Role of ERK1/2 in uterine contractility and preterm labor in rats

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Li, Yunping, Hyun-Dong Je, Sabah Malek, and Kathleen G. Morgan. Role of ERK1/2 in uterine contractility and preterm labor in rats. Am J Physiol Regul Integr Comp Physiol 287: R328–R335, 2004.—The present study tested the hypothesis that ERK activation is an essential step in the onset of labor in a rat model of preterm labor. The administration of RU-486, an anti-progesterone agent, to rats induced preterm delivery 22.2 ± 0.24 h after treatment. Changes in basal signaling events were studied in myometrial tissue from CO2- euthanized rats. Rats treated with RU-486 displayed a dramatically increased in vitro uterine contractility compared with gestational stage-matched, sham-treated rats. In vitro contractility was not significantly different from that during spontaneous delivery. During RU-486-induced preterm labor, as previously described for spontaneous labor, ERK phosphorylation levels increased, as did phosphorylation of caldesmon at Ser789, an ERK phosphorylation site. Also, a small but significant increase in 20-kDa myosin light chain phosphorylation was seen at a constant intracellular pCa of 7. When rats were chronically treated with an agent that prevents ERK activation, U-0126, the onset of RU-486-induced preterm labor was delayed in a statistically significant manner. Chronic in vivo treatment with U-0126 also significantly inhibited the RU-486-induced increase in in vitro contractility and ERK and caldesmon phosphorylation but did not alter the RU-486-induced increase in 20-kDa myosin light chain phosphorylation. These data indicate that ERK activation is a component of the multiple events leading to the development of labor in this rat model. We suggest that the ERK pathway could possibly be used to identify targets for the development of a novel class of tocolytic agents.

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In the United States, ~10% of all births are premature. Premature birth accounts for ~75% of all neonatal mortality and morbidity, including long-term disabilities (1, 29). However, there is no truly safe and effective agent approved in the United States for the selective suppression of preterm uterine contractions (40). A greater understanding of the mechanisms of uterine contraction during preterm and term labor is sorely needed for development of more effective and specific therapeutics.

Caldesmon (CaD) is an actin-binding protein that inhibits actin-activated ATPase activity in vitro and has been proposed to be a major regulator of smooth muscle actin-myosin interactions (30). In vitro, the binding of calmodulin or phosphorylation of CaD can reverse the inhibition (reviewed in Ref. 30). It is clear that ERK can phosphorylate CaD in vitro and in vivo (2); however, whether ERK regulates the inhibitory action of CaD and muscle contractility in vivo remains controversial (13, 17, 35). It has been difficult to determine a clear-cut, cause-and-effect relation between ERK activity and regulation of contractility in a physiological setting.

The ERK pathway is known to be activated during oxytocin-induced contraction (36) and prostaglandin F2α-induced contraction (37) of pregnant rat myometrium. Furthermore, we previously reported that spontaneous full-term labor in the rat is associated with a basal activation of ERK2 and subsequent phosphorylation of CaD that persists in vitro (28). Thus it is hypothesized that the change in ERK/CaD signaling may contribute to the change in uterine contractility during the initiation of parturition. The available ERK inhibitors are effective only when used as pretreatment to prevent the initial activation of ERK. Thus it has not been possible by simple in vitro experiments to determine whether there is a cause-and-effect relation between gestation-dependent activation of the ERK/CaD pathway and regulation of uterine contractility. To the best of our knowledge, the in vivo effect of ERK inhibition on modulating smooth muscle contractility has not been explored.

In the present study, we found that 1) RU-486-induced preterm labor displays an in vitro biochemical profile in uterine muscle tissue similar to that caused by spontaneous labor and 2) ERK inhibition by U-0126 delays RU-486-induced preterm labor in a statistically significant manner.

MATERIALS AND METHODS

Animals and treatment groups. All procedures were approved by our Institutional Animal Care and Use Committee. Sprague-Dawley primigravid pregnant rats (day 1 = sperm positive; Taconic, Germantown, NY; time 0 was arbitrarily taken as midnight) were used for the experiments. The female rats were mated overnight, and the vaginal plugs were checked on the following morning between 7 and 10 AM, including the mornings on the weekends. The rats were housed in a constant-temperature room with a 12:12-h light-dark cycle. Food and water were available ad libitum. The rats were divided into five groups: sham, RU-486 treated (RU), labor-matched U-0126 + RU-486 treated (U0 + RU), time-matched U0 + RU, and spontaneous labor (SL). The dosage schedule vs. gestational stage is illustrated in Fig. 1.

