephrine or PDBu. Tension was recorded with an on-line computer. To measure LC_{20} phosphorylation simultaneously in the same tissues, arterial rings were snap-frozen with liquid N_2-cooled clamps at the indicated times, and immersed in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM DTT mixture. Rings were stored at -80°C until used.

Measurement of LC_{20} 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% bromophenol blue, as previously described (8, 28). Samples (20 μl) were electrophoresed at 120 mA for 2.5 h after a 30 min pre-run in 1.0 mm mini-polyacrylamide gels containing 10% arcelamide-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 LC_{20} antibody (1:500, Sigma). Goat anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Calbiochem). Bands were detected with enhanced chemiluminescence (ECL), visualized on films, and analyzed with KODAK Electrophoresis Documentation and Analysis System and KODAK 1D Image Analysis Software. Moles phosphate per mole light chain was 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 $[Ca^{2+}]_i$ and tension. Simultaneous recordings of contraction and $[Ca^{2+}]_i$ in the same tissue were conducted as described previously (56).

Briefly, each arterial ring was attached to an isometric force transducer in a 5-ml tissue bath mounted on a CAF-110 intracellular Ca^{2+} analyzer (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 alternatively (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 photomultiplier tube. The fluorescence intensity at each excitation wavelength (F_{340} and F_{380}, respectively) and the ratio of these two fluorescence values ($R_{340/380}$) 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 PD098059 (30 μM) or vehicle alone in the organ bath (37 °C). After incubation, they were stimulated with PDBu (5 μM). The reaction was stopped at various times by snap-freezing the tissues in liquid nitrogen and stored at –80 °C until used. Protein levels of phosphorylated ERK_{42}, ERK_{44}, and caldesmon at Ser^{789} were determined by Western blot analysis as previously described (55). Briefly, samples with equal protein were loaded on 10% ($ERK_{42/44}$) and 7.5% (caldesmon) SDS-PAGE, separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were incubated with the antibodies against phospho-$ERK_{42/44}$ (Tyr^{202}/tyr^{204})

and phospho-caldesmon (Ser^{789}), followed by 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 KODAK Electrophoresis Documentation and Analysis System and KODAK 1D Image Analysis Software. The data were normalized by actin.

Materials. Phenylephrine, PDBu, PD098059, and monoclonal anti-myosin (light chains 20K) were obtained from Sigma (St. Louis, MO). Phospho-$ERK_{42/44}$ (Tyr^{202}/tyr^{204}) antibodies were from Cell Signaling Technology (Beverly, MA). Phospho-caldesmon (Ser^{789}) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All electrophoretic and immunoblot reagents were from Bio-Rad. Fura 2-AM was obtained from Molecular Probes (Eugene, OR). Other reagents were of analytical 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). Results were expressed as means ± SE obtained from the number (n) of experimental animals given. Differences were evaluated for statistical significance ($P < 0.05$) by one-way ANOVA and Student's *t*-test.

Results

Effect of PD098059 on phenylephrine-induced increases in $[Ca^{2+}]_i$, LC_{20} phosphorylation and tension. Phenylephrine produced time-dependent increases in $[Ca^{2+}]_i$, LC_{20} phosphorylation, and tension development in the uterine artery (Fig. 1). The tension development was preceded by both $[Ca^{2+}]_i$ and LC_{20} phosphorylation. Although the agonist-induced tension was sustained up to 5 min, both LC_{20} phosphorylation and $[Ca^{2+}]_i$ progressively declined to a steady state of 50% and 65%, respectively, of their peak values. As also illustrated in Fig. 1, PD098059 significantly inhibited phenylephrine-induced increase in $[Ca^{2+}]_i$ in the uterine artery, and decreased the peak level to 56% that of control. Likewise, PD098059 significantly decreased the rate of phenylephrine-mediated LC_{20} phosphorylation and the maximum phosphorylation in the uterine artery. The peak phosphorylation level was significantly decreased to 35.6 ± 8.7% that of control, and the time to the peak was delayed by 20 s, as compared to the control. Although PD098059 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, as compared to the control.

Effect of PD098059 on LC_{20} phosphorylation/$[Ca^{2+}]_i$ relation. Contractions of smooth muscle can be regulated by altering the dependence of LC_{20} phosphorylation on $[Ca^{2+}]_i$, or the dependence of force on LC_{20} phosphorylation. To determine the potential role of ERK in the regulation of Ca^{2+} sensitivity of LC_{20} phosphorylation at fixed $[Ca^{2+}]_i$, we examined the effect of PD098059 on the relation of phenylephrine-stimulated increases in $[Ca^{2+}]_i$ and LC_{20} phosphorylation in the uterine artery. As shown in Fig. 2,

PD098059 did not affect the slope ($LCp/R_{f340/380}$) (from control: 13.5 ± 2.8, n = 9 to the treatment group: 11.6 ± 1.2, n = 5; $P > 0.05$), but significantly decreased the intercept from 0.164 ± 0.054 to 0.0097 ± 0.0019 ($P < 0.05$). This downward shift in the curve has the effect of showing a decreased LC_{20} phosphorylation at any given intracellular Ca^{2+} level.

Effect of PD098059 on force/LC_{20} phosphorylation relation. Although the majority of stimuli result in a unique relation between LC_{20} phosphorylation and force in smooth muscle, collateral regulation *via* thin filaments in modulating contraction can either increase or decrease the force/LC_{20} phosphorylation ratio. We further examined the role of ERK in the regulation of contractions independent of changes in LC_{20} phosphorylation, by evaluating the effect of PD098059 on the relation between phenylephrine-stimulated increases in LC_{20} phosphorylation and force measured simultaneously in the same tissue. Phenylephrine-induced tensions were plotted against their corresponding phosphorylated LC_{20} levels of the initial rising phases in control and PD098059-treated tissues, respectively (Fig. 3A). As shown in Fig. 3A, PD098059 caused a leftward shift in the force/LC_{20} phosphorylation relation, suggesting that tension developed at a given level of LC_{20} phosphorylation in PD098059-treated tissues was increased, as compared with that in control tissues. Because the relation between phenylephrine-stimulated LC_{20} phosphorylation and force development was nonlinear in both control and PD098059-treated tissues, analysis was performed to determine the levels of LC_{20} phosphorylation of control and PD098059-treated tissues at corresponding tensions developed (from 0.4 to 2.8 g). As shown in Fig. 3B, there was a linear relation between the levels of LC_{20} phosphorylation of control and PD098059-treated tissues at

corresponding tensions, with the slope (0.456 ± 0.008) significantly lower than that of the line of identity, confirming that the amount of force produced per phosphorylated LC_{20} was enhanced by PD098059.

Effect of PD098059 on PDBu-stimulated $[Ca^{2+}]_i$, LC_{20} phosphorylation and tension. Compared to 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 either $[Ca^{2+}]_i$ (data not shown) or LC_{20} phosphorylation in the uterine artery (Fig. 4). Simultaneous measurement of $[Ca^{2+}]_i$ and contractions showed that PD098059 increased PDBu-induced contractions without changing $[Ca^{2+}]_i$ (data not shown). The effect of PD098059 on PDBu-induced contraction and LC_{20} phosphorylation was examined in the tissues in which contractile tension and LC_{20} phosphorylation were measured simultaneously in the same tissue. As shown in Fig. 4, PD098059 augmented the PDBu-mediated contractions over the time period examined, but did not change LC_{20} phosphorylation.

Effect of PD098059 on PDBu-stimulated phosphorylation of $ERK_{42/44}$ and caldesmon. PDBu produced time-dependent increases in phosphorylation of $ERK_{42/44}$ (Fig. 5), and ERK-dependent phosphorylation of caldesmon at Ser^{789} in the uterine artery (Fig. 6). PD098059 blocked PDBu-mediated phosphorylation of $ERK_{42/44}$ and caldesmon (Fig. 5 & Fig. 6).

Discussion

ERK and thick filament regulation. Smooth muscle contraction is regulated by both thick and thin filament regulatory pathways. Thick filament regulation is mediated by both Ca^{2+}-dependent mechanisms that lead to activation of myosin light chain kinase (MLCK) and LC_{20} phosphorylation, and Ca^{2+}-independent mechanisms that involve inactivation of myosin light chain phosphatase (MLCP) and a decrease in LC_{20} dephosphorylation. In the present study, we examined the temporal relationships among $[Ca^{2+}]_i$, LC_{20} phosphorylation, and isometric force in the pregnant uterine artery, and, consistent with previous findings, demonstrated that phenylephrine produced a time-dependent increase in $[Ca^{2+}]_i$ and LC_{20} phosphorylation that preceded the contraction. We found that the decrease in phenylephrine-induced force development caused by PD098059 was preceded with a reduction in LC_{20} phosphorylation, suggesting a role for ERK in thick filament regulation. Similar results were obtained in porcine carotid artery, in which PD098059-mediated inhibition of endothelin-1-induced contraction was associated with a reduction in LC_{20} phosphorylation (8).

Previous studies in isolated smooth muscle cells have suggested that ERK does not regulate $[Ca^{2+}]_i$ (38, 49). However, our recent studies in the intact arterial rings, in which $[Ca^{2+}]_i$ and contractile tension were measured simultaneously in the same tissue, demonstrated clearly that PD098059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in both $[Ca^{2+}]_i$ and Ca^{2+} sensitivity in the uterine artery (55). This suggests that, in addition to the Ca^{2+}-independent pathway as previously proposed (1, 2, 11, 17, 21, 33), the ERK signaling pathway also involves the

Ca^{2+}-dependent components of vascular contractions. Consistent with our previous findings, the present study demonstrated that PD098059 inhibited phenylephrine-induced increase in $[Ca^{2+}]_i$. The mechanisms involved in ERK-mediated regulation of $[Ca^{2+}]_i$ are not clear at present. Given the finding that PD098059 did not affect force development elicited by KCl depolarization (8, 52, 55), it is likely that ERK may regulate the release of Ca^{2+} from intracellular pools, but not Ca^{2+} entry *via* L-type Ca^{2+} channels. Because LC_{20} phosphorylation is primarily regulated by $[Ca^{2+}]_i$, our results suggest that ERK may regulate LC_{20} phosphorylation, in part, through modulating $[Ca^{2+}]_i$.

In addition, we found that PD098059 significantly decreased Ca^{2+} sensitivity of LC_{20} phosphorylation in response to phenylephrine, *i.e.* less LC_{20} phosphorylation at the given $[Ca^{2+}]_i$ in the presence of PD098059. Because alterations in the activities of MLCK or MLCP at fixed $[Ca^{2+}]_i$ will affect the Ca^{2+} sensitivity of LC_{20} phosphorylation, the results suggest that, in addition to the regulation of MLCK activity through changes in $[Ca^{2+}]_i$, ERK may also regulate either MLCK or/and MLCP activities independent of changes in $[Ca^{2+}]_i$. The finding that PD098059 decreased the intercept without affecting the slope of LC_{20} phosphorylation *vs.* $[Ca^{2+}]_i$ relation suggests that ERK may have a positive tonic effect on Ca^{2+} sensitivity of LC_{20} phosphorylation, but may not affect the agonist-mediated Ca^{2+} sensitivity. Both MLCK and MLCP can be involved in the regulation of Ca^{2+} sensitivity of LC_{20} phosphorylation, and MLCP may be primarily 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 both MLCK and LC20 phosphorylation in COS-7 cells, which were reversed by pretreatment with PD098059 (29).

ERK and thin filament regulation. The present findings that $[Ca^{2+}]_i$ and the degree of LC_{20} phosphorylation decline from their peak values to lower steady-state levels during phenylephrine-induced sustained uterine artery contraction, is in agreement with previous results (8, 12, 34, 54). Previously, the dissociation of force and LC_{20} phosphorylation was postulated to be a consequence of the "latch" state (25, 35). However, recent studies have suggested an additional thin filament regulation in smooth muscle contraction (21, 33). The present study demonstrated that PD098059 increased force development at given levels of LC_{20} phosphorylation mediated by phenylephrine. This suggests that in the uterine artery α_1-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 α_1-adrenoceptor-mediated thin filament regulation of contractions. Nevertheless, PD098059 inhibited the phenylephrine-induced contraction, suggesting that the thick filament regulatory pathway, *i.e.* LC_{20} phosphorylation, predominates in α_1-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 LC_{20} has been reported in phorbol ester-induced contractions (22, 41, 42, 43). However, the significance of phorbol ester-induced LC_{20} phosphorylation in the contraction is controversial, and there is an inconsistency in the dependence of LC_{20} phosphorylation on $[Ca^{2+}]_i$. For instance, it has been reported that phorbol-12,13-dibutyrate induces LC_{20} phosphorylation in Ca^{2+}-depleted rat aortae (22, 43), but 12-deoxyphorbol-13-isobutyrate does not induce LC_{20} phosphorylation in ferret aortae, even in the presence of extracellular Ca^{2+} (24). In addition, previous studies showed dissociation between LC_{20} phosphorylation and tension development in response to phorbol esters (14, 31, 47). The present study demonstrated that PDBu induced contractions independent of changes in either $[Ca^{2+}]_i$ or LC_{20} phosphorylation levels. This suggests that in the uterine artery PKC-induced contraction is mediated predominately through thin filament regulatory pathways. Phorbol ester-induced contractions have been shown to involve regulatory components, including caldesmon and calponin, associated with thin filaments (33, 45, 46, 53). Phosphorylation of caldesmon by PKC reverses its inhibitory effect on myosin ATPase (48). The present finding that PD098059 enhanced PKC-mediated contraction without changing either $[Ca^{2+}]_i$ or LC_{20} phosphorylation levels reenforces 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 caldesmon (CaD) kinase mediating CaD phosphorylation (2, 3, 4, 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 Ser^{789}) 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 interrelationship among ERK activity, phosphorylation of CaD and LC_{20}, and isometric force. They demonstrated that the inhibitory effect of PD098059 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 PD098059 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 $ERK_{42/44}$ and CaD at Ser^{789} and the phosphorylation was blocked by PD098059, suggesting ERK-mediated phosphorylation of Ser^{789} in CaD in the uterine artery. This is in agreement with previous findings (9). The novel finding of the present study is that PD098059 had opposite effects on PDBu-induced CaD phosphorylation at Ser^{789} and contractions. It blocked phosphorylation of CaD at Ser^{789},

but potentiated the contractions. This suggests that ERK-dependent phosphorylation of CaD at Ser^{789} may not be involved in the PDBu-induced contraction, but rather inhibit it in the uterine artery. Although we were unable to determine phosphorylation of CaD at Ser^{759} due to the lack of appropriate antibody, previous study demonstrated that the major site of ERK-dependent phosphorylation in CaD was at Ser^{789} (9). Although all of the phosphate incorporated into CaD by the ERK is in either Ser^{789} or Ser^{759}, not all of 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 different sites, as compared with those 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 both in native thin filaments and in the isolated state at multiple sites of Ser^{127}, Ser^{587}, Ser^{600}, Ser^{657}, Ser^{686} and Ser^{726}. PKC-mediated phosphorylation of both intact CaD and of its C-terminal fragment containing 658-756, significantly decreased their ability to inhibit acto-heavy meromyosin ATPase (51). Taken together, as shown in Fig. 7, we propose that in sheep uterine artery PKC induces phosphorylation of CaD at Ser^{789} through activation of ERK, as well as at other site(s) through unknown mechanisms. It is the phosphorylation of CaD at site(s) other than Ser^{789} that may be important in reversing the inhibitory effect of CaD on myosin ATPase and leading to contractions. Phosphorylation of ERK-dependent Ser^{789} may not lead to the reversal of

CaD inhibitory effects on myosin ATPase. Instead, phosphorylation of Ser^{789} may inhibit PKC-mediated phosphorylation of the other site(s).

In summary, we have shown in the pregnant uterine artery that α_1-adrenoceptor-mediated contraction is regulated through both thick and thin filament pathways, with the thick filament regulatory pathway, *i.e.* LC_{20} phosphorylation, predominating. However, PKC-mediated contraction is regulated predominately through thin filament pathways, *i.e.* independent of changes in LC_{20} phosphorylation. ERK activation differentially regulates thick and thin filament pathways in the uterine artery. ERK potentiates the thick filament regulatory pathway by elevating LC_{20} phosphorylation *via* increases in $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of LC_{20} phosphorylation. In contrast, the present study indicates that ERK attenuates thin filament regulatory pathways, and suppresses contractions independent of changes in LC_{20} phosphorylation in the uterine artery. Although it is not clear at present whether the ERK-mediated inhibition of the thin filament regulatory pathway is unique to the pregnant uterine artery, or it is a more generalized mechanism, this novel finding suggests an intriguing hypothesis that, among ERK's effects, caldesmon phosphorylation at ERK-specific sites may stabilize its inhibitory effect on actin-activated myosin ATPase.

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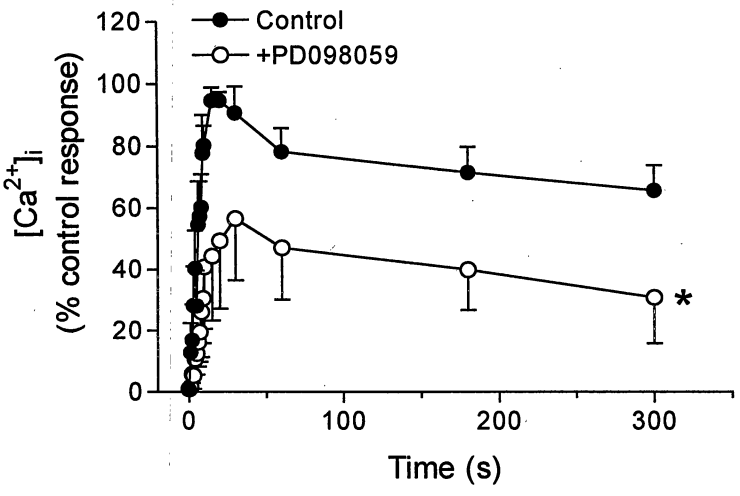
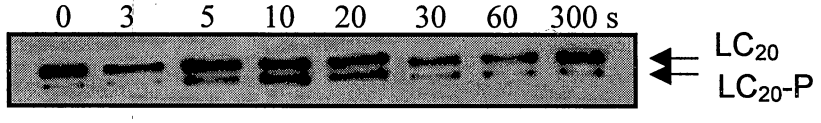
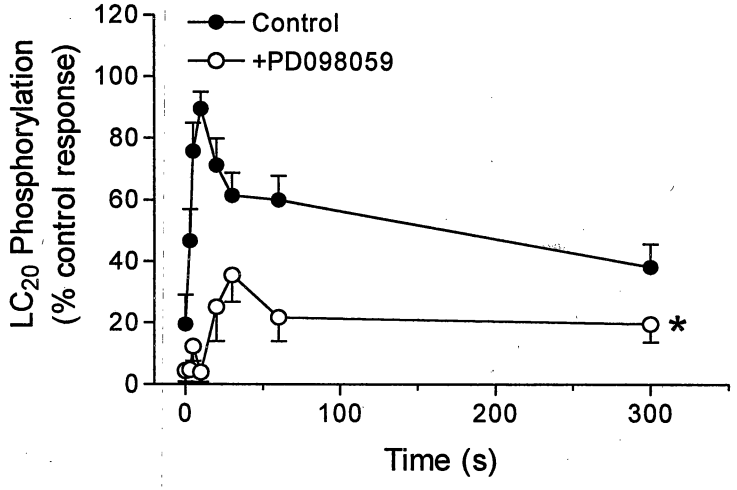
Figure 1. Effect of PD098059 on phenylephrine-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), LC_{20} phosphorylation, and contraction in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. $[Ca^{2+}]_i$, LC_{20} phosphorylation, and contraction were recorded simultaneously in the uterine artery. Representative Western immunoblot shows LC_{20} and phosphorylated LC_{20} (LC_{20}-P) induced by phenylephrine at indicated time points. Data are the means ± SEM from four to nine animals. All values of PD098059-treated tissues at and after 5 seconds are significantly decreased as compared to the control, and are indicated by * ($P < 0.05$).


Control
+PD098059
$[Ca^{2+}]_i$
(% control response)
120
100
80
60
40
20
0
0
100
200
300
Time (s)
*


0 3 5 10 20 30 60 300 s
LC_{20}
LC_{20}-P


Control
+PD098059
LC_{20} Phosphorylation
(% control response)
120
100
80
60
40
20
0
0
100
200
300
Time (s)
*

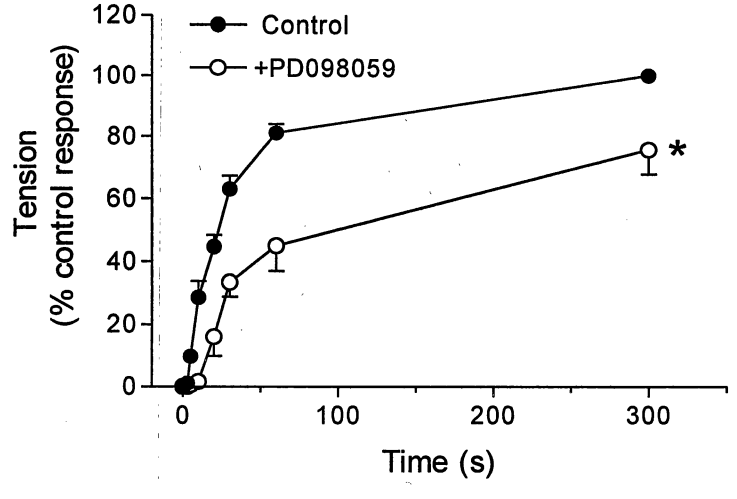

Control
+PD098059
Tension
(% control response)
120
100
80
60
40
20
0
0
100
200
300
Time (s)
*

Figure 2. Effect of PD098059 on phenylephrine-mediated LC_{20} phosphorylation/$[Ca^{2+}]_i$ relation in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Increases of LC_{20} phosphorylation stimulated by phenylephrine were plotted to show responses as a function of $[Ca^{2+}]_i$ (fura 2 signal, $R_{f340/380}$) at each corresponding time point (from 3 to 30 seconds). Data are the means ± SEM from four to nine animals.