Induction of preterm labor. Rats in the RU group (Fig. 1A; n = 6) were treated with RU-486 (2 mg/kg sc, 3 mg/ml in sunflower seed oil) at 10 AM on day 19 of pregnancy. Then they were closely observed for signs of labor on day 20 of pregnancy. U-0126 treatment: Rats in the labor-matched U0 + RU group (Fig. 1A; n = 6) were treated with U-0126 [100 mg/kg sc, maximal safe dose in rats (personal communication, James Trzaskos, Bristol Myers Squibb)] at 7 AM, 3 PM, and 11 PM on day 18 of pregnancy. U-0126 was dissolved at a concentration of 200 mg/ml in DMSO. On day 19 of pregnancy, RU-486 was administered at 10 AM for preterm labor induction. The U-0126 treatment continued until the delivery of the first pup.

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A: RU, labor-matched U0+RU or time-matched U0+RU groups

**RU0126 Injection, 100mg/kg, SC, q8h,**

<table>
<thead>
<tr>
<th>Day 18</th>
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**RU486 Injection, 2mg/kg, SC**

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<td>7AM</td>
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**B: Sham group**

**DMSO injection, 0.5ml/kg, SC, q8h,**

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<th>Day 18</th>
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**Sunflower seed oil, 0.67ml/kg, SC**

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<td>7AM</td>
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Fig. 1. Gestational stages and temporal sequence for rat injections. A: RU rats received an RU-486 injection at 10 AM on day 19 of pregnancy for induction of preterm labor. Labor-matched U0 + RU rats also received the same injection of RU-486, but in addition were treated with RU-0126, every 8 h, starting at 7 AM on day 18 of pregnancy until the onset of labor. Time-matched U0 + RU rats were injected with U-0126 and RU-486 exactly following the dose schedule used for the U0 + RU group, but the rats were euthanized exactly when labor commenced in the RU group. B: sham rats underwent a protocol similar to that used for the U0 + RU group but were injected with vehicle (DMSO and sunflower seed oil).

Rats in the time-matched U0 + RU group (Fig. 1A; n = 6) were injected with U-0126 and RU-486 on day 18 of pregnancy following the dose schedule for the labor-matched U0 + RU group, but the rats were euthanized exactly at the average time at which the RU group delivered the first pup, i.e., 22.2 h after RU-486 administration.

Sham control. On day 18 of pregnancy, rats in the sham control group (n = 6) were injected subcutaneously with the same amount of DMSO or sunflower seed oil used in the regimen described for the U0 + RU groups. The rats were euthanized via CO₂ inhalation at 11 AM on day 20 of pregnancy for sample collection to compare a nonlaboring state at an average stage of gestation similar to that at which the labor-matched U0 + RU and RU groups delivered.

**Tissue preparation and force recording.** For the collection of in-labor uterine smooth muscle samples, rats were closely observed at 15- to 30-min intervals. The delivery of the first pup was defined as an index of labor. Rats were euthanized by CO₂ inhalation followed by cervical dislocation. The pups were euthanized by CO₂, and all products of conception were removed from the uterine wall and placed on ice. The blotted weight of the pups was recorded. Excised uteri were immersed immediately into oxygenated Krebs solution at room temperature. The composition of Krebs solution was (mM) 120 NaCl, 5.9 KCl, 11.5 dextrose, 25 NaHCO₃, 1.2 NaH₂PO₄·H₂O, 1.2 MgCl₂·6H₂O, and 2.5 CaCl₂. Approximately 8 × 2 mm (length × width) whole-thickness uterine strips oriented parallel to the long axis of the longitudinal muscle bundles were dissected under a dissection microscope (Olympus VM).

Isometric force was recorded at 37°C as previously described (28, 42). All myometrial strips were 8 mm long at slack length and were gradually stretched to the optimal length with respect to spontaneous contractions (21). The contractile activity was digitally recorded with MacLab/8e, Chart version 3.5.4 (AD Instrument, Castle Hill, Australia). The area under the curve (AUC, mN·s·mg⁻¹) was obtained by integrating the force signal (in mN) over 15 min (in s) and normalized for tissue dry weight (in mg). The dry weight used in the present study was the weight of preparations after six acetone washes, which dehydrated the muscle strips.

**Western blot analysis.** The muscle strips were quick frozen in a dry ice-acetone slurry containing 10% TCA and 10 mmol/l DTT at the end of the force-recording experiments. Samples were homogenized in a buffer containing 20 mM MOPS, 4% SDS, 1% Triton X-100, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 μM leupeptin, 5.5 μM pepstatin A, 20 kallikrein-inactivating units of aprotinin, 2 mM Na₃VO₄, 1 mM NaF, 20 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 100 μM ZnCl₂. Protein-matched samples were subjected to SDS-PAGE, transferred to Immobilon-P membrane (Millipore, Bedford, MA), and subjected to immunostaining using the appropriate antibody. Blots were visualized with a SuperSignal West Pico peroxidase solution (Pierce, Rockford, IL). The images were detected with a chemiluminescence screen and quantified with a Bio-Rad Molecular Imager and Multi-Analyt software.

**Measurements of 20-kDa myosin light chain phosphorylation.** Tissue was prepared as described above. After equilibration and stretching, the myometrial strips were permeabilized with 20 μg/ml α-toxin (List Biological Lab, Campbell, CA) in pCa 6.4 buffer at 34°C for 30 min as previously described (28). The force development during α-toxin treatment reached a stable plateau phase, indicating the completion of permeabilization. Then the intracellular Ca²⁺ concentration ([Ca²⁺]) was clamped at pCa 7.0 for 12 min to prevent an artifactual triggering of contractions in response to the freezing solution. The measurement of 20-kDa myosin light chain (LC20) phosphorylation was performed by glycerol-urea gels and Western blots as previously reported (24). Moles of phosphate per mole of myosin light chain were calculated by dividing the density of the phosphorylated band by the sum of densities of the phosphorylated plus the unphosphorylated bands.

**Chemicals and antibodies.** Mifepristone (RU-486, Sigma; 11β-[4-dimethylamino]pheno1-17β-hydroxy-17-[1-propynyl]estra-4,9-dien-3-one) is a progesterone receptor antagonist. U-0126 (1,4-diamino-2,3-dicyclo-1,4-bis[2-aminophenylthio]butadiene) is a selective inhibitor of MAP kinase kinase (MEK1/2); it was a generous gift from DuPont Pharmaceuticals (Wilmington, DE). The phospho-p44/42 MAP kinase antibody (1:2,000) and p44/42 MAP kinase antibody (1:1,500) were purchased from Cell Signaling Technology (Beverly, MA). The CaD polyclonal antibody (1:30,000) was raised against full-length human myometrial CaD and was a gift from Dr. K. Mabuchi (Boston Biomedical Research Institute). The anti-phospho-CaD antibody (1:500; Upstate, Lake Placid, NY) was produced against a phosphopeptide containing the CaD sequence surrounding the Ser789 ERK phosphorylation site (12). The monoclonal anti-myosin light chain antibody (1:2,500) was a product of Sigma. General laboratory reagents were of analytic grade or better and were purchased from Sigma or Fisher Scientific.

**Statistics.** Values are means ± SE unless otherwise stated; n values represent the number of animals used in the experiments. Data were analyzed by ANOVA followed by Bonferroni’s post test or Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

RU-486 induces narrowly timed preterm labor in Sprague-Dawley rats. Under our standard housing conditions, control Sprague-Dawley pregnant rats (n = 13) went into spontaneous labor at a gestational stage of 22.93 days (SD 0.42). The individual deliveries showed a sizable range in their occurrence, with a cluster in the afternoon on day 22 of pregnancy and another cluster in the morning on day 23 of pregnancy (Fig. 2A). The average length of pregnancy in these rats was consistent with a previous report in the same model (43).
The antiprogesterone RU-486 has been used to induce a reliable model for preterm labor/birth in pregnant rats (6, 16, 43). In the present study, pregnant rats were injected subcutaneously with RU-486 at 10 AM on day 19 of gestation (Fig. 1). All rats \( (n=6) \) gave preterm birth 22.2 \( \pm \) 0.24 h after injection (Fig. 2B); thus delivery occurred in a much more narrow range, at a gestational stage of 20.35 days (SD 0.02), with a far smaller SD than for spontaneous labor.