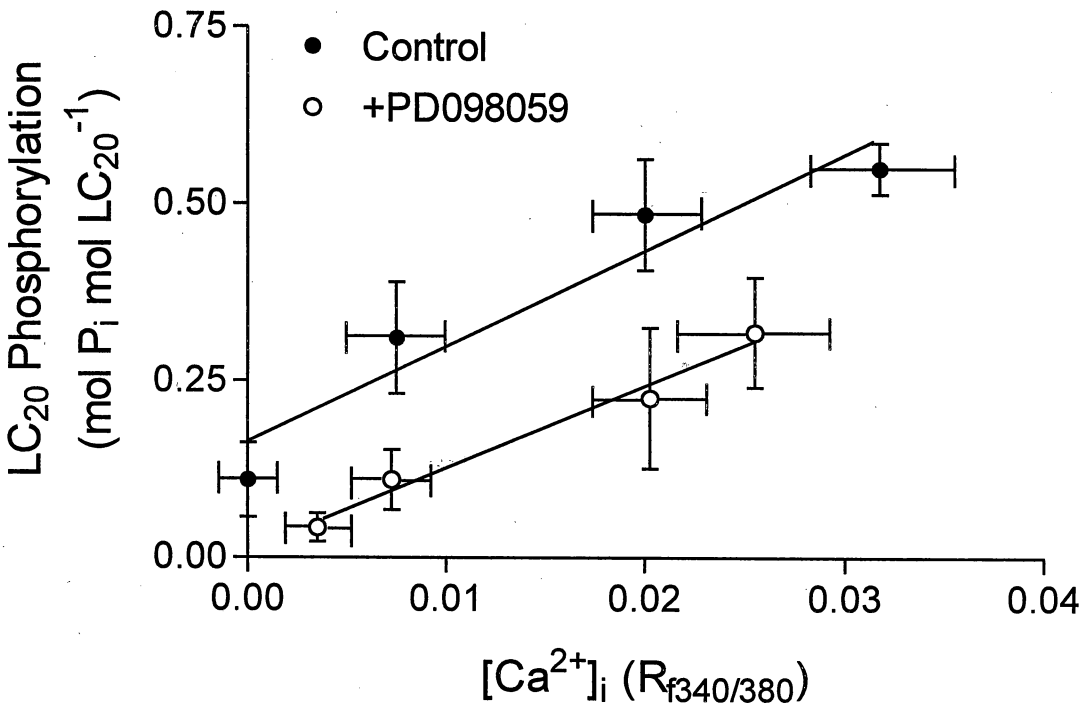
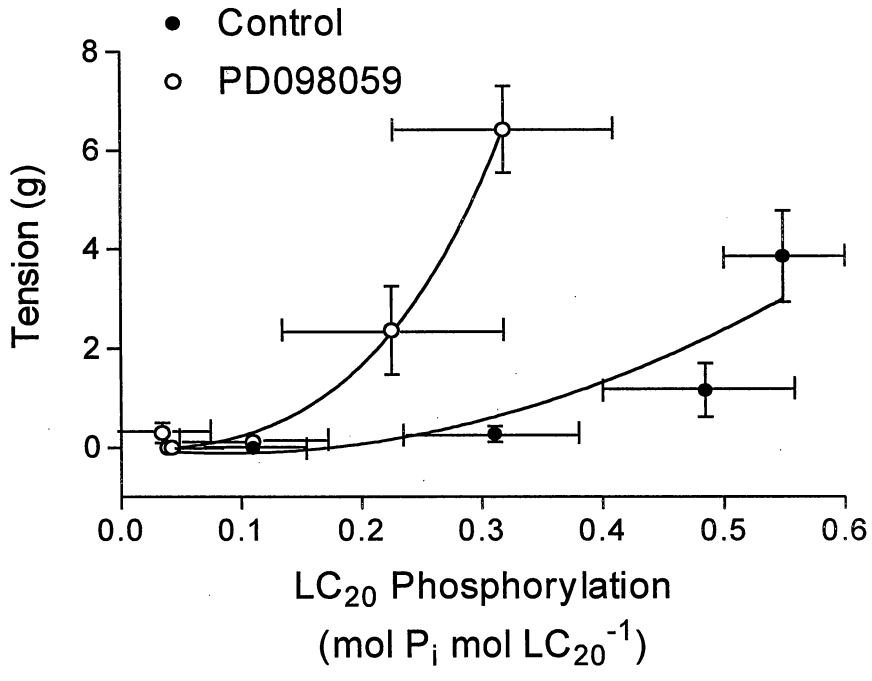

0.75
0.50
0.25
0.00
0.00
0.01
0.02
0.03
0.04
Control
+PD098059
LC20 Phosphorylation
(mol Pi mol LC20-1)
[Ca2+]i (Rf340/380)

Figure 3. Effect of PD098059 on phenylephrine-induced force/LC_{20} phosphorylation relation in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Increases of tension stimulated by phenylephrine were plotted to show responses as a function of phosphorylated LC_{20} measured simultaneously in the same tissue at each corresponding time point (0-10 s for the control, and 0-30 s for the PD098059 treated, shown in Fig. 1) (panel **A**). Panel **B** shows the relation between the levels of LC_{20} phosphorylation from control and PD098059-treated tissues at corresponding tensions developed, calculated from the curves in panel **A**. The dash line shows the line of identity. Data are the means ± SEM from four to nine animals.

A


Control
PD098059
Tension (g)
8
6
4
2
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
LC20 Phosphorylation
(mol Pi mol LC20-1)

B

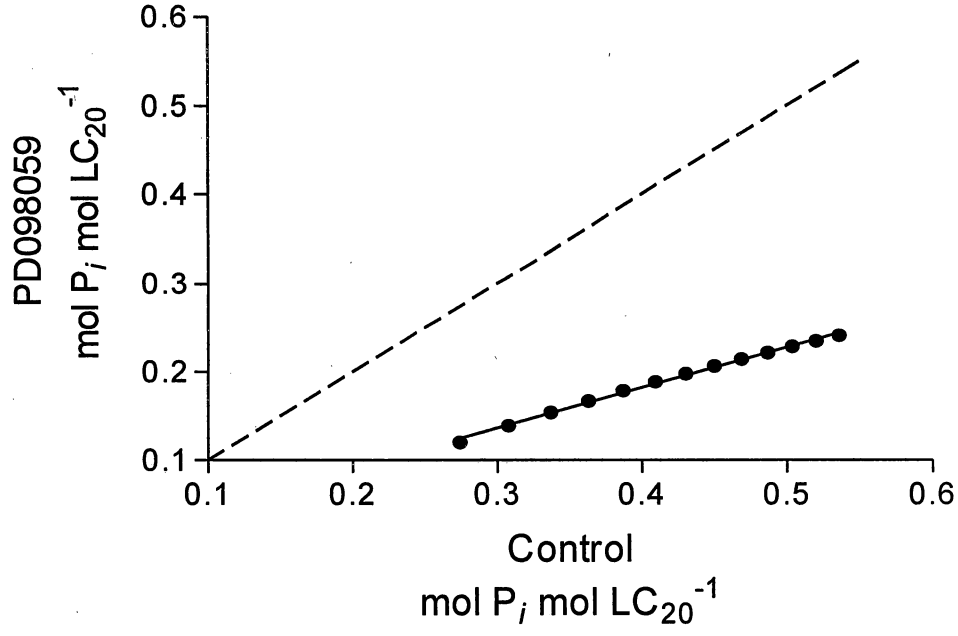

PD098059
mol Pi mol LC20-1
0.6
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.4
0.5
0.6
Control
mol Pi mol LC20-1

Figure 4. Effect of PD098059 on PDBu-stimulated tension and LC_{20} phosphorylation in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 5 μM PDBu. PDBu-induced contraction and LC_{20} phosphorylation (LC_{20}-P) were measured simultaneously in the same tissues. Representative Western immunoblot shows LC_{20} and no phosphorylated LC_{20} (LC_{20}-P) induced by PDBu at indicated time points. Phenylephrine (PE)-induced phosphorylated LC_{20} is shown, and serves as a positive control. Data are the means ± SEM from seven animals. * $P < 0.05$ *vs* control tensions.

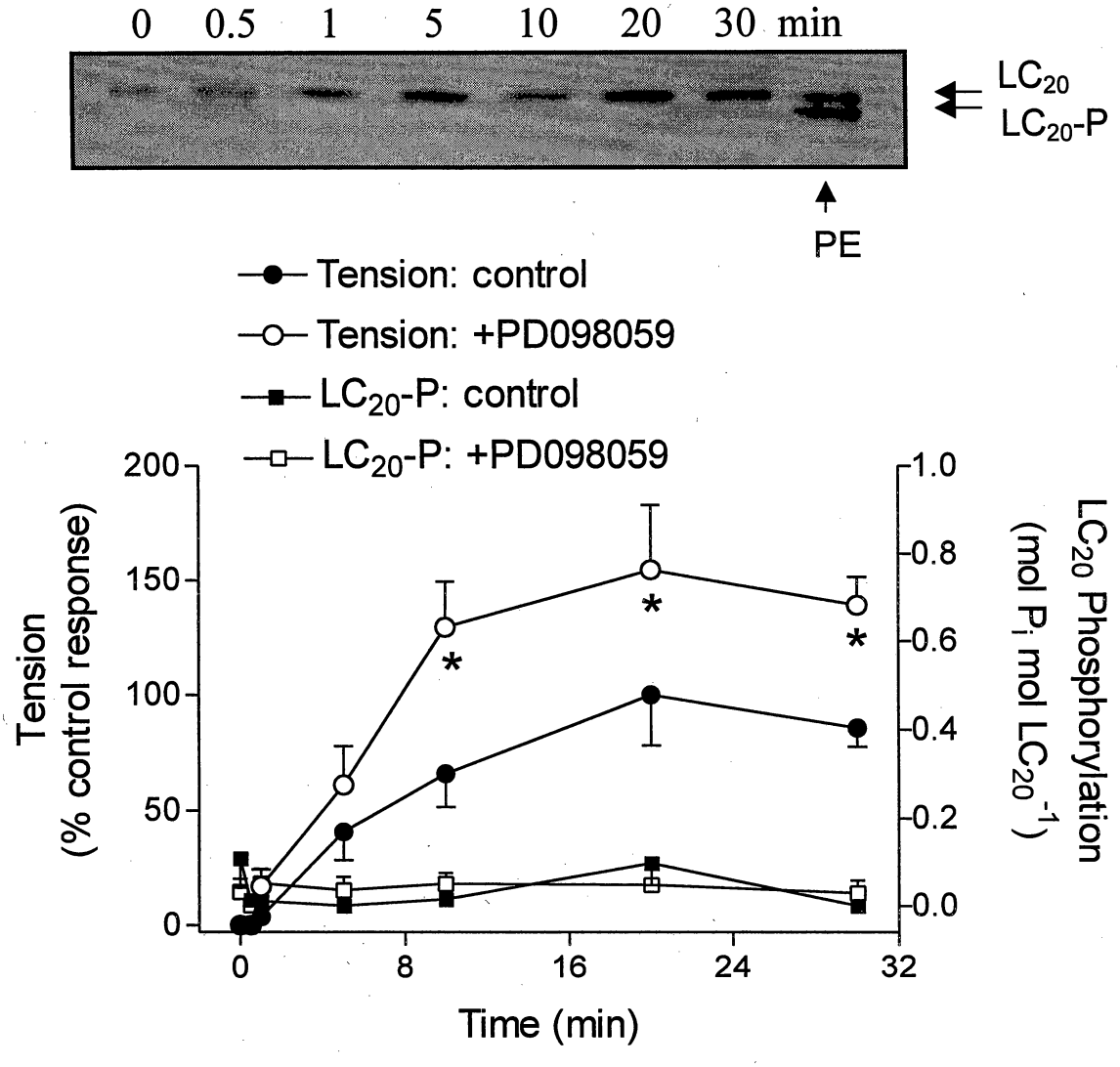

0 0.5 1 5 10 20 30 min
LC20
LC20-P
PE
Tension: control
Tension: +PD098059
LC20-P: control
LC20-P: +PD098059
Tension (% control response)
LC20 Phosphorylation (mol Pi mol LC20-1)
Time (min)
0
50
100
150
200
0.0
0.2
0.4
0.6
0.8
1.0
0
8
16
24
32
*
*
*

Figure 5. Effect of PD098059 on PDBu-stimulated phosphorylation of $ERK_{42/44}$ in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 5 μM PDBu. PDBu-induced increase in phosphorylated $ERK_{42/44}$ (p-ERK) was determined by Western immunoblot analysis. Data are the means ± SEM from six animals.

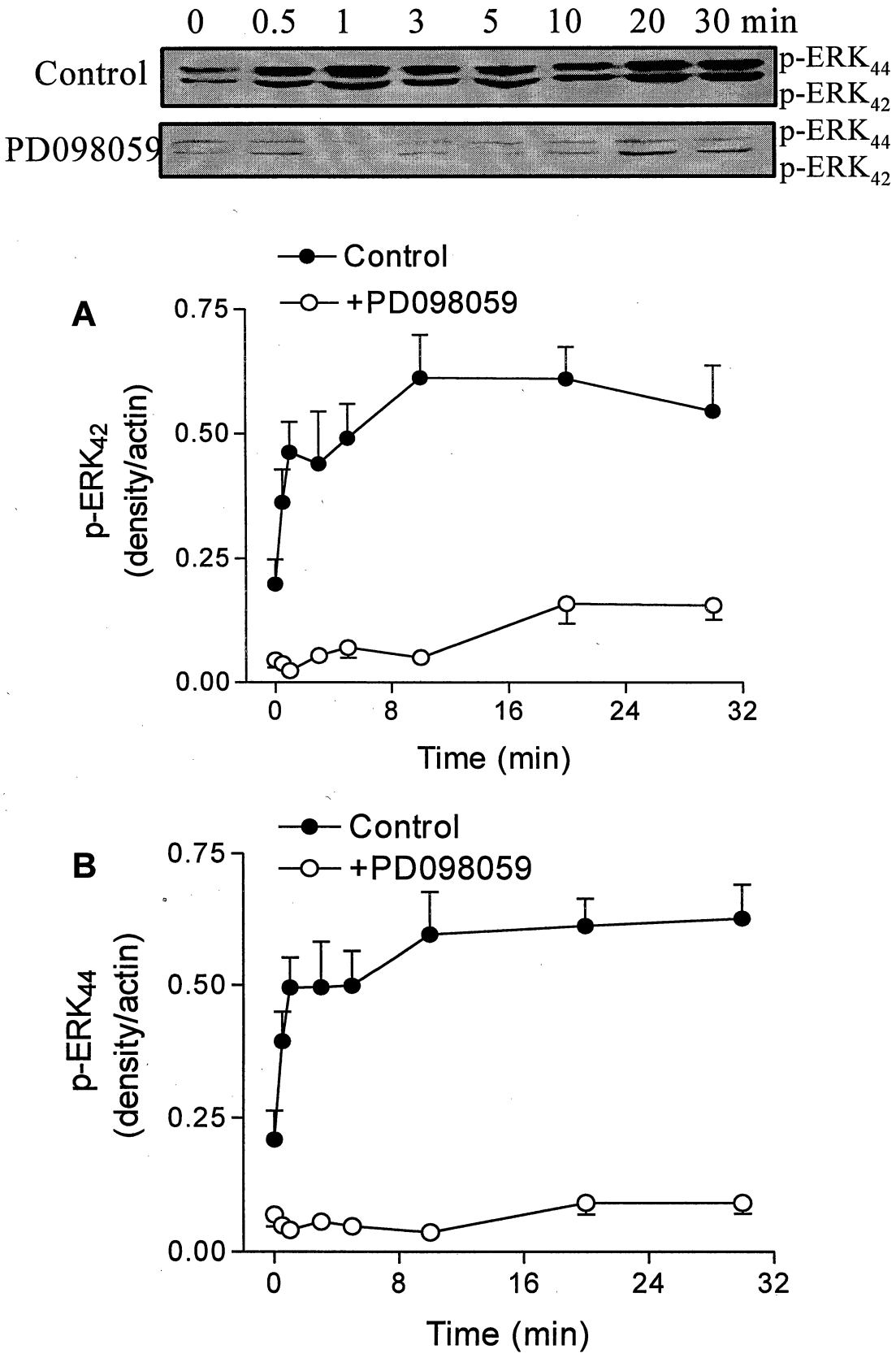
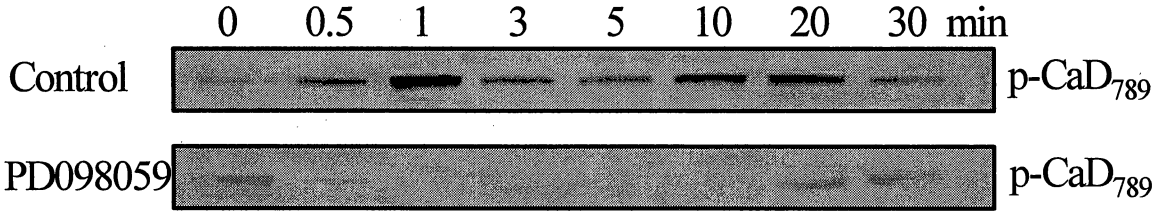

0 0.5 1 3 5 10 20 30 min
Control
PD098059
p-ERK44
p-ERK42
p-ERK44
p-ERK42
Control
+PD098059
A
p-ERK42 (density/actin)
0.75
0.50
0.25
0.00
0
8
16
24
32
Time (min)
Control
+PD098059
B
p-ERK44 (density/actin)
0.75
0.50
0.25
0.00
0
8
16
24
32
Time (min)

Figure 6. Effect of PD098059 on PDBu-stimulated phosphorylation of caldesmon (Ser^{789}) in the uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 5 μM PDBu. PDBu-induced increase in phosphorylated caldesmon at Ser^{789} ($p\text{-}CaD_{789}$) was determined by Western immunoblot analysis. Data are the means ± SEM from four to six animals.