Strips from RU-486-treated animals display increased myometrial contractility in vitro. The fact that RU-486 can induce preterm labor implies an effect on myometrial contractility; however, whether this increased contractility is associated with chronic changes in basal contractility that persist in vitro in the absence of the hormonal milieu of the intact rats has not been previously shown. Thus we compared the in vitro area under force curves (AUC) in sham, RU, and SL groups. As described previously (28), the in vitro myometrial strips were relatively quiescent on day 20 of pregnancy (Fig. 3A, sham group), displaying a lower AUC after normalization to tissue dry weight \( (1,532 \pm 152 \text{ mN} \cdot \text{s} \cdot \text{mg}^{-1}) \) than myometrial strips from the SL group (Fig. 3A). In vitro uterine contractility increased dramatically after in vivo RU-486 treatment (Fig. 3A, RU group, AUC = 3,815 \( \pm \) 449 mN\cdot s\cdot mg\(^{-1}\)), comparable to that during spontaneous labor (AUC = 4,201 \( \pm \) 924 mN\cdot s\cdot mg\(^{-1}\), \( P = 0.69 \)).

**ERK2 is activated during RU-486-induced preterm labor.** We previously reported that spontaneous labor is associated with phosphorylation and, hence, activation of ERK2 and subsequent phosphorylation of CaD and that these changes persist in vitro (28). The following question arises: Does RU-486-induced preterm labor cause the same changes in basal ERK2 and CaD phosphorylation that occur in spontaneous labor? Muscles were quick frozen in vitro after a force-recording experiment (see MATERIALS AND METHODS). ERK2 protein levels did not change in any group (Fig. 3D); however, ERK2 phosphorylation levels normalized to ERK2 protein levels significantly increased during RU-486-induced labor to a level comparable to that seen in spontaneous labor (Fig. 3B).

We previously reported that ERK1 protein levels and phospho-ERK1 levels in protein-matched samples are not detectably changed throughout pregnancy into spontaneous labor (28). Similarly, in the present study, there was no detectable change in ERK1 protein levels between sham, SL, and RU groups (Fig. 3D). Phospho-ERK1 levels, normalized to ERK1 protein levels, also did not change detectably during RU-486-induced preterm labor (Fig. 3C).

**h-CaD phosphorylation is increased during RU-486-induced preterm labor.** The effects of RU-486 on alterations at the level of the contractile apparatus have not been reported. To explore the subcellular mechanism(s) of the effect of RU-486 on myometrial contractility, we examined the protein and phosphorylation levels of CaD. We and other investigators previously reported that, compared with nonpregnant myometrium, CaD protein content is significantly increased at late pregnancy in the rat and human myometrial protein-matched samples (28, 45). We previously showed that CaD is phosphorylated at an ERK phosphorylation site in labor in the rat (28). No change in CaD protein levels between RU and SL groups was seen (Fig. 3F), but after normalization for the CaD protein levels, phospho-CaD signals were significantly increased in strips from rats in which RU-486 was used to induce preterm labor (Fig. 3E). The phospho-CaD antibody is specific for CaD phosphorylation at an ERK site, Ser\(^{789}\) (12). There was no significant difference in the levels of CaD phosphorylation between protein-matched samples from RU-486-induced labor and spontaneous labor (Fig. 3E).
Basal LC20 phosphorylation levels increased during RU-486-induced preterm labor. Smooth muscle contraction can be regulated by pathways that terminate on the thin filament and those that terminate on the thick filament (23). In our experience, it is not possible to measure consistent levels of LC20 phosphorylation in quick-frozen rat myometrial muscles, because the exposure to cold buffers triggers a contraction response, presumably as a result of Ca\(^{2+}\)/H\(_{\text{11001}}\) released from intracellular stores (5, 32). For this reason, we have permeabilized the muscles with \(-\)-toxin and clamped Ca\(^{2+}\)/H\(_{\text{11001}}\) at resting levels (pCa 7). This approach also gives a measure of the Ca\(^{2+}\)/H\(_{\text{11001}}\) sensitivity of LC20 phosphorylation, because the measurements are made at a constant [Ca\(^{2+}\)]. We previously reported a small, but significant, increase in basal LC20 phosphorylation. Thus the following question arises: Is a similar biochemical profile seen in vitro for LC20 phosphorylation in strips from RU-486-treated animals? LC20 phosphorylation levels were significantly increased from 0.12 ± 0.017 mol phosphate/mol LC20 (in the sham group) to 0.17 ± 0.013 in myometrium during RU-486-induced preterm labor (Table 1). An LC20 phosphorylation level of 0.40 ± 0.033 mol phosphate/mol LC20 in myometrical strips stimulated with 51 mM KCl for 4 min was used as a positive control. In general, an increase in basal LC20 phosphorylation would tend to increase contractility during labor.