0 0.5 1 3 5 10 20 30 min
Control
p-CaD789
PD098059
p-CaD789

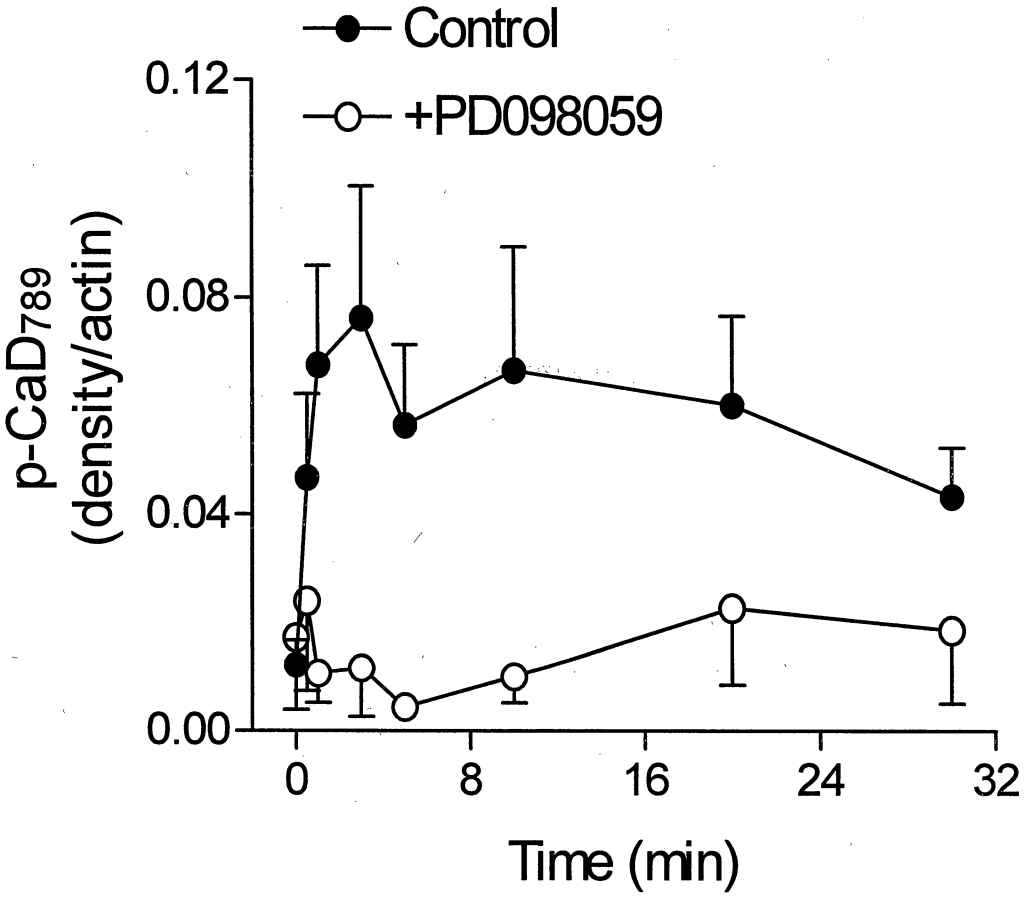
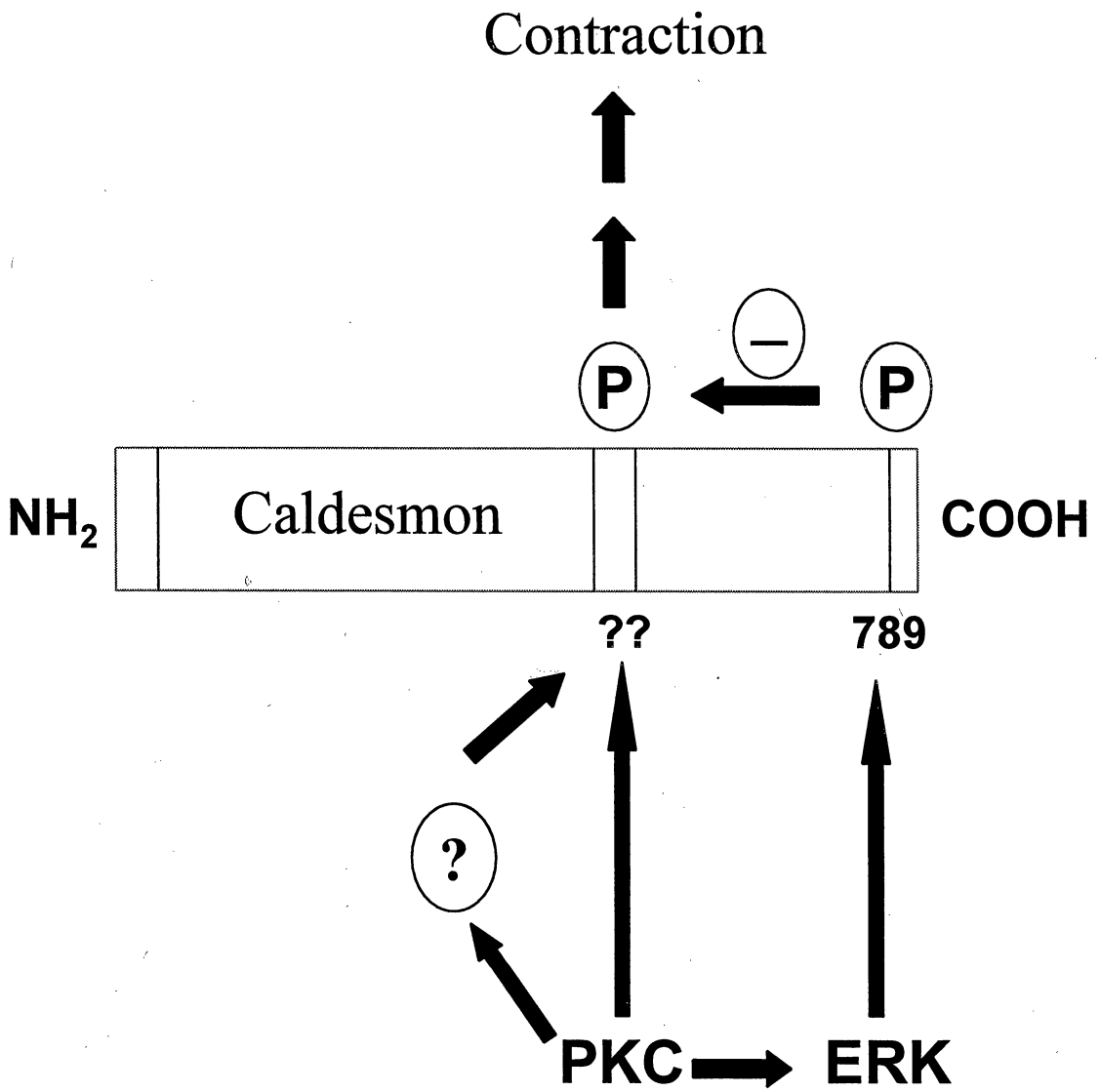

Control
+PD098059
0.12
0.08
0.04
0.00
p-CaD789 (density/actin)
0
8
16
24
32
Time (min)

Figure 7. Proposed model: ERK-dependent phosphorylation of CaD at Ser^{789} inhibits PKC-mediated contractions. We propose that PKC induces phosphorylation of CaD at Ser^{789} through activation of ERK, as well as at other site(s) through unknown mechanisms in sheep uterine artery. It is the phosphorylation of CaD at site(s) other than Ser^{789} that may be important in reversing the inhibitory effect of CaD on myosin ATPase and leading to contractions. Phosphorylation of ERK-dependent Ser^{789} may not lead to the reversal of CaD inhibitory effects on myosin ATPase. Instead, phosphorylation of Ser^{789} may inhibit PKC-mediated phosphorylation of the other site(s).


Contraction
P
P
NH2
Caldesmon
COOH
??
789
?
PKC
ERK

CHAPTER FOUR

Adaptation of Uterine Artery Thick and Thin Filament Regulatory Pathways to Pregnancy

DaLiao Xiao and Lubo Zhang

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Abstract

Little is known about the adaptation of contractile mechanisms of uterine artery smooth muscle to pregnancy. The present study tested the hypothesis that pregnancy differentially regulates thick and thin filament regulatory pathways in the uterine artery. Isometric tension, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and phosphorylation of 20 kDa myosin light chain (MLC_{20}) were measured simultaneously in uterine arteries isolated from nonpregnant and near-term (140 days gestation) pregnant sheep. Phenylephrine-mediated $[Ca^{2+}]_i$, MLC_{20} phosphorylation and contractions were significantly increased in pregnant, as compared with nonpregnant, uterine arteries. In contrast, phenylephrine-mediated Ca^{2+} sensitivity of MLC_{20} phosphorylation was decreased in the pregnant uterine arteries. Simultaneous measurement of phenylephrine-stimulated tension and MLC_{20} phosphorylation in the same tissue indicated a decrease in MLC_{20} phosphorylation-independent contractions in the pregnant uterine arteries. In addition, activation of protein kinase C (PKC) produced significantly lower sustained contractions in pregnant, as compared with nonpregnant, uterine arteries in the absence of changes in MLC_{20} phosphorylation levels in either vessels. In the nonpregnant uterine arteries, the ERK inhibitor PD098059 significantly increased phenylephrine-mediated, MLC_{20} phosphorylation-independent contractions. The results suggest that in the uterine artery pregnancy upregulates α_1-adrenoceptor-mediated Ca^{2+} mobilization and MLC_{20} phosphorylation. In contrast, pregnancy downregulates the Ca^{2+} sensitivity of myofilaments, which is mediated by both thick and thin filament pathways.

Introduction

Pregnancy is associated with a significant decrease in uterine vascular tone and a striking increase in uterine blood flow that optimizes the delivery of oxygen and substrates to the developing fetus *via* the placenta. Previous studies have focused on the endothelial adaptation, and have shown an increase in endothelial nitric oxide synthesis/release in the uterine artery during pregnancy (8, 35, 44, 46). However, little is known about the adaptation of contractile mechanisms of uterine artery smooth muscle to pregnancy. Pregnancy causes a transient and reversible sympathetic denervation of the uterine artery (21, 27, 28), and an increase in contractile reactivity to nonsynaptic α_1-adrenergic stimulation (3, 4, 11, 12, 30, 43, 47). In addition, recent studies have demonstrated that protein kinase C (PKC)-mediated sustained contraction is significantly attenuated in the pregnant uterine artery (47). PKC has been proposed to play an important role in the regulation of basal vascular tone and arterial caliber, the major determinants of blood flow (13, 26, 31).

Smooth muscle contraction is regulated through both thick and thin filament regulatory pathways. The thick filament regulation is mediated by both Ca^{2+}-dependent mechanisms that lead to activation of myosin light chain kinase (MLCK) and phosphorylation of 20-kD regulatory light chain of myosin (MLC_{20}), and Ca^{2+}-independent mechanisms that involve inactivation of myosin light chain phosphatase (MLCP) and a decrease in MLC_{20} dephosphorylation (18, 19, 32). In addition to the thick filament regulation, many studies have demonstrated dissociation between MLC_{20}

phosphorylation and cross-bridge cycling rates/tension development, suggesting a thin filament regulatory pathway (5, 25).

Our recent studies have shown in pregnant ovine uterine arteries that α_1-adrenoceptor-mediated contractions are regulated through both thick and thin filament pathways, with the thick filament regulatory pathway, *i.e.*, MLC_{20} phosphorylation, predominating (45). In contrast, activation of PKC induced contractions without changes in MLC_{20} phosphorylation levels (45). These studies have also shown that extracellular signal-regulated kinase (ERK) modulates contractile force in the pregnant uterine artery by a dual regulation of thick and thin filament pathways. ERK potentiated α_1-adrenoceptor-stimulated MLC_{20} phosphorylation *via* increases in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and the Ca^{2+} sensitivity of MLC_{20} phosphorylation. In contrast, ERK attenuated the thin filament regulatory pathway by suppressing the contractions independent of changes in MLC_{20} phosphorylation levels.

In the present study, we tested the hypothesis that pregnancy differentially regulates thick and thin filament regulatory pathways in the uterine artery. Isometric tension, $[Ca^{2+}]_i$ and MLC_{20} phosphorylation levels were measured simultaneously in uterine arteries isolated from nonpregnant and near-term (140 days gestation) pregnant sheep. The present study provides evidence that pregnancy upregulates α_1-adrenoceptor-mediated intracellular Ca^{2+} mobilization and MLC_{20} phosphorylation, but downregulates the Ca^{2+} sensitivity of MLC_{20} phosphorylation. In addition, pregnancy significantly downregulates MLC_{20} phosphorylation-independent contractions in the uterine artery.

Methods

Tissue preparation. Nonpregnant and 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% to 2.0% halothane in O_2 throughout the surgery. An incision in the abdomen was made and the uterus exposed. The uterine arteries were isolated and removed without stretching, and were placed into a modified Krebs solution (pH 7.4) of the following composition (in mM): 115.2 NaCl, 4.7 KCl, 1.80 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22.14 $NaHCO_3$, 0.03 EDTA, and 7.88 dextrose. The Krebs solution was oxygenated with a mixture of 95% O_2-5% CO_2. After the tissues were removed, animals were killed with euthanasia solution (T-61, Hoechst-Roussel; Somervile, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University, and followed the guidelines in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Contraction studies. The third (nonpregnant) and fourth (pregnant) branches of the main uterine arteries with a similar external diameter were separated from the surrounding tissue, and were cut into 2-mm ring segments. Isometric tension was measured in the Krebs' solution in tissue bath at 37 °C, as described previously (45). 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 then stimulated with phenylephrine or phorbol 12,13-dibutyrate (PDBu) (Sigma, St. Louis, MO), and contractile tensions and MLC_{20} phosphorylation

levels were measured simultaneously in the same tissues. Tensions developed were continuously recorded with an on-line computer. To measure phosphorylated MLC_{20} simultaneously in the same tissue, arterial rings were snap-frozen with liquid N_2-cooled clamps at the indicated times, and rapidly immersed in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM DTT mixture. Tissues were then stored at -80°C until the analysis of MLC_{20} phosphorylated. In certain experiments, tissues were pretreated with PD098059 (30 μM) (Sigma, St. Louis, MO) or vehicle (DMSO) for 30 min, followed by stimulation with phenylephrine.

Measurements of MLC_{20} phosphorylated. Tissue MLC_{20} phosphorylated levels were measured as described previously (45). Briefly, tissues were brought to room temperature in a dry ice-acetone-TCA-DTT mixture and 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% bromophenol blue, as previously described. Sample (20 μl) were electrophoresed at 12 mA for 2.5 h after a 30 min pre-run in 1.0 mm mini-polyacrylamide gels containing 10% arcelamide-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 MLC_{20} antibody (1:500, Sigma, St. Louis, MO). Goat anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Calbiochem). Bands were detected with enhanced chemiluminescence (ECL), visualized on films and were analyzed with the Kodak 1D image analysis software. Moles of phosphate per mole of myosin light chain (fractional MLC_{20} phosphorylated) were calculated by dividing the density of the phosphorylated band by the sum of the densities

of the phosphorylated plus the unphosphorylated bands. In some experiments, phosphorylated MLC_{20} was corrected for protein content differences between nonpregnant and pregnant uterine arteries, and was expressed as mg MLC_{20} phosphorylated/g tissue wet weight, as previously described in ovine uterine arteries by Annibale et al. (4).

Simultaneous measurement of $[Ca^{2+}]_i$ and tension. Simultaneous recordings of contraction and $[Ca^{2+}]_i$ in the same tissue were conducted as described previously (49). Briefly, the arterial ring was attached to an isometric force transducer in a 5-ml tissue bath mounted on a CAF-110 intracellular Ca^{2+} 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 (Molecular Probes, Eugene, OR) 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 alternatively (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 a photomultiplier tube. The fluorescence intensity at each excitation wavelength (F_{340} and F_{380}, respectively) and the ratio of these two fluorescence values ($R_{f340/380}$) were recorded with a time constant of 250 ms and stored with the force signal on a computer.

Data analysis. Data were analyzed by computer-assisted linear or nonlinear regression to fit the data using GraphPad Prism (GraphPad software; San Diego, CA).

Results were expressed as means ± SEM. Differences were evaluated for statistical significance ($P < 0.05$) by one-way ANOVA and Student's *t*-test.

Results

Phenylephrine-induced $[Ca^{2+}]_i$ and tension. Phenylephrine produced dose-dependent increases in $[Ca^{2+}]_i$ and contractions in both nonpregnant and pregnant uterine arteries (Fig. 1). As shown in Fig.1A, the phenylephrine-induced concentration-contraction curve was shifted to the left in the pregnant uterine artery (pD_2: 5.86 ± 0.13), as compared with that in the nonpregnant uterine artery (pD_2: 5.15 ± 0.18) ($P < 0.05$). The maximal responses were 2.51 ± 0.14 g and 1.79 ± 0.19 g in the pregnant and nonpregnant uterine arteries, respectively ($P < 0.05$). In accordance, phenylephrine produced a dose-dependent increase in $[Ca^{2+}]_i$ (fura-2, $R_{f340/380}$) in both nonpregnant (pD_2: 5.6 ± 0.14) and pregnant (pD_2: 6.0 ± 0.12) uterine arteries ($P = 0.07$) (Fig. 1B). The maximal response was significantly increased in the pregnant (0.103 ± 0.009), as compared with that in the nonpregnant (0.032 ± 0.004) uterine arteries ($P < 0.05$).

Phenylephrine-induced MLC_{20} phosphorylation. Fig. 2 shows the time courses of phenylephrine-induced increases in phosphorylated MLC_{20} in nonpregnant and pregnant uterine arteries. As shown in Fig. 2, the initial rate of phenylephrine-stimulated MLC_{20} phosphorylation (MLC_{20}-P mg/g wet wt/sec) and the peak phosphorylation levels (MLC_{20}-P mg/g wet wt) were significantly increased in pregnant uterine arteries (the rate: 0.071 ± 0.017; the peak level: 0.881 ± 0.056), as compared with those in the nonpregnant vessels (the rate: 0.022 ± 0.004; the peak level: 0.629 ± 0.061) ($P < 0.05$). As shown in Fig. 3, phenylephrine-induced dose-dependent increases in phosphorylated MLC_{20} were significantly increased in pregnant, as compared with nonpregnant, uterine arteries.

MLC_{20} phosphorylation-$[Ca^{2+}]_i$ relation. Regulatory pathways in smooth muscle that modulate the Ca^{2+} dependence of force may exert their effects by altering the dependence of MLC_{20} phosphorylation on $[Ca^{2+}]_i$, or the dependence of force on MLC_{20} phosphorylation. To determine the effect of pregnancy on the regulation of the Ca^{2+} sensitivity of MLC_{20} phosphorylation at given $[Ca^{2+}]_i$, we examined the relation of phenylephrine-induced dose-dependent increases in $[Ca^{2+}]_i$ and phosphorylated MLC_{20} in both nonpregnant and pregnant uterine arteries. As shown in Fig. 4, there was a significant decrease in the slope (MLC_{20}-P/$[Ca^{2+}]_i$) in pregnant arteries (7.7 ± 2.2), as compared with that in nonpregnant vessels (20.7 ± 7.6) ($P < 0.05$).

Force-MLC_{20} phosphorylation relation. To further examine whether pregnancy regulated contractions independent of changes in MLC_{20} phosphorylation, we measured the phosphorylated MLC_{20} and contractions simultaneously in the same tissues stimulated with different doses of phenylephrine. Phenylephrine-induced tensions were plotted against their corresponding phosphorylated MLC_{20} levels in both nonpregnant and pregnant tissues. As shown in Fig. 5, there was a significant rightward shift in the tension-MLC_{20} phosphorylation relation curve in the pregnant, as compared with nonpregnant, uterine arteries. The slopes obtained were not different between nonpregnant and pregnant uterine arteries.

Effect of PD098059 on phenylephrine-induced MLC_{20} phosphorylation and contractions. Our previous studies demonstrated that the ERK inhibitor, PD098059, significantly inhibited phenylephrine-induced increases in MLC_{20} phosphorylation and contractions, but increased MLC_{20} phosphorylation-independent contractions in pregnant ovine uterine arteries (45). To determine whether PD098059 had similar effects in the

nonpregnant uterine arteries, we examined the effect of PD098059 on phenylephrine-induced increases in phosphorylated MLC_{20} and contractions in nonpregnant uterine arteries. As shown in Fig. 6, PD098059 inhibited the initial increases in MLC_{20} phosphorylation and tension development induced by phenylephrine, but significantly enhanced the contractions independent of changes in phosphorylated MLC_{20} by increasing the slope (tension/MLC_{20}-P) from 6.8 ± 1.7 to 12.6 ± 1.1 ($P < 0.05$) (Fig. 6C).

PDBu-stimulated MLC_{20} phosphorylation and tension. We previously demonstrated that PDBu-induced contractions were significantly decreased in pregnant, as compared with nonpregnant, uterine arteries (47). To determine the mechanism of MLC_{20} phosphorylation in PDBu-mediated contractions, we measured PDBu-induced contractile tensions and phosphorylated MLC_{20} levels simultaneously in the same tissues of nonpregnant and pregnant uterine arteries. As shown in Fig. 7, PDBu produced a significantly decreased contraction in pregnant, as compared with nonpregnant, uterine arteries, in the absence of changes in phosphorylated MLC_{20} levels in either vessels.

Discussion

The present study offers the following new findings. First, α_1-adrenoceptor-mediated Ca^{2+} mobilization and MLC_{20} phosphorylation are significantly greater in pregnant than nonpregnant uterine arteries. Second, α_1-adrenoceptor-mediated Ca^{2+} sensitivity of MLC_{20} phosphorylation, and MLC_{20} phosphorylation-independent contractions are significantly less in pregnant than nonpregnant uterine arteries. Third, activation of PKC induces contractions in the absence of changes in MLC_{20} phosphorylation in both nonpregnant and pregnant uterine arteries, and pregnancy is associated with a decrease in MLC_{20} phosphorylation-independent contractions in the uterine artery. Fourth, in agreement with the finding in the pregnant uterine artery (45), the ERK inhibitor PD098059 decreases α_1-adrenoceptor-mediated initial rising in MLC_{20} phosphorylation and contractions, but increases the MLC_{20} phosphorylation-independent contractions in the nonpregnant uterine arteries.

The present finding that α_1-adrenoceptor-mediated contractions of isolated uterine arteries were increased in the pregnant animals is in agreement with previous studies (3, 4, 11, 12, 30, 43, 47). This is probably due in part to a transient and reversible sympathetic denervation of the uterine artery during pregnancy (21, 27, 28), resulting in a sensitization of postsynaptic α_1-adrenoceptor-mediated responses. In contrast, pregnancy did not result in changes in postsynaptic α_1-adrenoceptor-mediated contractions in systemic vessels (3, 17). In addition, 5-HT- and KCl-stimulated contractions were the same between nonpregnant and pregnant uterine arteries (48). These findings suggest a specific effect of pregnancy on α_1-adrenoceptors in the uterine artery, and are consistent with the notion of sympathetic denervation-mediated

sensitization of postsynaptic α_1-adrenoceptor-mediated responses. Previous studies demonstrated that the increased α_1-adrenoceptor-mediated contractions in pregnant ovine uterine arteries were not the result of increased cellularity in the arterial cross section, but rather were related to properties of the vascular smooth muscle themselves (4). In addition, uterine arterial wall thickness and actin-to-myosin ratio were found unaltered during pregnancy (4).

Consistent with the sensitization of postsynaptic α_1-adrenoceptor-mediated Ca^{2+} mobilization and contractions, we have demonstrated that the density of α_1-adrenoceptors and norepinephrine binding affinity to α_1-adrenoceptors are significantly increased in pregnant, as compared with nonpregnant, ovine uterine arteries, resulting in increased inositol 1,4,5-trisphosphate (IP_3) synthesis (42, 43). The role of IP_3 as the messenger of agonist-mediated Ca^{2+} mobilization in smooth muscle has been firmly established (36). In addition to the possible sensitization of postsynaptic α_1-adrenoceptors due to sympathetic denervation in the pregnant uterine artery, pregnancy and steroid hormones may also have direct effects on α_1-adrenoceptor-mediated responses in the uterine artery. In isolated female rat aorta, estrogen pretreatment increased the number of α_1-adrenoceptors and the amount of intracellular Ca^{2+} available for contraction (6). In myometrial smooth muscle, progesterone had no effect on IP_3-dependent or -independent Ca^{2+} release from intracellular Ca^{2+} stores (15), but significantly increased α_1-adrenoceptor-mediated synthesis of inositol phosphates (33).

The increased Ca^{2+} mobilization is likely to play an important role in the increased MLC_{20} phosphorylation by activation of α_1-adrenoceptors in the pregnant uterine artery in the present study. This is consistent with the previous finding in ovine

uterine arteries that phenylephrine-stimulated total amount of MLC_{20} phosphorylated was increased in pregnant as compared with nonpregnant vessels (4). In contrast, studies in human myometrial smooth muscle demonstrated that KCl-mediated MLC_{20} phosphorylation was significantly reduced in pregnant as compared with nonpregnant tissues (41). This further suggests that increased MLC_{20} phosphorylation observed in the pregnant uterine artery is specific to α_1-adrenoceptor-mediated pathway. As discussed above, this selective sensitization occurred in the upstream of signal transduction pathway at the receptor levels, *i.e.* α_1-adrenoceptor density and endogenous agonist binding affinity. Unlike α_1-adrenoceptors, KCl-mediated MLC_{20} phosphorylation depends on Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Both estrogen and progesterone have been demonstrated to inhibit Ca^{2+} influx in smooth muscle (15, 34).

In addition to intracellular Ca^{2+} concentrations, phosphorylation of MLC_{20} is also regulated by activities of MLCK and MLCP. Although it is unknown at present whether MLCK activity is altered in the uterine artery during pregnancy, the specific activity of MLCK was not different between nonpregnant and pregnant myometrium (41). In the present study, we have demonstrated that pregnancy is associated with a significant decrease in the Ca^{2+} sensitivity of MLC_{20} phosphorylation in response to phenylephrine, *i.e.* less MLC_{20} phosphorylation at given $[Ca^{2+}]_i$ in the pregnant uterine artery, suggesting an increase in MLCP activity in the pregnant vessels. It has been demonstrated that steroid hormones regulate the expression of MLCP catalytic (PP1-δ) and large regulatory subunit (MYPT), and increase the levels of PP1-δ and MYPT in pregnant myometrium (39). In addition, estrogen and progesterone treatment inhibited agonist- and GTPγS-

induced Ca^{2+} sensitization of smooth muscle by increasing the expression of Rnd1 and Rnd3, which inhibited the RhoA-dependent pathways (9, 24).

The present finding that α_1-adrenoceptor-mediated contractions at given levels of MLC_{20} phosphorylation were significantly deceased in pregnant as compared with nonpregnant uterine arteries suggests that pregnancy enhances the thin filament-mediated inhibition of contractions in the uterine artery. This is further supported by the results of PKC-mediated contractions in the uterine artery. In the present study, we have demonstrated that PKC-induced contractions are independent of changes in MLC_{20} phosphorylation in both nonpregnant and pregnant uterine arteries, indicating that in the uterine artery PKC-induced contractions were mediated predominately through thin filament regulatory pathways. Although many studies have demonstrated that PKC mediates Ca^{2+}-independent contractions by increasing myofilament Ca^{2+} sensitivity in vascular smooth muscle, the significance of MLC_{20} phosphorylation in PKC-induced contractions remains controversial, and may be tissue-dependent. Consistent with the present finding, the dissociation between MLC_{20} phosphorylation and tension development in response to phorbol esters has been demonstrated previously (16, 37). In addition to the thick filament regulation, *i.e.* MLC_{20} phosphorylation, through MLCP, the Ca^{2+} sensitivity of myofilaments is also regulated through smooth muscle thin filament actin-binding proteins such as caldesmon that inhibits myosin ATPase activity. Studies using the antagonist peptide GS17C and the antisense have strongly suggested an important and physiologically relevant role for caldesmon in suppressing smooth muscle tone (25). It has been demonstrated that PKC induces phosphorylation of caldesmon, which reverses its inhibitory effect on myosin ATPase (38, 40, 45). The finding that PKC-mediated

contractions were attenuated in the pregnant uterine artery in the present study further suggests an adaptation of the thin filament regulatory pathway to pregnancy. Indeed, it has been reported that caldesmon levels increase in both human and rat myometrium during pregnancy, which contributes to a suppression of contractility of the pregnant myometrium (23, 41).

In agreement with our previous findings in the pregnant uterine artery, the present study has demonstrated that the ERK inhibitor PD098059 causes a significant leftward shift in the force-MLC_{20} phosphorylation relation in the nonpregnant uterine artery. This suggests that ERK plays a key role in maintaining the thin filament-mediated inhibitory effect on contractions of the uterine artery. We have previously demonstrated that pregnancy selectively enhances the role of ERK in α_1-adrenoceptor-mediated contractions and its effect in suppressing PKC-mediated contractions in the uterine artery (47). In vascular smooth muscle, ERK has been demonstrated as a physiologically relevant caldesmon kinase, and phosphorylates caldesmon at Ser^{789} (1, 2). However, whether caldesmon phosphorylation at ERK specific sites induces smooth muscle contraction remains controversial (10, 22, 29). Our recent studies in the pregnant uterine artery have suggested that ERK-mediated phosphorylation of caldesmon may stabilize the inhibitory effect of caldesmon on actin-activated myosin ATPase (45). Given that pregnancy significantly increases ERK protein levels and signaling pathway in the uterine artery (7, 14, 47), ERK is likely to play an important role in the adaptation of uterine artery contractility to pregnancy by increasing the thin filament-mediated inhibitory effect on contractions of the uterine artery.

In the perspective of physiology, during pregnancy the uterine vasculature acts as a low-resistance shunt to accommodate the large increase in uteroplacental blood flow required for normal fetal development. In addition to increased endothelial nitric oxide synthesis/release, pregnancy is associated with a transient and reversible sympathetic denervation of the uterus and uterine artery, which is associated with a profound decrease in contractions of smooth muscle to electric field stimulation (21, 28). Although the decreased sympathetic innervation, combined with increased nitric oxide release, maintain low uterine vascular tone in pregnancy, sympathetic denervation is likely to sensitize postsynaptic α-adrenergic pathway, and to increase ability of nonsynaptic α-adrenergic-mediated contractions in the uterine artery. This may be important for the mother to protect herself under stresses, and allow a redistribution of blood by contracting the uterine artery to circulating catecholamines. This is achieved in part by enhanced intracellular Ca^{2+} mobilization through activation of α_1-adrenoceptors resulting in activation of myosin light chain kinase and phosphorylation of MLC_{20}. On the other hand, the decreased Ca^{2+} sensitivity of myofilaments contributes to maintaining low basal vascular tone of the uterine artery during pregnancy, and helps to maintain low vascular tone in response to increased blood flow through the uterine artery during pregnancy. The cell and molecular mechanisms involving both thick and thin filament regulatory pathways in the adaptation of uterine artery myofilament Ca^{2+} sensitivity to pregnancy present important avenues for future investigation.

Acknowledgment

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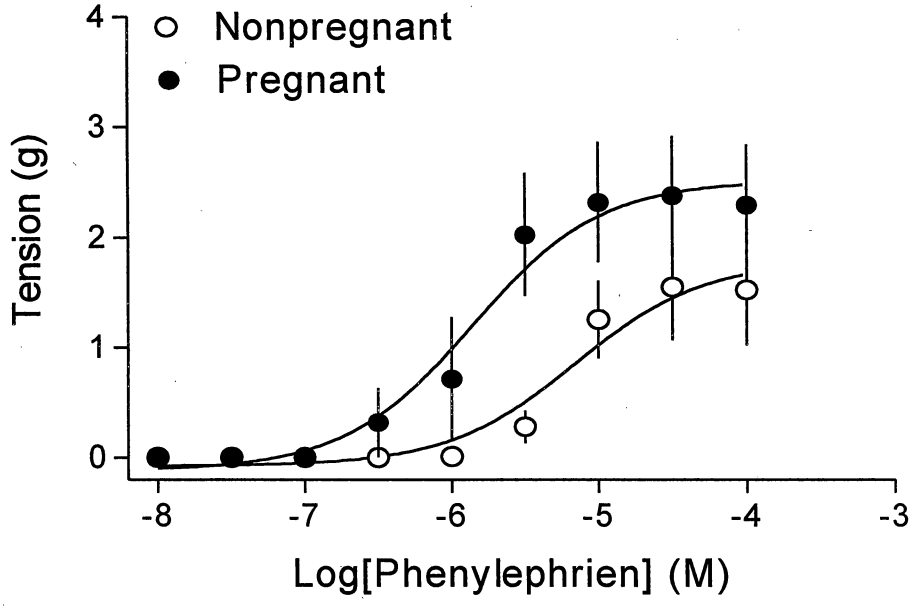
Figure 1. Phenylephrine-induced intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and contractions in the uterine artery. Cumulative concentration-response curves of phenylephrine-induced increases in $[Ca^{2+}]_i$ and contractions were measured simultaneously in the same tissues of uterine arteries obtained from nonpregnant and pregnant ovine. Data are means ± SEM of tissues from 4 animals. The values of pD_2 and the maximal response were presented in the text.


Nonpregnant
Pregnant
Tension (g)
4
3
2
1
0
-8
-7
-6
-5
-4
-3
Log[Phenylephrien] (M)

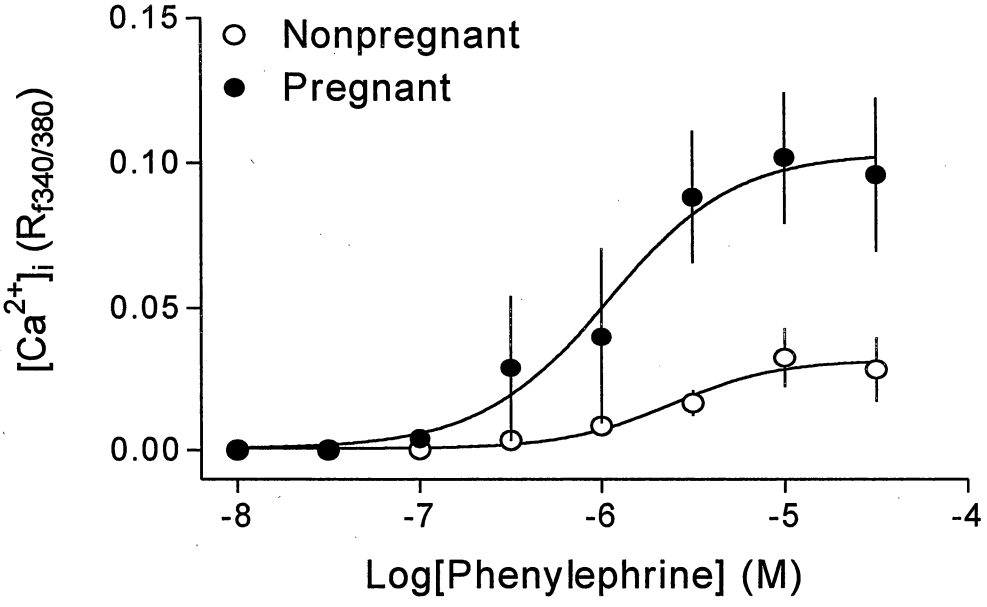
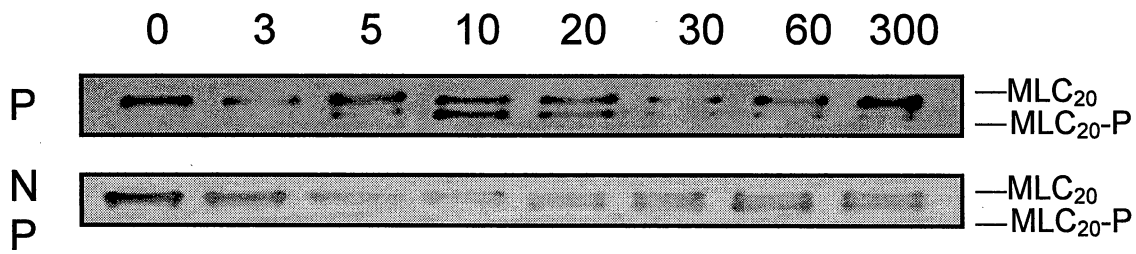

Nonpregnant
Pregnant
$[Ca^{2+}]_i$ ($R_{f340/380}$)
0.15
0.10
0.05
0.00
-8
-7
-6
-5
-4
Log[Phenylephrine] (M)

Figure 2. Phenylephrine-induced time courses of MLC_{20} phosphorylation in the uterine artery. Arterial rings of nonpregnant (NP) and pregnant (P) uterine arteries were stimulated with 3 μM phenylephrine. Phosphorylation of MLC_{20} was detected by Western immunoblot as described in the Methods. Representative immunoblot shows unphosphorylated MLC_{20} and phosphorylated MLC_{20} (MLC_{20}-P) induced by phenylephrine at 0-300 s. The levels of phosphorylated MLC_{20} were expressed as mg phosphorylated MLC_{20} per g tissue wet weight. Data are means ± SEM of tissues from 4 animals.


0
3
5
10
20
30
60
300
P
N
P
—MLC20
—MLC20-P
—MLC20
—MLC20-P

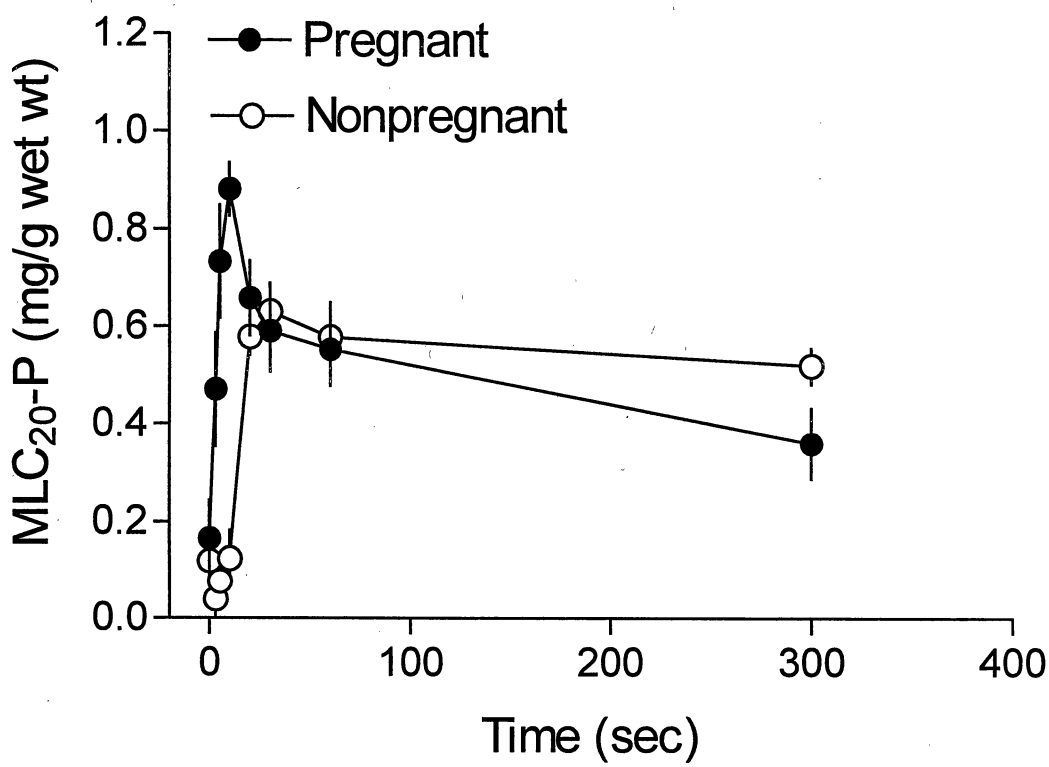

Pregnant
Nonpregnant
MLC20-P (mg/g wet wt)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
Time (sec)

Figure 3. Phenylephrine-induced dose-dependent increases in MLC_{20} phosphorylation in the uterine artery. Arterial rings of nonpregnant and pregnant uterine arteries were stimulated with increasing doses of phenylephrine. Phosphorylation of MLC_{20} was detected by Western immunoblot as described in the Methods, and was expressed as mg phosphorylated MLC_{20} per g tissue wet weight. Data are means ± SEM of tissues from 4 animals. * $P < 0.05$, pregnant *vs*. nonpregnant.

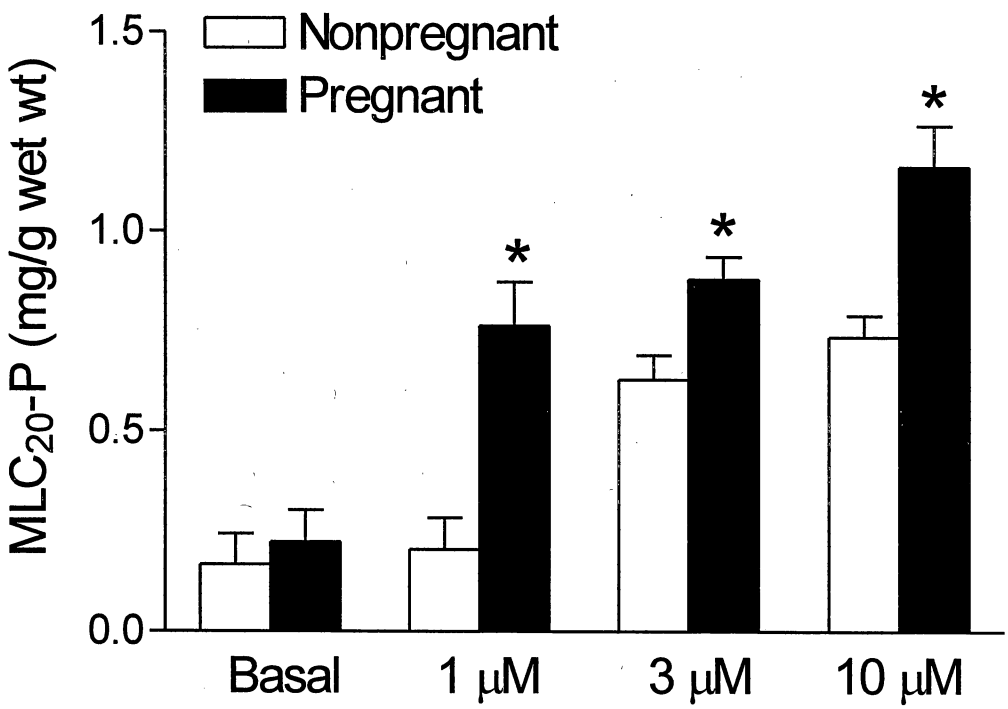

Nonpregnant
Pregnant
MLC20-P (mg/g wet wt)
1.5
1.0
0.5
0.0
*
*
*
Basal
1 μM
3 μM
10 μM

Figure 4. Phenylephrine-induced MLC_{20} phosphorylation-$[Ca^{2+}]_i$ relation in the uterine artery. Arterial rings of nonpregnant and pregnant uterine arteries were stimulated with increasing doses of phenylephrine (0.3 to 30 μM). Increased levels of phosphorylated MLC_{20} stimulated by phenylephrine were plotted to show responses as a function of $[Ca^{2+}]_i$ (fura-2 signal, $R_{f340/380}$) at each corresponding concentrations. Data are means ± SEM of tissues from 4 animals.

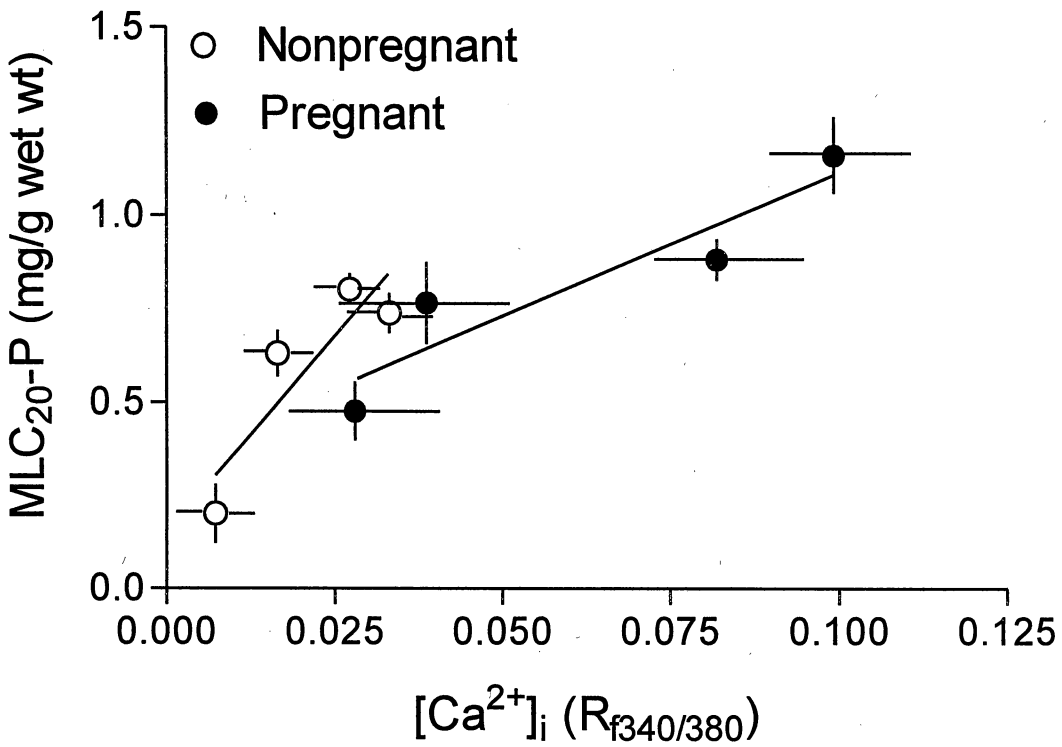

Nonpregnant
Pregnant
MLC20-P (mg/g wet wt)
1.5
1.0
0.5
0.0
0.000
0.025
0.050
0.075
0.100
0.125
[Ca2+]i (Rf340/380)

Figure 5. Phenylephrine-induced force-MLC_{20} phosphorylation relation in the uterine artery. Arterial rings of nonpregnant and pregnant uterine arteries were stimulated with increasing doses of phenylephrine (0.3 to 30 μM). Increased tension stimulated by phenylephrine was plotted to show responses as a function of phosphorylated MLC_{20} measured simultaneously in the same tissue at each corresponding concentrations. Data are means ± SEM of tissues from 4 animals.