**MEK inhibitor U-0126 delays RU-486-induced preterm labor in rats.** To test the idea that there is a cause-and-effect relation between activation of the ERK/CaD pathway and the onset of labor contraction, we pretreated the pregnant rats with the MEK inhibitor U-0126 (14) before the administration of RU-486 (see MATERIALS AND METHODS). U-0126 is a potent and specific inhibitor of the mitogen-activated protein kinase kinases MEK-1 and MEK-2, upstream kinases for ERK1/ERK2 (9, 14). ERK inhibitors PD-98059 and U-0126 have been studied extensively in vitro. However, relatively few studies have used ERK inhibitors in vivo because of the low potency of PD-98059 and expense of U-0126 for chronic treatment (33, 41). Treatment with U-0126 delayed the onset of parturition in a statistically significant manner to an average

### Table 1. LC20 phosphorylation levels

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<tr>
<th>Group</th>
<th>Sham-Vehicle (n = 5)</th>
<th>Time-Matched U0 + RU (n = 5)</th>
<th>RU (n = 4)</th>
<th>51 mM KCl (n = 4)</th>
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<td></td>
<td>0.12 ± 0.017</td>
<td>0.16 ± 0.013†</td>
<td>0.17 ± 0.014†</td>
<td>0.40 ± 0.033</td>
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Values are means ± SE in mol phosphate/mol 20-kDa myosin light chain (LC20). U0, U-0126; RU, RU-486. *P < 0.05 vs. sham; †P = 0.7 vs. RU.
of 25.2 ± 0.64 h after RU-486 administration (Fig. 4, P < 0.01 compared with animals treated with RU alone). There was no overlap of the labor onset times between the RU and U0 + RU groups (Fig. 4).

Interestingly, despite the fact that U-0126 delayed RU-486-induced preterm labor, once labor commenced (albeit at a later point in time), the myometrial AUC for the labor-matched U0 + RU group (3,466 ± 624 mN·s·mg⁻¹) was not significantly different from that for the laboring animals treated with RU-486 alone (3,815 ± 449 mN·s·mg⁻¹) or the SL group (4,201 ± 924 mN·s·mg⁻¹). Similarly, phospho-ERK-to-ERK ratios and phospho-CaD-to-CaD ratios in the labor-matched U0 + RU group (10.7 ± 1.6 and 22.6 ± 2.9, respectively) were indistinguishable from those parameters in laboring animals treated with RU-486 alone (11.1 ± 1.3 and 24.6 ± 3.8, respectively) or the SL group (13.6 ± 3.4 and 32.2 ± 3.0, respectively). Thus increased in vitro contractility, increased ERK activation, and increased CaD phosphorylation appear to be diagnostic of labor whenever it occurs.

U-0126 inhibits the RU-486-induced increase in ERK and CaD phosphorylation and in vitro contractility in time-matched animals. As stated above, once labor commenced, the in vitro contractilities and phospho-ERK, phospho-CaD, and phospho-LC20 levels of labor-matched groups (SL, U0 + RU, and RU groups) were significantly increased compared with the sham group. A key question is whether the MEK inhibitor actually prevented ERK activation. Thus we also studied an additional control group of animals treated with U-0126 and RU-486 for which the animals were killed at exactly the average time point after RU-486 administration at which the animals receiving RU-486 alone went into labor (see MATERIALS AND METHODS). In these time-matched groups, there was indeed a significant effect of U-0126 to inhibit the RU-486-induced increase in in vitro contractility (Fig. 5A), ERK2 phosphorylation (Fig. 5B), and CaD phosphorylation (Fig. 5E) during the time that labor was delayed. Neither ERK1 nor ERK2 protein levels (Fig. 5D) nor h-CaD protein levels (Fig. 5F) nor ERK1 phosphorylation levels (Fig. 5C) were significantly changed between these groups.

U-0126 does not prevent the RU-486-induced increase in the LC20 phosphorylation level. As shown in Table 1, RU-486-induced preterm labor is associated with a small, but significant, increase in LC20 phosphorylation level. Others have suggested that ERK may be able to regulate LC20 phosphorylation levels by using myosin light chain kinase as a substrate (11, 25, 31, 34). However, U-0126 pretreatment did not prevent the RU-486-induced increase in the LC20 phosphorylation level. The LC20 phosphorylation levels in the RU group are not statistically significantly different from those in the time-matched U0 + RU group (Table 1). These results are consistent with the idea that the main target of the ERK-mediated pathway in this system is CaD, rather than myosin light chain kinase. These results, in contrast, are not consistent with the idea, cited above, that ERK is regulating LC20 phosphorylation in this system.