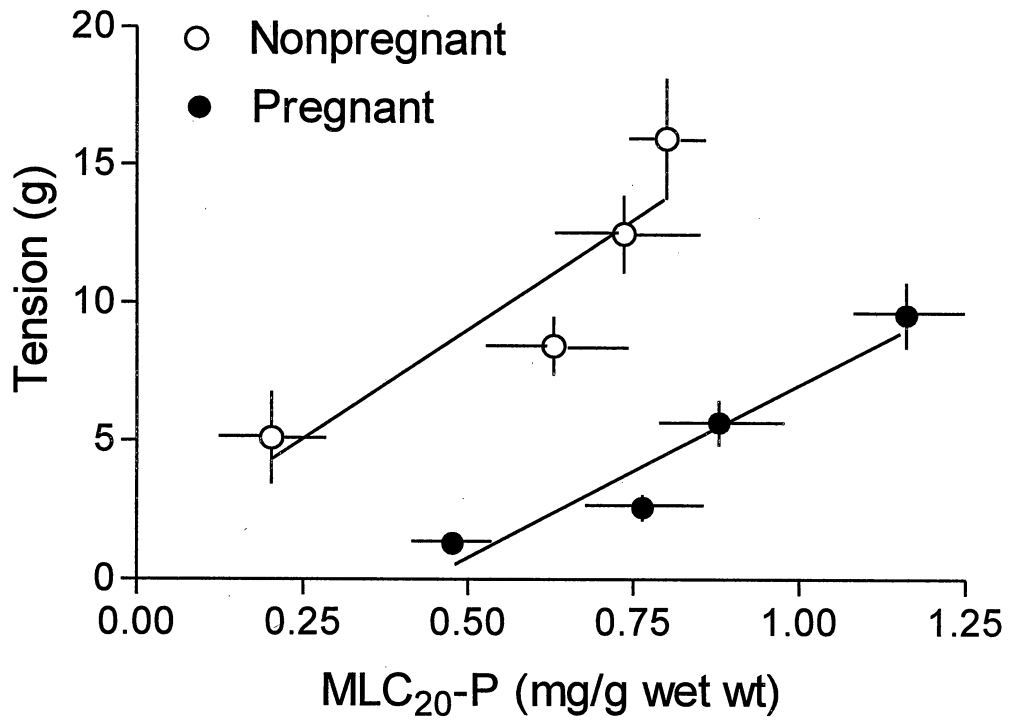
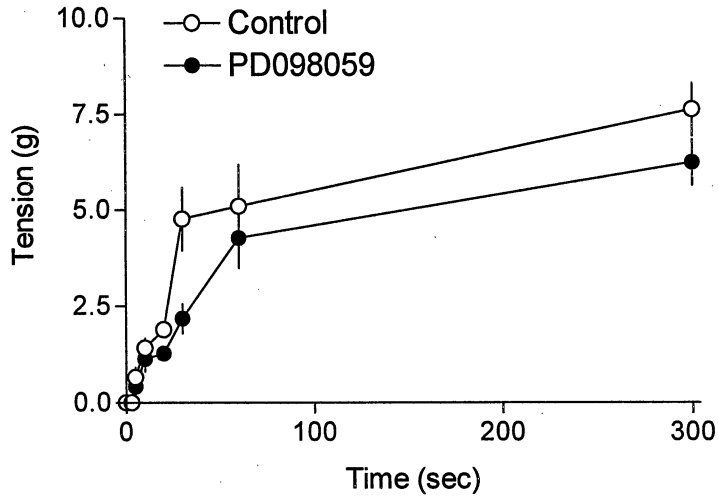
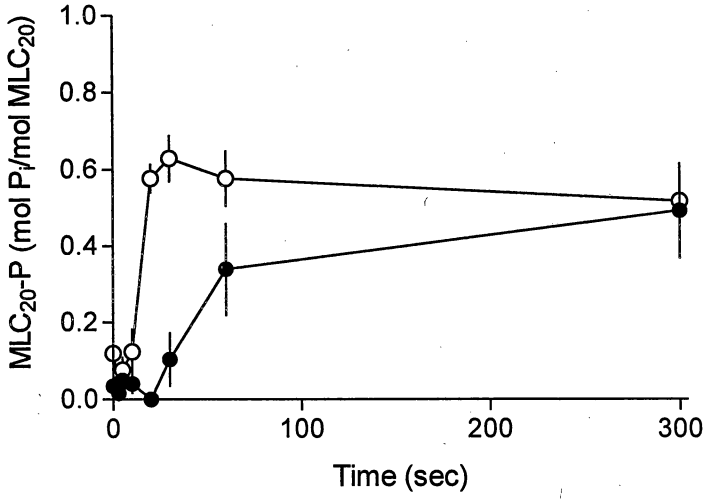

Nonpregnant
Pregnant
Tension (g)
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
1.25
MLC20-P (mg/g wet wt)

Figure 6. Effect of PD098059 on phenylephrine-induced contractions and MLC_{20} phosphorylation in the nonpregnant uterine artery. Arterial rings were pretreated with 30 μM PD098059 or with the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Contractions (panel A) and MLC_{20} phosphorylation (panel B) were measured simultaneously in the uterine artery. Increased tension stimulated by phenylephrine was plotted to show responses as a function of phosphorylated MLC_{20} measured simultaneously in the same tissue at each corresponding time point (panel C). Data are means ± SEM of tissues from 5 animals.


Control
PD098059
Tension (g)
10.0
7.5
5.0
2.5
0.0
0
100
200
300
Time (sec)


MLC20-P (mol Pi/mol MLC20)
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (sec)

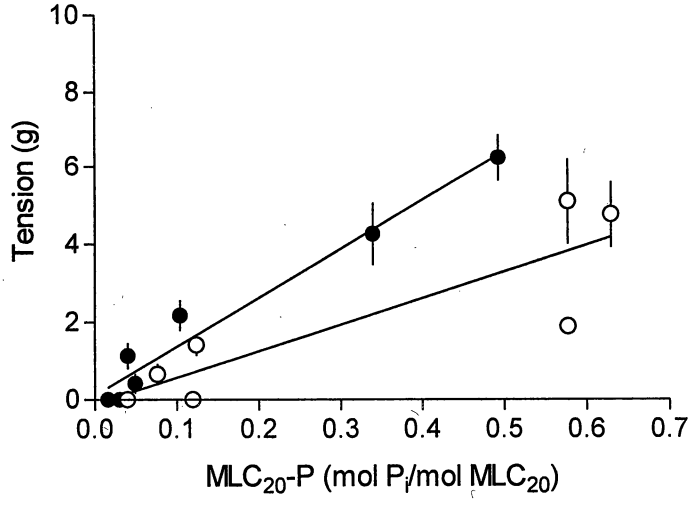
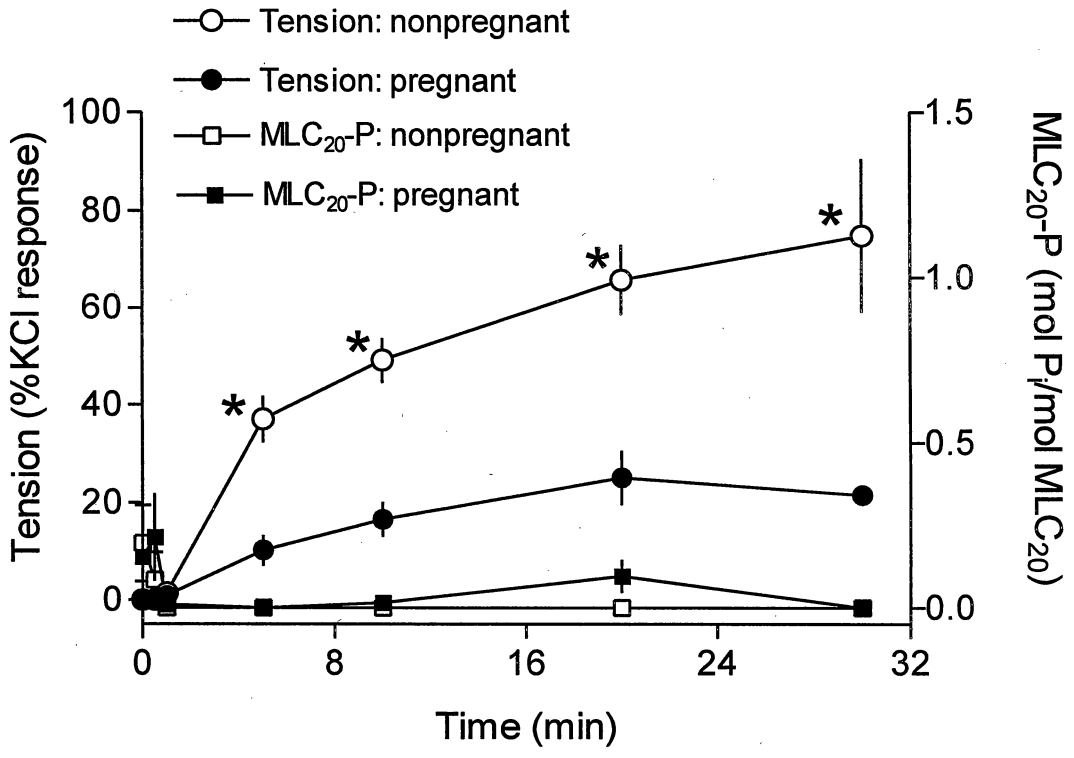

Tension (g)
10
8
6
4
2
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
MLC20-P (mol Pi/mol MLC20)

Figure 7. PDBu-induced contractions and MLC_{20} phosphorylation in the uterine artery. Arterial rings of nonpregnant and pregnant uterine arteries were stimulated with 5 μM PDBu. PDBu-induced contractions and MLC_{20} phosphorylation were measured simultaneously in the same tissues. Data are the means ± SEM of tissues from 4 to 8 animals. * $P < 0.05$, pregnant *vs.* nonpregnant.


Tension: nonpregnant
Tension: pregnant
MLC20-P: nonpregnant
MLC20-P: pregnant
Tension (%KCl response)
MLC20-P (mol Pi/mol MLC20)
Time (min)
0
8
16
24
32
0
20
40
60
80
100
0.0
0.5
1.0
1.5
*
*
*
*

CHAPTER FIVE

α_1-Adrenoceptor-Mediated Phosphorylation of MYPT1 and CPI-17 in the Uterine Artery: Role of ERK/PKC

DaLiao Xiao and Lubo Zhang

This chapter will be submitted for publication to the American Journal of Physiology

Abstract

We have previously demonstrated that ERK/PKC signaling pathways play a key role in the regulation of Ca^{2+} sensitivity and contractility of the uterine artery. The present study tested the hypothesis that ERK and PKC differentially regulated myosin light chain phosphatase activity by phosphorylation of MYPT1 and CPI-17. Agonist-induced contractions and phosphorylation of MYPT1/Thr^{696}, MYPT1/Thr^{850}, and CPI-17/Thr^{38} were measured simultaneously in the same tissues of isolated near-term pregnant ovine uterine arteries. Phenylephrine produced time-dependent concurrent increases in the phosphorylation of $ERK_{44/42}$ and MYPT1/Thr^{850}, which preceded contractions. In addition, phenylephrine induced phosphorylation of CPI-17/Thr^{38} that was concurrent with the contractions. In contrast, phenylephrine did not induce phosphorylation of MYPT1/Thr^{696} in the uterine artery. PD098059 inhibited phosphorylation of $ERK_{44/42}$ and the initial peak phosphorylation of MYPT1/Thr^{850}, but did not affect CPI-17/Thr^{38} phosphorylation. Activation of PKC by PDBu induced a time-dependent phosphorylation of CPI-17/Thr^{38} that preceded the contractions of the uterine artery. In addition, PDBu activated PKCα, and induced a co-immunoprecipitation of PKCα with caldesmon. The results suggest that phosphorylation of MYPT1/Thr^{850} and CPI-17/Thr^{38} play important roles in the regulation of agonist-mediated Ca^{2+} sensitivity in the uterine artery, which are regulated in part by ERK and PKC, respectively. In addition, phosphorylated CPI-17 may regulate the Ca^{2+} sensitivity by interacting with caldesmon and reversing its inhibitory effect on myosin ATPase.

Introduction

Smooth muscle contraction is predominantly regulated by phosphorylation of myosin light chain, which is determined by the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (15,19). Increases in intracellular Ca^{2+} concentrations activate MLCK resulting in phosphorylation of 20 kDa myosin light chain (MLC_{20}) and contractions. However, the force development of smooth muscle is not simply determined by intracellular Ca^{2+} concentrations (21, 35, 41, 58). Ca^{2+} sensitivity of myofilaments also plays an important role in the regulation of smooth muscle contraction. Regulation of Ca^{2+} sensitivity may include at least two components, thick filament regulation (*i.e.* Ca^{2+} sensitivity of MLC_{20} phosphorylation) and thin filament regulation (*i.e.* MLC_{20} phosphorylation-independent contractions). MLCP plays a key role in regulating Ca^{2+} sensitivity of MLC_{20} phosphorylation (13, 27, 28, 46). MLCP consists of three subunits: a 110 to 130 kDa regulatory subunit (MYPT1), an ~37 kDa catalytic subunit (PP1c), and a 20 kDa subunit of unknown function. Inhibition of MLCP is regulated by at least three mechanisms: *a.* phosphorylation of MYPT1, *b.* arachidonic acid-induced dissociation of the holoenzyme, and *c.* PKC-mediated phosphorylation of CPI-17 at Thr^{38}, which becomes a potent inhibitor of PP1c. Two phosphorylation sites of MYPT1 have been identified at Thr^{696} and Thr^{850}.

In addition to the thick filament regulation, Ca^{2+} sensitivity is also regulated by the smooth muscle actin-binding protein caldesmon that inhibits myosin ATPase activity (34). Studies using the antagonist peptide GS17C and the antisense have strongly suggested an important and physiologically relevant role for caldesmon in

suppressing smooth muscle tone (7, 23, 36, 39). Removal of caldesmon-mediated inhibition can be achieved by phosphorylation of caldesmon and/or a binding of other regulatory proteins, e.g. Ca^{2+}/calmodulin, which reverse its inhibitory effect on myosin ATPase.

Recently, we have demonstrated in the pregnant uterine artery that α_1-adrenoceptor-mediated contractions are regulated through both thick and thin filament pathways, with the thick filament regulatory pathway, *i.e.* MLC_{20} phosphorylation, predominating. However, PKC-mediated contractions were regulated predominately through the thin filament pathway, *i.e.* independent of changes in MLC_{20} phosphorylation (55). We have demonstrated that ERK plays an important role in the regulation of α_1-adrenoceptor-mediated contractions of the uterine artery (55,56). Inhibition of ERK by PD098059 significantly decreased the Ca^{2+} sensitivity of MLC_{20} phosphorylation in response to phenylephrine, *i.e.*, less MLC_{20} phosphorylation at a given intracellular Ca^{2+} concentration (55). However, ERK-mediated phosphorylation of the thin filament binding protein caldesmon at Ser^{789} may not lead to the reversal of caldesmon inhibitory effects on actin-activated myosin ATPase in pregnant ovine uterine arteries. In contrast, our previous studies have suggested that in the pregnant uterine artery PKC-induced, thin filament-dependent contractions may be mediated by a direct phosphorylation of caldesmon or by phosphorylation of other regulatory protein(s) that may interact with caldesmon.

In the present study, we determined the temporal relationships between phosphorylation of MYPT1/Thr^{696}, MYPT1/Thr^{850}, and CPI-17/Thr^{38} and contractions induced by phenylephrine, and tested the hypothesis that ERK played a role in inhibition

of MLCP in the uterine artery. Given that CPI-17 is a PKC substrate (8) and phosphorylated CPI-17 may serve as a regulatory binding protein, the present study also determined the temporal relationship between PKC-mediated phosphorylation of CPI-17 and contractions. In addition, we determined the potential direct interaction between PKC and caldesmon by examining the co-immunoprecipitation of PKCα isozyme with caldesmon in the uterine artery.

Methods

Tissue preparation. Near-term 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% to 2.0% halothane in O_2 throughout the surgery. An incision in the abdomen was made and the uterus exposed. The uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution (pH 7.4) of the following composition (in mM): 115.21 NaCl, 4.7 KCl, 1.80 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 22.14 $NaHCO_3$, 0.03 EDTA, and 7.88 dextrose. The Krebs solution was oxygenated with a mixture of 95% O_2-5% CO_2. After the tissues were removed, 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 guidelines in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Contraction Studies. Fourth branches of the main uterine arteries were separated from the surrounding tissue, and cut into 2-mm ring segments. The arterial rings were attached to an isometric force transducer and bathed in the Krebs' solution under 95% O_2-5%CO_2 at 37 °C. Isometric tensions were measured as described previously (56). After 60-min of equilibration in the tissue bath, 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 then stimulated with phenylephrine and/or phorbol 12,13-dibutyrate (PDBu). Tension developed was recorded with an online

computer. At the indicated times, arterial rings were snap-frozen with liquid N_2-cooled clamps, followed by immersion in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM DTT. The rings were stored at -80°C until analysis. In certain experiments, tissues were pretreated with PD098059 (30 μM) or the vehicle (DMSO) for 30 min, followed by stimulation with phenylephrine.

Western immunoblotting analysis. Tissues were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, PH 7.4. Homogenates were then centrifugation at 4 °C for 5 min at 6,000 g and the supernatants were collected. Protein was quantified in the supernatant by the method of Bradford (1976). Samples with equal protein were loaded on 7.5% (MYPT1/Thr^{696} and MYPT1/Thr^{850}) or 10% (CPI-17/Thr^{38} and p-$ERK_{42/44}$) polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS), and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred on to nitrocellulose membrane. Nonspecific binding sites in the membranes were blocked by an overnight incubation at 4 °C in a Tris-buffered saline solution (TBS) containing 5% dry milk. The membranes were incubated with primary antibodies and then with secondary antibodies. 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.

Measurement of PKCα translocation. Uterine arteries were mounted in tissue baths and equilibrated for at least 60 min in the Krebs' solution under 95% O_2-5%CO_2 at 37 °C. The rings were then exposed to PDBu (3 μM) or the vehicle (DMSO) for 30 min.

The uterine artery rings were snap-frozen and homogenized in ice-cold homogenization buffer containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, PMSF 1 mM, β-mercaptoethanol 10 mM, and benzamide 1 mM. The homogenate was centrifuged at 100,000g for 60 minutes at 4 °C, and the supernatant was used as the cytosolic fraction. The pellet corresponding to the membrane particulate fraction was solubilized in a buffer containing Triton X-100 at a final concentration of 0.1% by stirring on ice for 45 minutes at 4 °C, followed by centrifugation at 100,000g for 60 minutes at 4 °C to remove insoluble membrane particles. The supernatant was collected and was referred to as the membrane particulate fraction. Immunoreactive bands for PKCα in cytosolic and particulate fractions were determined by Western blotting using PKCα antibody (1:500; Santa Cruz) as described above.

Immunocytochemistry. Smooth muscle cells were freshly isolated from the uterine arteries using a collagenase-elastase digestion mixture with gentle shaking as described previously (43). After enzyme incubation, tissues were rinsed with the Hanks solution, and dissociated cells were placed over un-siliconized coverslips. The cells were settled by gravity and spontaneously adhered to the glass. Immunocytochemical staining of PKCα in freshly isolated cells was determined as previous described (24). Briefly, after treatment, freshly isolated cells were fixed with 4% paraformaldehyde in phosphate–buffered saline (pH 7.4). The excess fixative was quenched with 0.1% mM glycine in 1% Bovine serum albumin (BSA)-Hanks solution. The cells were then permeabilized with 0.1% Triton X-100 in 1% BSA-Hanks and washed in 1% BSA-Hanks. Cells were blocked with 10% bovine serum in 1% BSA-Hanks, reacted with the PKCα antibody, washed in 1% BSA-Hanks with 0.05% Triton X-100, labeled with a

secondary antibody conjugated with FITC. Cells were then washed in 1% BSA-Hanks to remove excess label. The cell nuclei were stained with Hoechest 33258. Cells were then examined using a fluorescent microscope with the SPOT digital camera.

Co-immunoprecipitation. Co-immunoprecipitation of PKCα and caldesmon was determined as described previously (33). Tissues were homogenized in a buffer containing 50 mM Tris, pH 7.4, 10% glycerol, 5 mM EGTA, 140 mM NaCl, 1% Triton, and the protease inhibitors leupeptin and pepstatin (5.5 μM), aprotinin 20 kallikrein-inactivating unit, 1 mM Na_3VO_4, 10 mM NaF, 0.25% (w/v) sodium deoxycholate, 100 μM $ZnCl_2$, 20 mM β-glycerophosphate, and 2 μM PMSF. After centrifugation, the protein concentration was determined using the Bradford method (BioRad). An equal amount of protein was incubated with the caldesmon antibody overnight at 4 °C with gentle rotation. Then, protein A/G PLUS-agarose (Santa Cruz) was added and incubated for an additional 2 hours. Afterward, beads were washed extensively with the homogenization buffer and immune complex was eluted in SDS-PAGE sample buffer. Total immune complex samples and protein samples from total tissue homogenates were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with the antibody against PKCα. After washing and incubating with secondary antibody, immunoreactive proteins were visualized with the ECL detection system.

Materials. Phenylephrine, PDBu, and PD098059 were obtained from Sigma (St. Louis, MO). Phosphorylated MYPT1/Thr^{696} and MYPT1/Thr^{850} antibodies and caldesmon antibody were obtained from Upstate (Lake Placid, NY). Phosphorylated $ERK_{42/44}$ (Thr^{202}/Tyr^{204}) antibodies were from Cell Signaling Technology (Berverly, MA). Phosphorylated CPI-17/Thr^{38} antibody and PKCα antibody were from Santa Cruz

Biotechnology (santa Cruz, CA). All electrophoretic and immunoblot reagents were from Bio-Rad. General laboratory reagents were of analytical grade or better and were purchased from Sigma and Fisher Scientific. All drugs were prepared fresh each day and kept on ice throughout the experiment.

Data analysis. Data were analyzed using GraphPad Prism (GraphPad software; San Diego, CA). Values are means ± SEM. Differences were evaluated for statistical significance ($P < 0.05$) by two-way ANOVA and Student's *t*-test.

Results

Phenylephrine-induced contractions and phosphorylation of ERK, MYPT1, and CPI-17. Fig. 1 shows temporal relationships between phenylephrine-induced contractions and phosphorylation of ERK, MYPT1/Thr^{850} and CPI-17/Thr^{38}, measured simultaneously in the same tissues of pregnant uterine arteries. Phenylephrine produced time-dependent and concurrent increases in phosphorylation of ERK and MYPT1/Thr^{850} that preceded the contractions. Phenylephrine-induced phosphorylation of CPI-17/Thr^{38} trailed phosphorylations of ERK and MYPT1/Thr^{850}, but was concurrent with the contractions.

Effect of PD098059 on phenylephrine-induced phosphorylation of $ERK_{44/42}$. Our previous studies (55) have demonstrated that PKC activation stimulates a time-dependent increase in $ERK_{44/42}$ phosphorylation in the pregnant uterine artery, which is blocked by PD098059, the MEK/ERK inhibitor. As shown in Fig. 2, phenylephrine-induced time-dependent increases in phosphorylation of both ERK_{44} and ERK_{42} were also blocked by PD098059.

Effect of PD098059 on phenylephrine-induced phosphorylation of MYPT1/Thr^{850} and MYPT1/Thr^{696}. Previous studies suggested a differential regulation of phosphorylation of MYPT1/Thr^{850} and MYPT1/Thr^{696} in smooth muscle. Agonists stimulated phosphorylation of MYPT1/Thr^{850} but not MYPT1/Thr^{696} (46). Basal phosphorylation levels of MYPT1/Thr^{696} were detected in the uterine artery (Fig. 3). Consistent with previous studies, phenylephrine did not increase MYPT/Thr^{696} phosphorylation in the uterine artery (Fig. 3). This was not altered by inhibition of ERK

with PD098059. In contrast, phenylephrine induced a time-dependent increase in phosphorylation of MYPT1/Thr^{850}, which was blocked in the presence of PD098059 (Fig. 4).

Effect of PD098059 on phenylephrine-induced phosphorylation of CPI-17/Thr^{38}. Phenylephrine produced a time-dependent increase in phosphorylation of CPI-17 in the pregnant uterine artery (Fig. 5). PD098059 showed a tendency to decrease phenylephrine-induced CPI-17 phosphorylation, which did not reach a statistically significant level (Fig. 5).

PDBu-induced contraction and phosphorylation of CPI-17. Given that CPI-17 is a PKC substrate and phosphorylated CPI-17 plays an important role in the regulation of Ca^{2+} sensitivity of myofilaments, we determined the temporal relationship between PDBu-induced phosphorylation of CPI-17/Thr^{38} and contractions measured simultaneously in the same tissues. As shown in figure 6, PDBu produced a time-dependent increase in phosphorylation of CPI-17/Thr^{38} that preceded the contractions.

PDBu-induced translocation of PKCα isozyme. We have previously demonstrated that PDBu increases particulate-to-cytosolic PKC activity ratio in the uterine artery, which is blocked by staurosporine (56). To determine the isozyme involved in PDBu-stimulated PKC activity, we determined the effect of PDBu on the distribution of PKCα in cytosolic and particulate fractions. The tissues were pretreated with 0.1 μM staurosporine for 30 min before the addition of PDBu (3 μM for 30 min). PKCα levels in the cytosolic and particulate fractions were determined by Western blotting using the PKCα antibody. Fig. 7 shows that PDBu significantly increased particulate-to-cytosolic PKCα ratio in the uterine artery, which was blocked by

staurosporine. To confirm the translocation of PKCα was from cytosol to cell plasma membrane, we examined the immunohistochemical staining of PKCα in freshly isolated smooth muscle from the uterine artery. As shown in Fig. 8, in the resting cells, PKCα was distributed in the cytosol. PDBu induced a translocation of PKCα from cytosol to plasma membrane, which was accompanied by contractions of the cells.

Co-immunoprecipitation of PKCα and caldesmon. Our recent studies have suggested that PKC may directly phosphorylate caldesmon in the uterine artery (55). The direct interaction of PKCα and caldesmon was determined by co-immunoprecipitation of PKCα and caldesmon. As shown in Fig. 9, PKCα was detected in the caldesmon immunoprecipitates in PDBu-stimulated tissues, but not in the control, suggesting a direct interaction between the activated PKCα with caldesmon in the uterine artery.

Discussion

The main findings of the present study are as follows: 1) phenylephrine induces phosphorylation of ERK and MYPT1/Thr^{850} that precedes the contractions, 2) phenylephrine-induced phosphorylation of MYPT1/Thr^{850} is mediated in part by activation of ERK, 3) phenylephrine produces concurrent phosphorylation of CPI-17/Thr^{38} and contractions, and the phosphorylation of CPI-17/Thr^{38} is not mediated by ERK activation, 4) phenylephrine does not increase phosphorylation of MYPT1/Thr^{696}, 5) PDBu activates PKCα isozyme and induces a time-dependent increase in CPI-17/Thr^{38} phosphorylation that precedes the contractions, 6) activated PKCα co-immunoprecipitates with the actin-binding protein caldesmon, in the uterine artery.

It has been demonstrated in many studies that different agents, which produce smooth muscle contraction, activate ERK at the same time (2, 5, 11, 12, 53). Recently, we have shown that the MEK/ERK inhibitor PD098059 selectively inhibits phenylephrine-induced contractions in the pregnant, but not in nonpregnant, uterine arteries, suggesting that pregnancy enhances the role of ERK in α_1-adrenoceptor-mediated contractions in the uterine artery (56). In the present study, we found that phenylephrine produced a time-dependent increase in phosphorylation of both ERK_{44} and ERK_{42}, which was inhibited by PD098059. This supports a role ERK in the regulation of α_1-adrenoceptor-mediated contractions in the uterine artery. Different signaling transduction mechanisms have been reported in the ERK-mediated regulation of smooth muscle contraction. Previous studies have shown that PD098059 does not affect agonist-induced $[Ca^{2+}]_i$ in isolated smooth muscle cells but inhibits agonist-induced smooth

muscle contraction by decreasing Ca^{2+} sensitivity of contractile proteins (1, 2, 5, 12, 17). Our previous studies have also demonstrated that inhibition of ERK by PD098059 significantly decreases the Ca^{2+} sensitivity of MLC_{20} phosphorylation in response to phenylephrine, *i.e.*, less MLC_{20} phosphorylation at a given intracellular Ca^{2+} concentration in the uterine artery (55). It has been proposed that ERK mediates smooth muscle contraction through the thin filament regulatory pathway by phosphorylation of caldesmon (2, 17, 34). However, our recent studies have demonstrated that in the pregnant uterine artery phosphorylation of ERK-specific site Ser^{789} of caldesmon may not lead to the reversal of caldesmon inhibitory effect on myosin ATPase. Instead, phosphorylation of caldesmon/Ser^{789} may stabilize its inhibitory effect on actin-activated myosin ATPase (55).

In the present study, we have demonstrated that phosphorylation of MYPT1/Thr^{850} precedes phenylephrine-mediated contractions. The finding that MYPT1/Thr^{850} consisted of two bands in the present study is in agreement with previous findings. It has been shown that MYPT1 in smooth muscle consists of two isoforms and bands, but often the separation is not distinct (42, 54). The present finding that the time course of MYPT1/Thr^{850} phosphorylation resembled that of phenylephrine-induced MLC_{20} phosphorylation (55) and preceded contractions suggests that phenylephrine-mediated Ca^{2+} sensitization is partly regulated though phosphorylation of MYPT1/Thr^{850} resulting in an inhibition of MLCP activity in the uterine artery. Although MYPT1/Thr^{850} was originally shown not an inhibitory site *in vitro* (9), recent study demonstrated that its phosphorylation state might affect localization of MLCP and its catalytic subunit on myosin (51). The present finding that PD098059 blocked

both ERK and MYPT1/Thr^{850} phosphorylation induced by phenylephrine suggests that ERK-mediated regulation of Ca^{2+} sensitivity of MLC_{20} phosphorylation is mediated in part though MYPT1/Thr^{850} phosphorylation in the uterine artery. It has been shown that Rho-kinase (ROK) is activated in response to G-protein activation, and is responsible for the phosphorylation of MYPT1/Thr^{850} and inhibition of MLCP (9, 25, 26). ROK is activated by the Ras-related monomeric GTPase, Rho, which increases force at constant intracellular Ca^{2+} concentrations (14). However, the precise mechanism of ROK activation that leads to phosphorylation of MYPT1 in smooth muscle is not yet known. There are concerns as to the access of ROK to its substrate MYPT1 because both Rho and ROK have to be recruited from a cytosolic pool to the cell membrane for activation (10, 49). The present finding suggests that ERK may be a link between activated ROK and phosphorylation of MYPT1 in the uterine artery. Consistent with the present finding, previous studies demonstrated that RhoA was involved in the Angiotensin II–induced ERK activation and contractions in intact rat mesenteric resistance arteries (32). In addition, Krepinsky et al. (30) also reported that the early activation of RhoA was essential for stretch-induced ERK activation in mesangial cells.

At least two phosphorylation sites of MYPT1 have been identified at Thr^{696} and Thr^{850} (46). The present finding that phenylephrine did not increase phosphorylation of MYPT1/Thr^{696} in the uterine artery is consistent with previous findings that MYPT1/Thr^{850} is phosphorylated by Rho-kinase through agonist activation, and MYPT1/Thr^{696} is constitutively phosphorylated, by kinases other than Rho-kinase, and dose not respond to agonists (26, 37, 46). These findings suggest an exciting hypothesis that the different phosphorylation sites may regulate MLCP activities differently at

basal and agonist-stimulated states. Basal levels of phosphorylated MYPT1/Thr^{696} have been demonstrated in the uterine artery in the present study, yet the mechanisms involved in the regulation of MYPT1/Thr^{696} in the uterine artery is not clear at present.

The finding that phenylephrine-induced phosphorylation of CPI-17 was concurrent with the contractions suggests that phosphorylated CPI-17 may also be important in phenylephrine-mediated Ca^{2+} sensitization of myofilaments. Previous studies suggested that phosphorylated CPI-17/Thr^{38} was a potent inhibitor of catalytic subunit (PP1c) of MLCP (46). However, the finding that phenylephrine-induced CPI-17 phosphorylation trailed phenylephrine-induced MLC_{20} phosphorylation (55) suggests that it may not be important in the regulation of Ca^{2+} sensitivity of MLC_{20} phosphorylation in the uterine artery. This is further supported by the results of PKC-mediated responses in the uterine artery. In the present study, we found that activation of PKC by PDBu induced phosphorylation of CPI-17 that preceded the contractions. CPI-17 was initially recognized as a PKC substrate and phosphorylated by PKC (8). However, interestingly, our previous studies have demonstrated that activation PKC produces contractions without changes in MLC_{20} phosphorylation levels in the uterine artery (55). These findings suggest that PKC-mediated phosphorylation of CPI-17 may not contribute to regulation of MLCP activity and MLC_{20} phosphorylation in the uterine artery. Woodsome et al. (54) reported that PDBu significantly increased both CPI-17/Thr^{38} phosphorylation and MLC_{20} phosphorylation in femoral artery, but increased CPI-17/ Thr^{38} phosphorylation without increase in MLC_{20} phosphorylation in vas deferens smooth muscle. These studies suggest that the role of phosphorylated CPI-17 in the regulation of MLCP activity and Ca^{2+} sensitivity of MLC_{20} phosphorylation is tissue-

specific. It is not clear whether or how phosphorylated CPI-17 regulates Ca^{2+} sensitivity in the uterine artery. In addition to the thick filament regulation through MLCP, Ca^{2+} sensitivity is also regulated by the smooth muscle actin-binding protein caldesmon that inhibits myosin ATPase activity (34). Remove of caldesmon-mediated inhibition can be achieved by phosphorylation of caldesmon and/or a binding of other regulatory proteins, e.g. Ca^{2+}/calmodulin, which reverse its inhibitory effect on myosin ATPase. Given that phosphorylated CPI-17 is a regulatory binding protein and binds to target proteins, e.g. the catalytic subunit of MLCP, it is speculated that it may regulate Ca^{2+} sensitivity in the uterine artery by binding to caldesmon and reversing its inhibitory effect on myosin ATPase.

In the present study, we demonstrated that PDBu activated PKCα isozyme in the uterine artery by stimulating its translocation from cytosol to cell plasma membranes. This is consistent with our previous finding that PDBu increased particulate-to-cytosolic PKC activity ratio in the uterine artery (56). PKCα has been implicated in smooth muscle contractions, and is involved in the regulation of myogenic tone of vascular smooth muscle (6, 57). Previous studies have demonstrated that activation of PKC may increase phosphorylation of caldesmon at sites other than ERK-dependent phosphorylation sites (47, 50, 52). In sheep aorta, PKC phosphorylated caldesmon both in native thin filaments and in the isolated state at multiple sites of Ser^{127}, Ser^{587}, Ser^{600}, Ser^{657}, Ser^{686} and Ser^{726}, decreased caldesmon's inhibitory effect on myosin ATPase (52). Our recent studies in pregnant ovine uterine arteries suggested that PKC induced phosphorylation of caldesmon at Ser^{789} indirectly through activation of ERK, as well as at other site(s) through unknown mechanisms (55). It is the phosphorylation of

caldesmon at site(s) other than Ser^{789} that may be important in reversing the inhibitory effect of caldesmon on myosin ATPase and leading to contractions. In the present study, we demonstrated a co-immunoprecipitation of activated PKCα with caldesmon, indicating a direct interaction between the activated PKCα with caldesmon in the uterine artery. This provides a support for PKC-mediated direct phosphorylation of caldesmon in the uterine artery.

In summary, we have demonstrated in pregnant ovine uterine arteries that α_1-adrenoceptor-mediated Ca^{2+} sensitization of MLC_{20} phosphorylation is regulated in part through the ERK-dependent phosphorylation of MYPT1/Thr^{850}. α_1-Adrenoceptor- and PKC-mediated phosphorylation of CPI-17/Thr^{38} may not be involved in the regulation of MLCP activity and MLC_{20} phosphorylation. The potential role of phosphorylated CPI-17/Thr^{38} in the regulation of Ca^{2+} sensitivity through the thin filament regulation in the uterine artery presents an intriguing avenue for future investigation. The present study has also demonstrated a role of PKCα isozyme in the PKC-mediated responses, and suggested a role of PKCα in a direct phosphorylation of caldesmon in the uterine artery.

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Figure 1. Time courses of phenylephrine-induced phosphorylation of ERK_{44}, MYPT1/Thr^{850}, CPI-17/Thr^{38} and contractions in the uterine artery. Uterine arterial rings from pregnant sheep were stimulated with 3 μM phenylephrine. Contractile responses were terminated at the various time points indicated, and phosphorylated levels of ERK_{44}, MYPT1/Thr^{850} and CPI-17/Thr^{38} were determined in the same tissue by Western blotting. Data are means ± SEM of tissues from 4 animals.

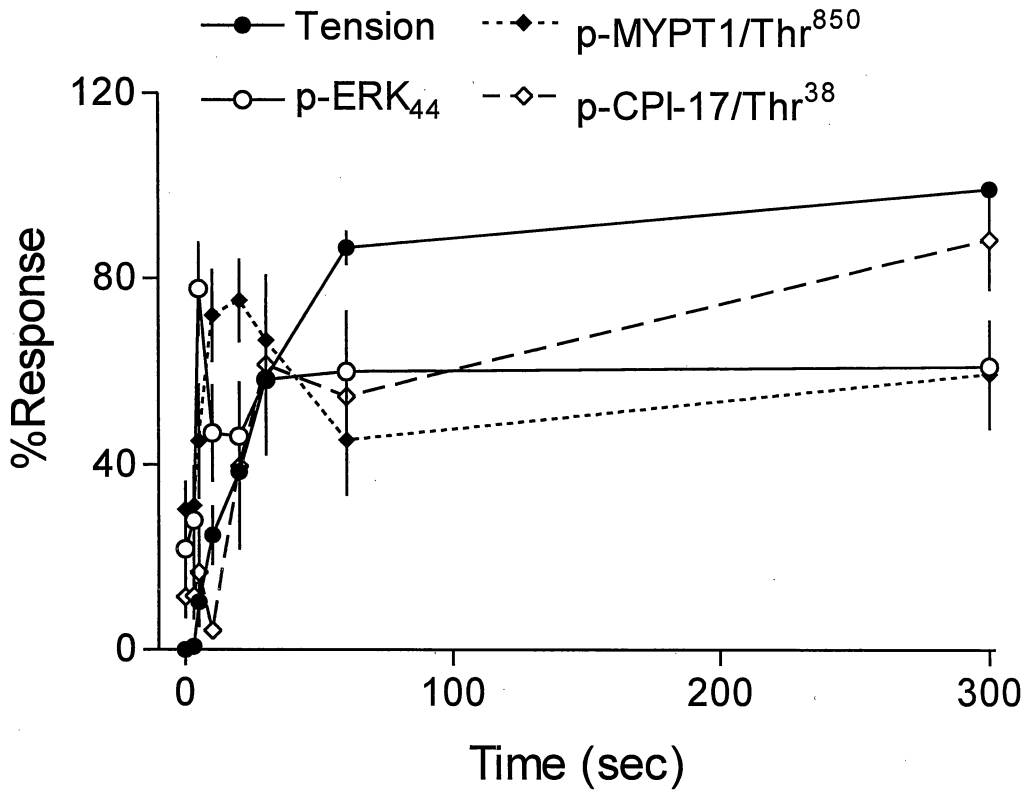
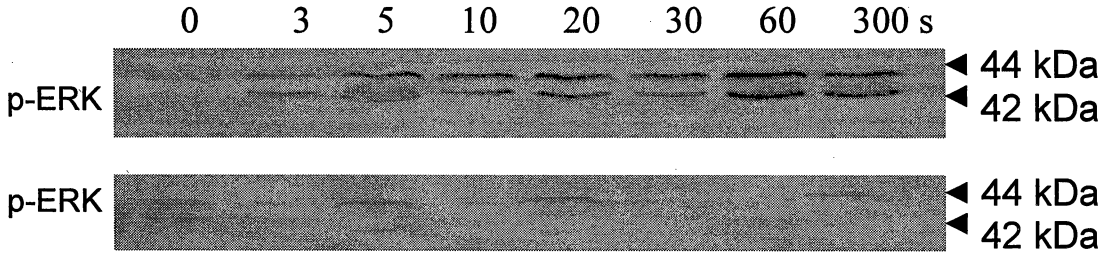
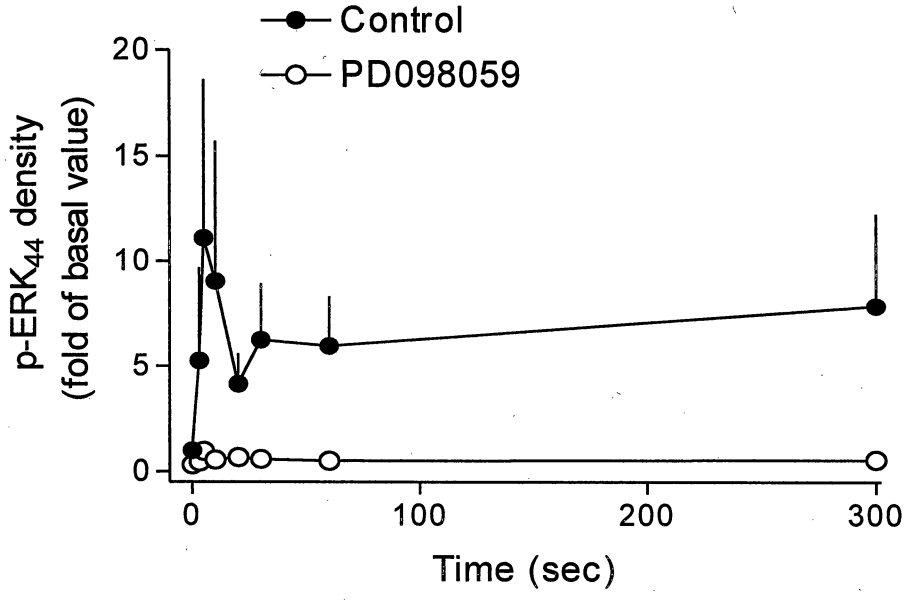

Tension
p-MYPT1/Thr850
p-ERK44
p-CPI-17/Thr38
120
80
40
0
%Response
0
100
200
300
Time (sec)

Figure 2. Effect of PD098059 on phenylephrine-induced phosphorylation of ERK $_{44/42}$ in the uterine artery. Uterine arterial rings from pregnant sheep were pretreated with 30 μM PD098059 or the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Representative Western immunoblots show phosphorylated $ERK_{44/42}$ induced by phenylephrine in the absence or presence of PD098059 at 0-300 s. Data are means ± SEM of tissues from 6 animals. Two-way ANOVA analysis indicates a significant difference between control and PD098059 treated samples ($P < 0.05$).


0
3
5
10
20
30
60
300 s
p-ERK
44 kDa
42 kDa
p-ERK
44 kDa
42 kDa


Control
PD098059
p-ERK44 density
(fold of basal value)
20
15
10
5
0
0
100
200
300
Time (sec)

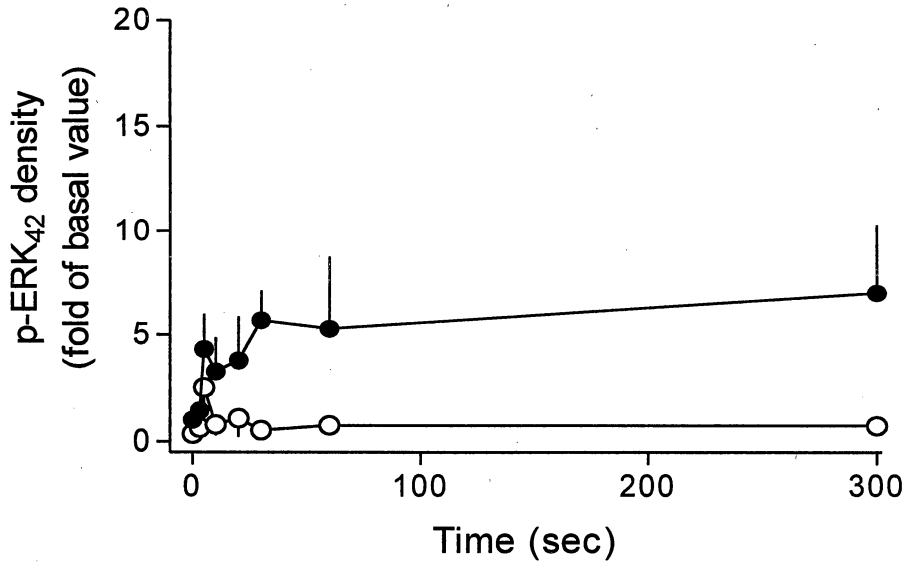
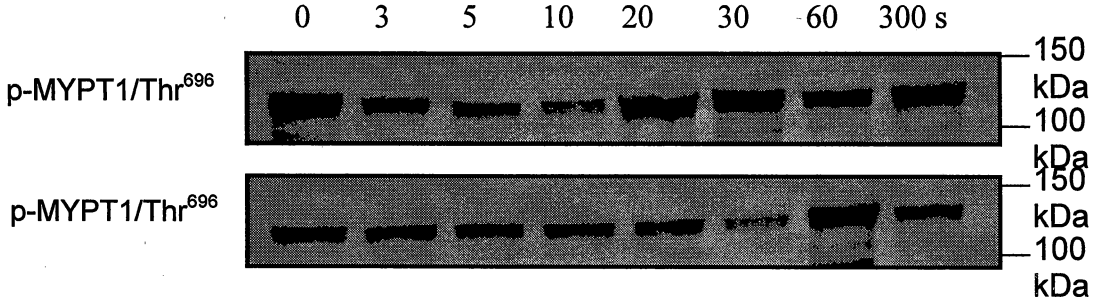

p-ERK42 density
(fold of basal value)
20
15
10
5
0
0
100
200
300
Time (sec)

Figure 3. Effect of PD098059 on phenylephrine-induced phosphorylation of MYPT1/Thr^{696} in the uterine artery. Uterine arterial rings from pregnant sheep were pretreated with 30 μM PD098059 or the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Representative Western immunoblots show phosphorylated MYPT1/Thr^{696} induced by phenylephrine in the absence or presence of PD098059 at 0-300 s. Data are means ± SEM of tissues from 3 animals.


0
3
5
10
20
30
60
300 s
p-MYPT1/Thr696
150 kDa
100 kDa
p-MYPT1/Thr696
150 kDa
100 kDa

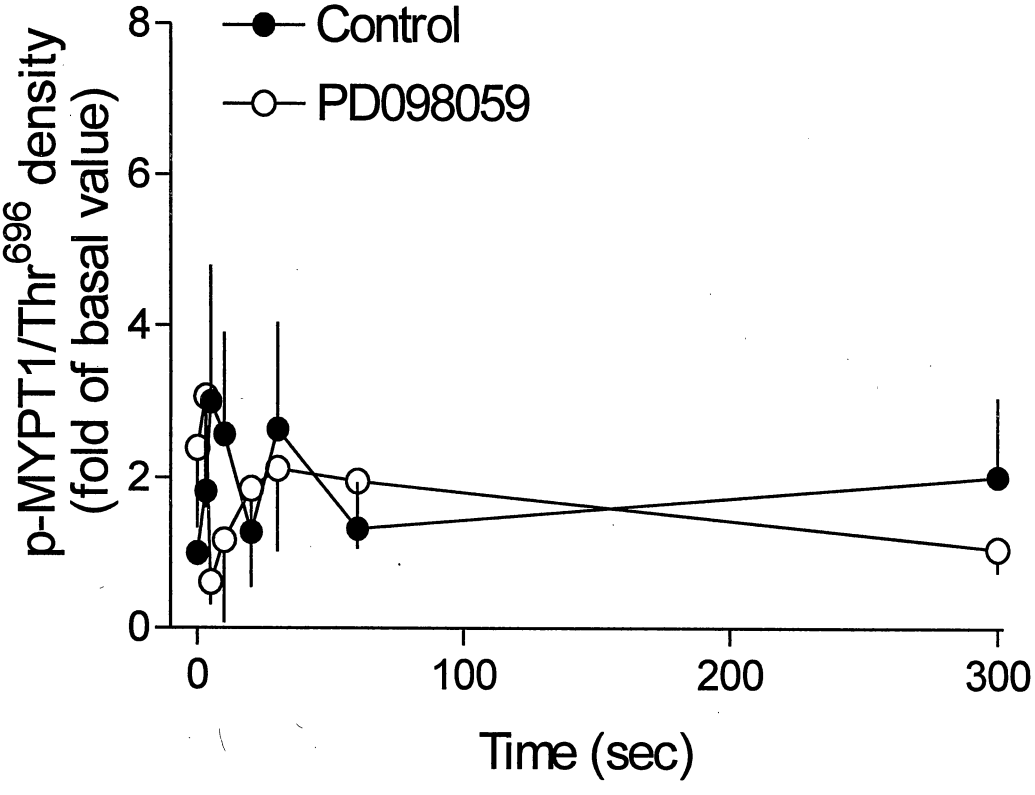
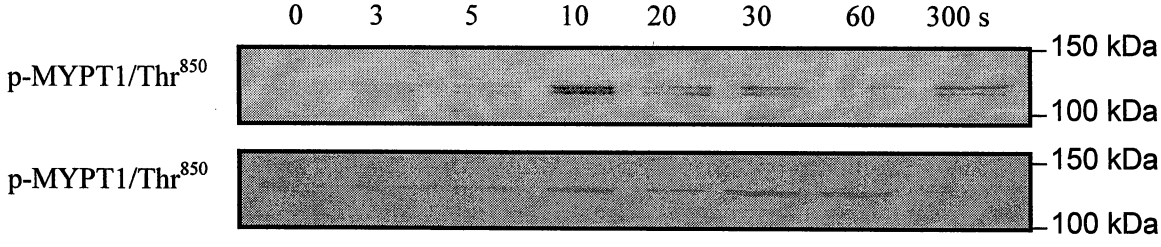

Control
PD098059
p-MYPT1/Thr696 density (fold of basal value)
8
6
4
2
0
0
100
200
300
Time (sec)

Figure 4. Effect of PD098059 on phenylephrine-induced phosphorylation of MYPT1/Thr^{850} in the uterine artery. Uterine arterial rings from pregnant sheep were pretreated with 30 μM PD098059 or the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Representative Western immunoblots show phosphorylated MYPT1/Thr^{850} induced by phenylephrine in the absence or presence of PD098059 at 0-300 s. Data are means ± SEM of tissues from 6 animals. Two-way ANOVA analysis indicates a significant difference between control and PD098059 treated samples ($P < 0.05$).


0
3
5
10
20
30
60
300 s
p-MYPT1/Thr850
150 kDa
100 kDa
p-MYPT1/Thr850
150 kDa
100 kDa

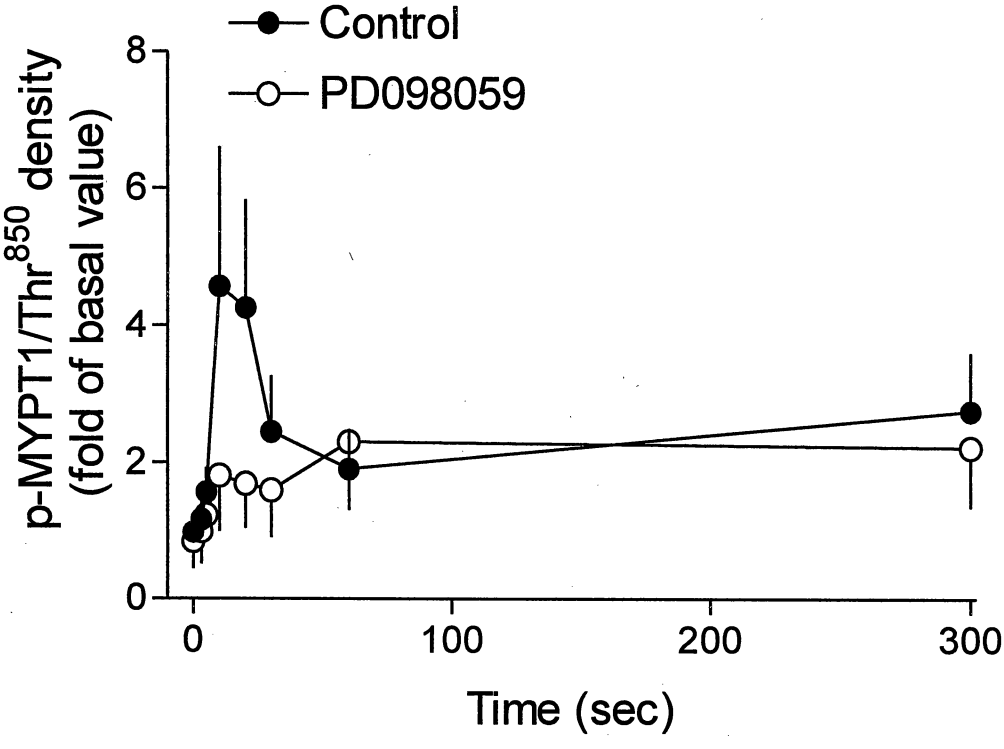
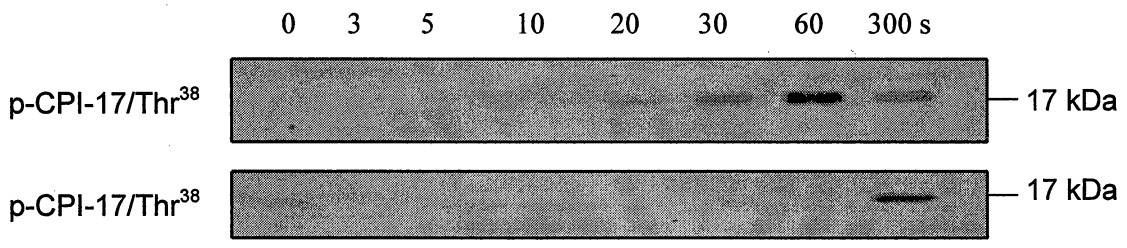

Control
PD098059
p-MYPT1/Thr850 density
(fold of basal value)
0
2
4
6
8
0
100
200
300
Time (sec)

Figure 5. Effect of PD098059 on phenylephrine-induced phosphorylation of CPI-17/Thr^{38} in the uterine artery. Uterine arterial rings from pregnant sheep were pretreated with 30 μM PD098059 or the vehicle DMSO (control) for 30 min, followed by stimulation with 3 μM phenylephrine. Representative Western immunoblots show phosphorylated CPI-17/Thr^{38} induced by phenylephrine in the absence or presence of PD098059 at 0-300 s. Data are means ± SEM of tissues from 4 to 5 animals. Two-way ANOVA analysis indicates no significant difference between control and PD098059 treated samples ($P > 0.05$).


0 3 5 10 20 30 60 300 s
p-CPI-17/Thr38
17 kDa
p-CPI-17/Thr38
17 kDa

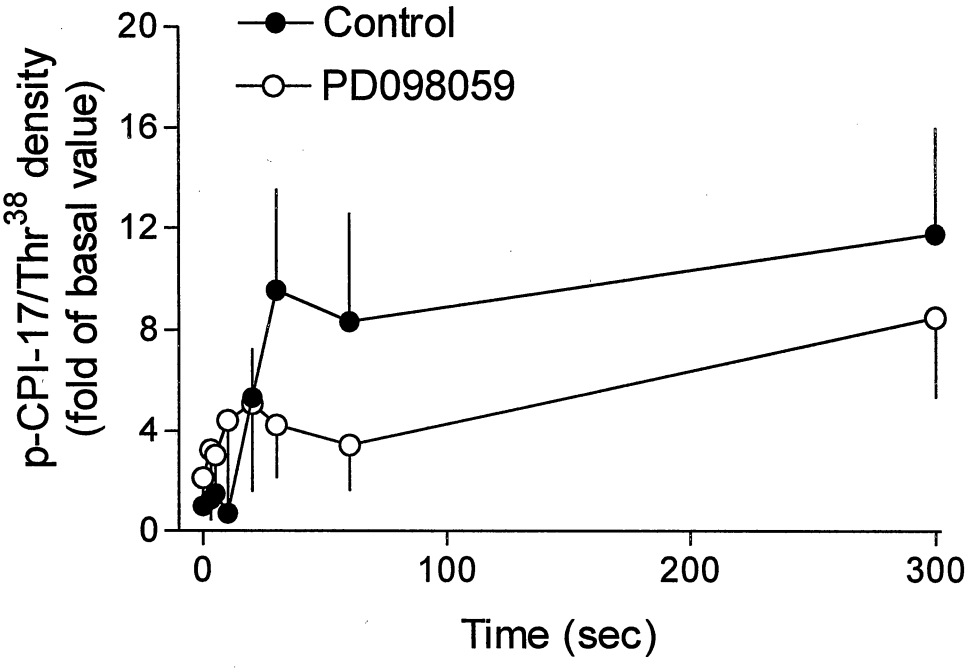

Control
PD098059
p-CPI-17/Thr38 density
(fold of basal value)
20
16
12
8
4
0
0
100
200
300
Time (sec)

Figure 6. Time courses of PDBu-induced phosphorylation of CPI-17/Thr^{38} and contractions in the uterine artery. Uterine arterial rings from pregnant sheep were stimulated with 5 μM PDBu. Contractile responses were terminated at the various time points indicated, and phosphorylated levels of CPI-17/Thr^{38} were determined in the same tissue by Western blotting. Data are means ± SEM of tissues from 5 animals.

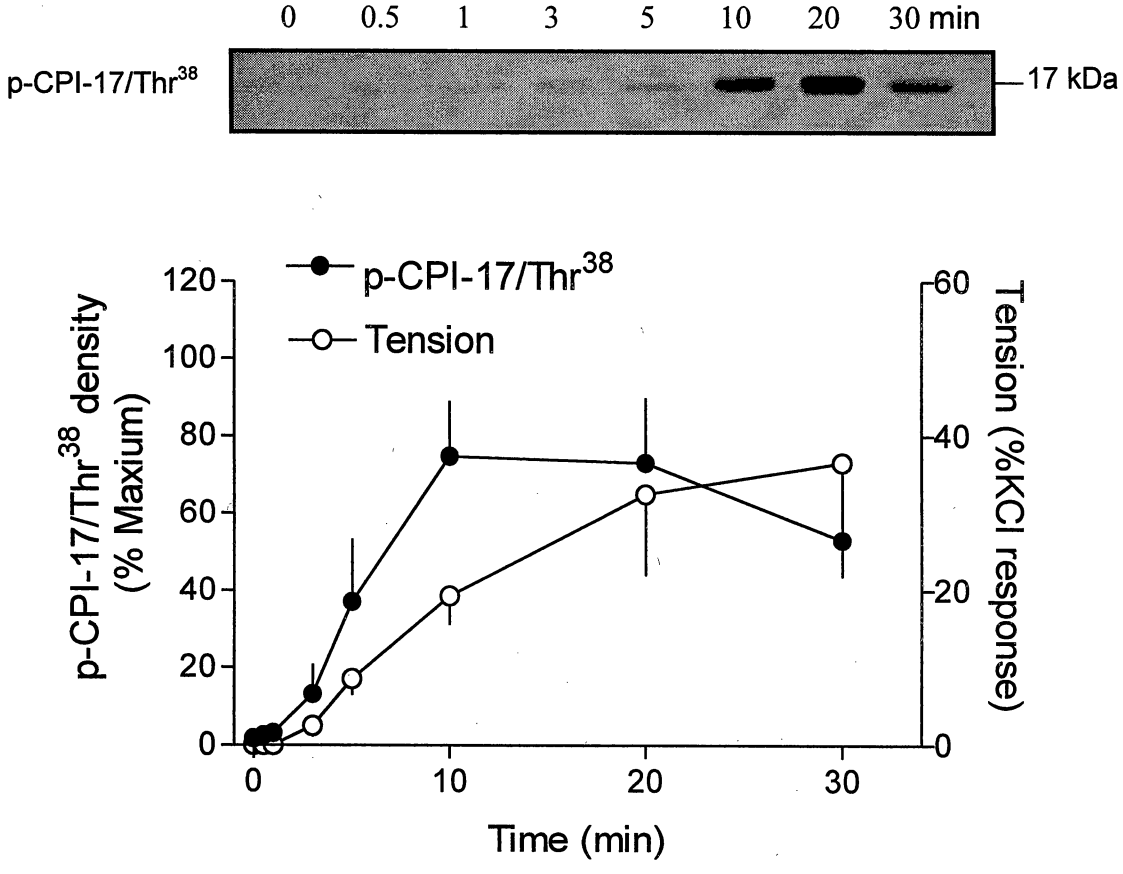

0
0.5
1
3
5
10
20
30 min
p-CPI-17/Thr38
17 kDa
p-CPI-17/Thr38
Tension
p-CPI-17/Thr38 density
(% Maxium)
Tension (%KCl response)
120
100
80
60
40
20
0
60
40
20
0
0
10
20
30
Time (min)

Figure 7. PDBu-induced PKCα translocation in the uterine artery. Uterine arterial rings from pregnant sheep were pretreated in the absence or presence of 0.1 μM staurosporine (Stau) for 30 min, followed by stimulation with 3 μM PDBu for 30 min. Representative Western immunoblots show PKCα determined by Western blotting in the cytosol and membrane particulate fractions. Data are means ± SEM of tissues from 5 animals. * $P < 0.05$, *vs.* control.

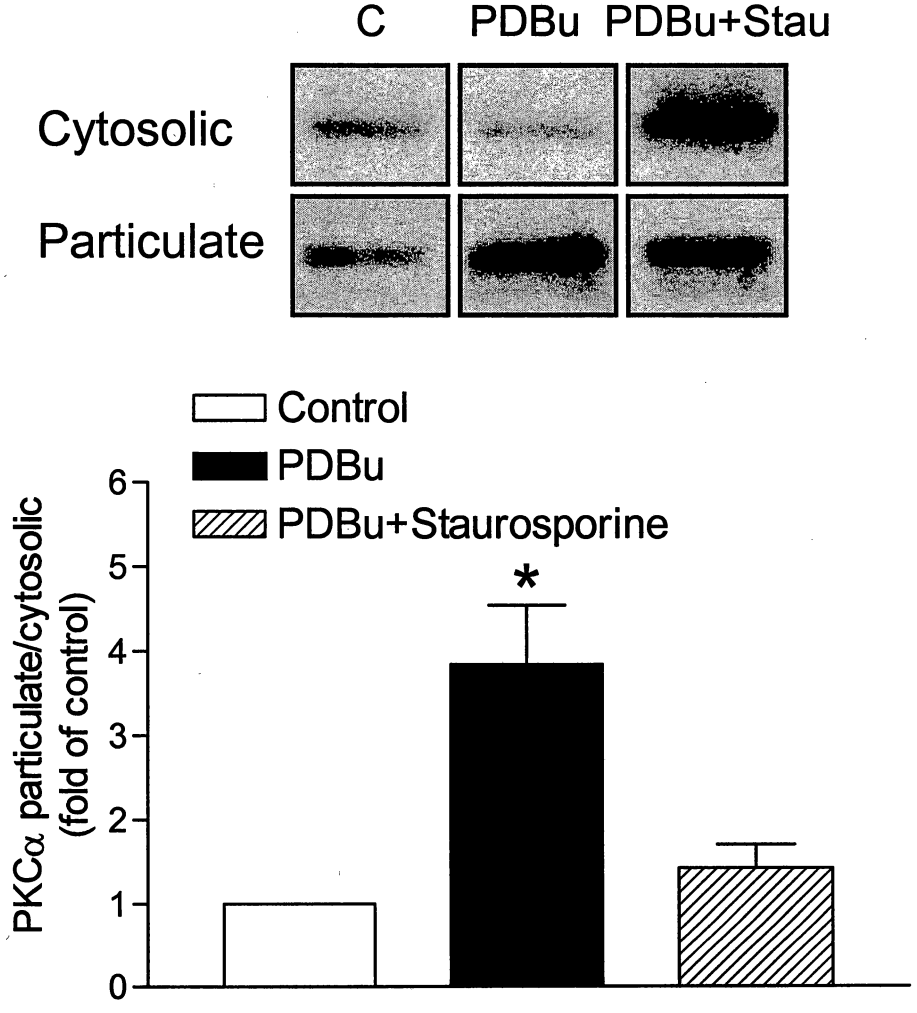

C
PDBu
PDBu+Stau
Cytosolic
Particulate
Control
PDBu
PDBu+Staurosporine
PKCα particulate/cytosolic
(fold of control)
0
1
2
3
4
5
6
*

Figure 8. PDBu-induced redistribution of PKCα in uterine artery smooth muscle cells. Freshly isolated smooth muscle cells from pregnant uterine arteries were treated with PDBu (5 μM) or vehicle control for 20 min. After fixation, cells were labeled with anti-PKCα antibody as described in Methods. Representative fluoro-microscopy images show the cells stained for PKCα (green) and nuclei (blue). At resting condition, uterine artery smooth muscle cells showed typical elongated spindle shape, and a relatively homogeneous cytosolic staining for PKCα. PDBu stimulated a translocation of PKCα from cytosol to the cell plasma membrane, which was accompanied with a shortening of smooth muscle cells. The result was confirmed by three experiments.

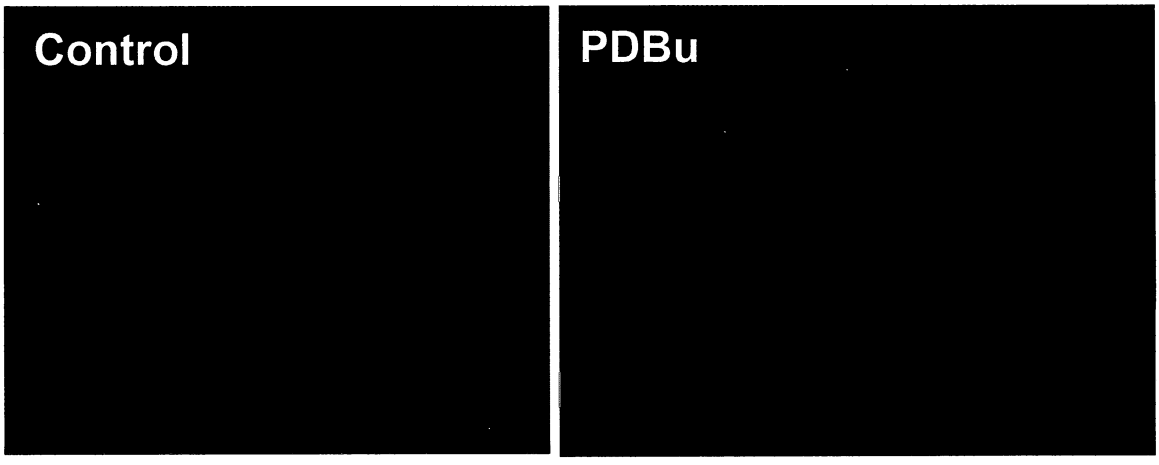
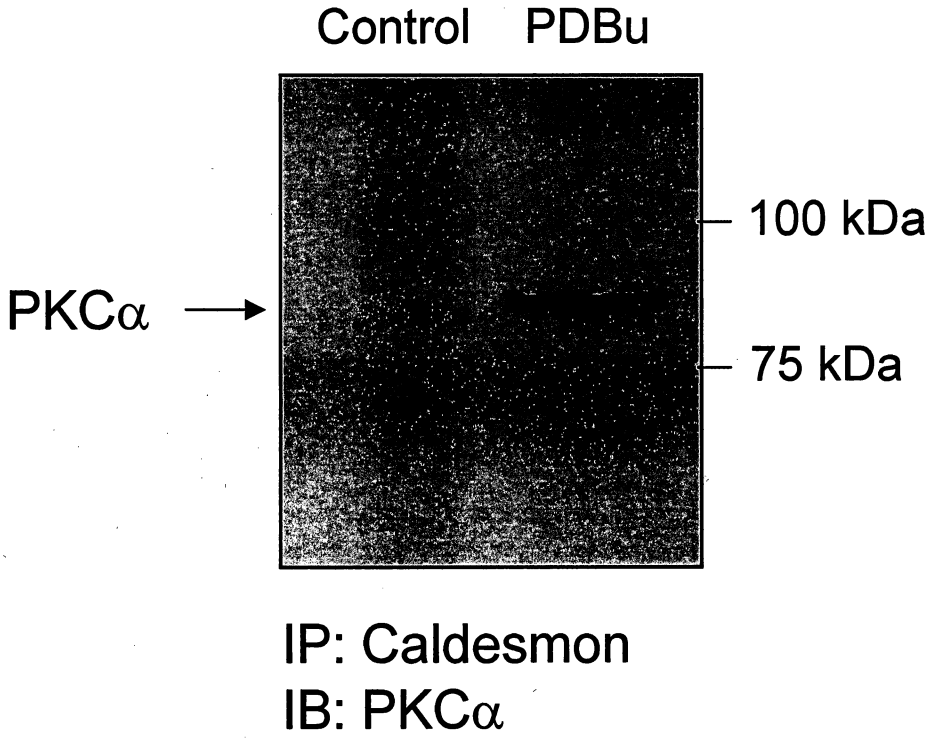

Control
PDBu

Figure 9. Co-immunoprecipitation of PKCα and caldesmon in the uterine artery. Uterine arterial rings from pregnant sheep were treated with 5 μM PDBu or the vehicle (control) for 5 min. Immunoprecipitation of caldesmon was performed using the caldesmon antibody. Immune complex were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with the PKCα antibody. The representative Western immunoblot shows that PKCα in the caldesmon immunoprecipitates was detected in PDBu stimulated samples, but not in the control. The result was confirmed by three experiments.


Control
PDBu
100 kDa
75 kDa
PKCα
IP: Caldesmon
IB: PKCα

CHAPTER SIX

Summary

Potential role of ERK in regulation of uterine artery contraction

It has been demonstrated in many studies that different agents that produce contractions of smooth muscle, activate ERK at the same time (3, 7, 8, 12, 14). However, conflicting results were obtained regarding a role for ERK in smooth muscle contractile regulation. The preceding serial studies have been an in-depth look at the potential role of ERK in regulation of uterine artery contraction, the interactions among α_1-adrenergic receptor agonist, PKC and ERK, and their mediated changes in the down stream signaling pathways during pregnancy.

In the studies of chapter two, we investigated the potential role of ERK in uterine artery contraction, and tested the hypothesis that pregnancy up-regulated ERK-mediated function in the uterine artery. Our report, for the first time, provides direct evidence that ERK plays an important role in the regulation of uterine artery contractility, and its effect is agonist-dependent. More importantly, pregnancy selectively enhances the role of ERK in α_1-adrenoceptor-mediated contractions and its effect in suppressing protein kinase C-mediated contraction in the uterine artery. In addition, both the Ca^{2+}-dependent and independent components are involved in the ERK pathway in the uterine artery. The lack of effect of PD-98059 on KCl-induced contractions in both nonpregnant and pregnant uterine arteries suggests that the function of Ca^{2+} channels may not be regulated by the ERK pathway in uterine arteries. This is in agreement with several previous findings in different animal models and arterial types (8, 11, 24). Our finding that PD-98059

inhibited 5-HT-induced contractions of the uterine artery is in agreement with the previous study in which 5-HT-mediated contractions were inhibited by PD-98059 in rat aorta, mesenteric artery, and tail artery (36). The results suggest the involvement of the ERK pathway in 5-HT-induced contractions of the uterine artery. The finding that the inhibitions of PD-98059 on 5-HT-induced contractions were not different in pregnant and nonpregnant uterine arteries suggests that the effect of ERK on the 5-HT-mediated contraction is not regulated by pregnancy. Unlike its effect on the 5-HT-mediated contraction, PD-98059 showed no effect on the phenylephrine-induced contractions in nonpregnant uterine artery. Nevertheless, PD-98059 did inhibit phenylephrine-mediated contractions in pregnant uterine artery. These results suggest that pregnancy up-regulates the coupling of the ERK pathway to α_1-adrenoceptor-mediated contractions in the uterine artery. Given that both 5-HT and α_1-adrenoceptor-mediated contractions share a common downstream signal, *i.e.* inositol 1,4,5-trisphosphate, in the uterine artery, it is intriguing that PD-98059 differentially regulated 5-HT and phenylephrine-induced contractions in uterine arteries. This would suggest a specific coupling of the ERK pathway to individual receptor signaling pathways. The finding that the PDBu-induced contraction was significantly attenuated in pregnant uterine artery is in agreement with previous results in rat thoracic aorta (22), suggesting that the role of PKC in the regulation of uterine vascular tone is down-regulated during pregnancy. PD-98059 had no effect on the PDBu-induced contraction in nonpregnant uterine arteries but significantly increased PDBu-induced contractions in pregnant uterine arteries. To our knowledge, it has not been reported previously that PD-98059 increases contractions to any agonists examined. The finding that PD-98059 increased PDBu-induced

contractions of pregnant uterine arteries and eliminated its difference between nonpregnant and pregnant uterine arteries is likely to have physiological significance, and suggests that ERK may play a very important role in increased uterine blood flow by suppressing the PKC-mediated contraction during pregnancy. Consistent with this notion, the present study demonstrated a significant increase in ERK-2 protein levels in pregnant, as compared with nonpregnant, uterine arteries. This is in agreement with recent findings in uterine artery endothelial cells in which pregnancy is associated with a dramatic enhancement in ERK-2 signaling pathway (6, 12).

ERK regulates uterine artery contraction through both thick and thin filament regulatory pathways

In chapter three, we further explored the mechanisms ERK-mediated uterine artery contraction. We found that ERK regulates contraction through both thick and thin filament regulatory pathways in the uterine artery.

Our findings reported in chapter three, that phenylephrine-induced contraction was initiated by an increase in $[Ca^{2+}]_i$ and LC20 phosphorylation, and that PDBu-induced contraction was not involved in changes of $[Ca^{2+}]_i$ and LC20 phosphorylation, suggest that both thick filament and thin filament regulation play an important role in regulation of uterine arterial smooth muscle contractility. To address the possible role of ERK involvement in thick filament regulation of the uterine arterial smooth muscle contraction, we examined the temporal relationships among isometric tension, $[Ca^{2+}]_i$ and LC20 phosphorylation-stimulated by PE after pretreatment of ERK inhibitor in the uterine artery. We found that inhibition of ERK MAP kinase resulted in a decrease in

LC20 phosphorylation, thus lead to reduce the contractile tension, suggesting a role for ERK in thick filament regulation. Similar results were obtained in porcine carotid artery, in which PD098059-mediated inhibition of endothelin-1-induced contraction was associated with a redustion in LC20 phosphorylation (9).

Our data (chapter two) demonstrated clearly that PD098059-mediated inhibition of phenylephrine-induced contraction was associated with a decrease in $[Ca^{2+}]_i$ and Ca^{2+} sensitivity in the uterine artery. This suggests that, in addition to the Ca^{2+}-independent pathway as previously proposed (1, 3, 11), the ERK signaling pathway also involves the Ca^{2+}-dependent components of vascular contractions. In addition, we found that PD098059 significantly decreased Ca^{2+} sensitivity of LC_{20} phosphorylation in response to phenylephrine, *i.e.* less LC_{20} phosphorylation at the given $[Ca^{2+}]_i$ in the presence of PD098059. Because alterations in the activities of MLCK or MLCP at fixed $[Ca^{2+}]_i$ will affect the Ca^{2+} sensitivity of LC_{20} phosphorylation, the results suggest that, in addition to the regulation of MLCK activity through changes in $[Ca^{2+}]_i$, ERK may also regulate either MLCK or/and MLCP activities independent of changes in $[Ca^{2+}]_i$.

The notion that ERK may regulate thin filament regulatory pathways has been supported by the results of PKC-mediated contractions in the uterine artery. Our data indicated that PDBu induced contractions independent of changes in either $[Ca^{2+}]_i$ or LC_{20} phosphorylation levels. This suggests that PKC-induced contraction in the uterine artery is mediated predominately through thin filament regulatory pathways. Moreover, our finding that PD098059 enhanced PKC-mediated contraction without changing either $[Ca^{2+}]_i$ or LC_{20} phosphorylation levels reenforces the conclusion that ERK inhibits the thin filament regulatory pathway in the uterine artery.

Taken together, these findings suggest 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.

ERK-dependent phosphorylation of caldesmon inhibits PKC-mediated contraction

In chapter three, we demonstrated that PDBu produced parallel time courses in increasing phosphorylation of $ERK_{42/44}$ and caldesmon (CaD) at Ser^{789} and the phosphorylation was blocked by PD098059, suggesting ERK-mediated phosphorylation of Ser^{789} in CaD in the uterine artery. This is in agreement with previous findings (10). The novel finding of the present study is that PD098059 had opposite effects on PDBu-induced CaD phosphorylation at Ser^{789} and contractions. It blocked phosphorylation of CaD at Ser^{789}, but potentiated the contractions. This suggests that ERK-dependent phosphorylation of CaD at Ser^{789} may not be involved in the PDBu-induced contraction, but rather inhibit it in the uterine artery.

CaD functions as a thin filament regulatory protein and exerts an inhibitory effect on vascular smooth muscle contractions (13, 23, 34). 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 (18, 20, 31). Nonetheless, the importance of CaD phosphorylation at ERK specific sites (particularly at Ser^{789}) in smooth muscle contraction remains controversial (9, 29, 35). From our findings in

chapter three, we propose that in sheep uterine artery PKC induces phosphorylation of CaD at Ser^{789} through activation of ERK, as well as at other site(s) through unknown mechanisms. It is the phosphorylation of CaD at site(s) other than Ser^{789} that may be important in reversing the inhibitory effect of CaD on myosin ATPase and leading to contractions. Phosphorylation of ERK-dependent Ser^{789} may not lead to the reversal of CaD inhibitory effects on myosin ATPase. Instead, phosphorylation of Ser^{789} may inhibit PKC-mediated phosphorylation of the other site(s).

Role of ERK/PKC in the regulation of MYPT1 and CPI-17 phosphorylation

It has demonstrated that the increase in force induced by agonist at a given $[Ca^{2+}]_i$, is due to the inhibition of myosin light chain phosphatase (MLCP). MLCP inhibition leads to an increase in both myosin light chain phosphorylation and contractile force of smooth muscle without any changes in Ca^{2+}, hence causing an enhancement of the contractile Ca^{2+} sensitivity (25, 26). We have previously demonstrated that ERK/PKC signaling pathways play a key role in regulation of Ca^{2+} sensitivity and contractility in uterine artery. We speculate that ERK may regulate uterine artery contraction, at least, partly through inhibition of MLCP activity. Inhibition of MLCP is regulated by at least three mechanisms: *a*. phosphorylation of MYPT1, *b*. arachidonic acid-induced dissociation of the holoenzyme, and *c*. PKC-mediated phosphorylation of CPI-17, which becomes a potent inhibitor of PP1c (a catalytic subunit of MLCP). At least two phosphorylation sites of MYPT1 have been identified at Thr^{696} and Thr^{850} (34), which are regulated through the RhoA/ROK pathway in a variety cells, including smooth muscle (21).

In chapter five, we have demonstrated that basal levels of phosphorylated MYPT1/Thr^{696} had been detected, but the phosphorylation level was not increased in response to α_1-adrenoceptor activation in the uterine artery. This implies that there is no α_1-adrenoceptor mediated G-protein-coupled signaling pathway leading to Thr^{696} phosphorylation in the uterine artery smooth muscle. In contrast to Thr^{696}, MYPT1/Thr^{850} is significantly phosphorylated in response to activation of α_1-adrenoceptor in the uterine artery. These findings suggest an exciting hypothesis that the different phosphorylation sites may regulate MLCP activities differently at basal and agonist-stimulated states. Our finding that PD098058 inhibited phenylephrine-induced MYPT1/Thr^{850} phosphorylation suggests that ERK might involve the phosphorylation of MYPT1/Thr^{850} in the uterine artery. A previous report demonstrated that Rho A was involved in the Ang II–induced ERK activation and contraction in intact rat mesenteric resistance arteries (30). Krepinsky et al. (28) also reported that the early activation of RhoA is essential for stretch-induced actin stress fiber formation and ERK activation in mesangial cells. These findings suggest that ROK is upstream of ERK and imply that MEK/ERK might be a link between activated ROK and MYPT1 and may be responsible for α_1-adrenoceptor-mediated Ca^{2+} sensitization in the uterine artery contraction.

CPI-17 was initially recognized as a PKC substrate and phosphorylated by PKC (15), and also phosphoryalted by ROK kinase in vitro (27). Thus, both PKC and ROK signaling pathways may contribute to regulation of MLCP activity through increase in CPI-17 phosphorylation. Our results are consistent with earlier other reports that activation of α_1-adrenoceptor and PKC result in an increase of CPI-17 phosphoryaltion at

Thr^{38} in the uterine artery. However, interestingly, our previous study (Chapter three) has demonstrated that PKC produces contraction without significantly increase in myosin light chain phosphorylation levels in the uterine artery. This clearly suggests that PKC-CPI-17 pathway might not contribute to regulation of MLCP activity and LC20 phosphorylation in the uterine artery. We speculate that phosphorylated CPI-17 may facilitate or localize PKC binding to caldesmon and regulate Ca^{2+} sensitivity of contractions through the thin filament pathway.

Pregnancy differentially regulates thick and thin filament regulatory pathway

We have demonstrated (chapter three) in the pregnant uterine artery that α_1-adrenoceptor-mediated contraction is regulated through thick and thin filament pathways, with the thick filament regulatory pathway predominating. However, PKC-mediated contraction is regulated predominantly through thin filament pathways. Little is known about the adaptation of contractile mechanisms of uterine artery smooth muscle to pregnancy. We speculate that pregnancy differentially regulates thick and thin filament regulatory pathways in uterine artery.

In chapter four, we have demonstrated that α_1-adrenoceptor-mediated contractions are increased by pregnancy, which is in agreement with previous studies (4, 5). This is probably due in part to a transient and reversible sympathetic denervation of the uterine artery during pregnancy (32, 33), resulting in a sensitization of postsynaptic α_1-adrenoceptor-mediated responses. It is likely that multiple mechanisms are contributed to this increase of α_1-adrenoceptor-mediated contractions. Previous studies demonstrated that the density of α_1-adrenoceptors and norepinephrine binding affinity to

α_1-adrenoceptors are significantly increased in pregnant uterine artery (38). Further more, the present finding that pregnancy significantly increased phenylephrine-induced $[Ca^{2+}]_i$ and LC20 phosphorylation suggests that pregnancy upregulates α_1-adrenoceptor-mediated thick filament regulatory pathway.

The present study demonstrated that pregnancy decreased force development at given levels of LC_{20} phosphorylation. This suggests that pregnancy mediated contraction is also regulated, in addition to the thick filament pathway, by thin filament pathways in the uterine artery smooth muscle cells. The finding that pregnancy potentiated α_1-adrenoceptor-mediated contraction in the uterine artery suggests that the thick filament regulatory pathway, i.e., LC_{20} phosphorylation, predominates in α_1-adrenoceptor-mediated contractions. The idea that pregnancy may inhibit thin filament regulatory pathways is further supported by the results of PKC-mediated contractions in the uterine artery. In the present study, we have demonstrated that PKC-induced contractions are independent of changes in MLC_{20} phosphorylation in both nonpregnant and pregnant uterine arteries, indicating that in the uterine artery PKC-induced contractions were mediated predominately through thin filament regulatory pathways. The finding that PKC-mediated contractions were attenuated in the pregnant uterine artery in the present study further suggests an adaptation of the thin filament regulatory pathway to pregnancy.

Conclusion and implications

Progressively increasing uterine blood flow is essential for fetal growth and maturation. Uterine blood flow during pregnancy is controlled by two distinct phenomena: phasic contractility and tone. Phasic contractility, which mediates short-

term contractions, functions through catecholamine activation of α_1-adrenergic receptors, leading to generate inositol triphosphate (IP_3) and Diacylglycerol (DAG). The phasic contractions proceed as IP_3 stimulates the release of intracellular calcium stores that activate the calmodulin-MLCK pathway, increasing LC20 phsophorylation and causing myosin filaments to slide past actin filaments (i. e., activating thick filament pathway); however, the resulting contraction is phasic, because the myosin is rapidly dephosphorylated by phosphatases, and the free cytosolic calcium is quickly reduced. A tonic contraction is initiated when the DAG generated during PIP_2 hydrolysis binds to and activates protein kinase C. The activated PKC then initiates a sustained contraction through phosphophorylation of the actin-associated binding proteins and leading to shortening of the actin filments (i. e., activating thin filament pathway).

During pregnancy, arterial contractility is a transient reduction in luminal diameter in response to nerve stimulation or to an α_1-adrenergic agonist, leading to short-term reduction in uterine blood flow. However, at the same time, arterial tone is progressively decreasing (i.e., increasing diameter) during pregnancy. It has reported that progesterone is the only sex steroid that directly modulates the α_1-AR/calmodulin-MLCK pathway in the uterine artery (17). Progesterone of endogenous or exogenous origin increases α_1-AR numbers on uterine arterial smooth muscle membranes. This is consisten with the high and maintained phasic contractility of the uterine arterial bed throughout gestation (19). It retains its ability to phasically contract to shunt blood away from the uterus to other vascular beds during times of acute maternal stress to facilitate maternal survival. On the other hand, the progressive decrease in uterine arterial tone during pregnancy seems to result from the metabolism of placental estrogens to

vasoactive catechol forms (16). Catechol estrogens directly inhibit calcium uptake through potential sensitive calcium channel, resulting in a reduced activity of membrane-bound PKC-DAG complexes. Any factor that inhibits the PKC pathway would be expected to decrease uterine arterial tone, resulting in increased vessel diameter and blood flow. The goal of our research was to determine the mechanism(s) during pregnancy that allow for progressive uterine arterial dilation and yet allow for brief periods of constriction to facilitate maternal survival. Understanding these mechainisms should allow development of therapies to facilitate fetal growth and survival as well as correct abnormalities of the uterine arterial vasulature resulting from diseas of toxicity.

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