Effect of U-0126 on fetal weight. Even though ERKs are present ubiquitously in most cells of the body, we observed no obvious signs of toxicity of U-0126 to the mothers or pups in this rat model. There were no differences in daily weight gain or general behavior in pregnant rats between the U0 + RU and RU groups (data not shown). U-0126 treatment did not affect fetal weight (Table 2).

DISCUSSION

Three major findings of the present study are as follows: 1) RU-486-induced labor is associated with increased ERK2, CaD, and LC20 phosphorylation and increased contractility detected in vitro, 2) the MEK inhibitor U-0126 can delay RU-486-induced preterm labor in a statistically significant manner, and 3) the delay in RU-486-induced preterm labor is associated with the inhibition of ERK and CaD phosphorylation by U-0126. These findings are of considerable interest at the basic and clinical levels.

At the basic level, it is important that the ERK/CaD pathway may regulate myometrial contractility. In time-matched groups, there is a clear-cut effect of chronic in vivo treatment with U-0126 to inhibit myometrial contractility (Fig. 5A), ERK2 phosphorylation (Fig. 5B), and CaD phosphorylation (Fig. 5E). To our knowledge, this is the first in vivo evidence for the involvement of ERK in smooth muscle regulation. There has been considerable controversy concerning whether ERK phosphorylation regulates the inhibitory action of CaD. On the basis of the finding that the sites of CaD phosphorylation in vivo are identical to those phosphorylated by ERK in vitro (2, 4, 7), it has been suggested that ERK is the endogenous CaD kinase (3, 10, 20, 47). What has been far less clear is whether ERK-mediated phosphorylation of CaD alters CaD’s actions. Childs et al. (8) reported that ERK-dependent phosphorylation of CaD only slightly attenuated its actin binding. Krymsky et al. (27) found some reversal of CaD’s inhibition of myosin ATPase activity in the absence of tropomysosin but little effect in the presence of tropomysosin. However, more recently, ERK-dependent phosphorylation of CaD has been reported to cause local conformational changes, which markedly diminish actin binding at one of the two actin-binding sites of CaD (39). Others have reported that ERK-mediated phosphorylation of CaD reverses its inhibitory action in an in vitro motility assay (17). In a permeabilized smooth muscle preparation, the addition of activated ERK produced
positive results in one case and negative results in another case. Similarly, ERK inhibitor studies have produced positive results in some cases and negative results in another case. It is quite likely that the ERK/CaD pathway is tissue, species, and agonist dependent. The results of the present study, combined with our past study, strongly suggest a gestation-dependent alteration of the ERK/CaD pathway, in that ERK and CaD are phosphorylated during spontaneous and RU-486-induced labor and U-0126 pretreatment delays the RU-486-induced preterm labor.

At the clinical level, the magnitude of the delay of preterm labor in the rat model by U-0126 is similar to that caused by L-366509, an oxytocin receptor antagonist. The similarity of the efficacy of U-0126 in the rat model to that of an oxytocin receptor antagonist in the same model is especially of interest, because atosiban, an oxytocin receptor antagonist, is approved for use in humans in the United Kingdom and is under investigation in clinical trials in the United States. Targeting the ERK/CaD pathway may possibly yield alternative therapeutics or agents that could be used in combined therapies.

The duration of pregnancy and labor is much shorter in the rat model than in the human (averaging 23 days vs. 266 days, 12 times longer in the human). Thus a 3-h delay in the rat model can be thought of as being roughly equivalent to \( \pm 36 \) h in the human. Indeed, in the case of the oxytocin receptor antagonist cited above, this agent caused a prolongation of pregnancy of up to 7 days. This degree of delay in delivery of a premature infant allows sufficient time to transfer the mother and fetus to the appropriate hospital and implement betamethasone treatment, which will induce maturation of the fetal lungs and significantly decrease the likelihood of respiratory distress syndrome.

Thus the MEK/ERK pathway may provide useful targets for the future development of therapeutic agents. However, even though we did not detect obvious toxicity of U-0126 to the rats in this study, we also did not attempt to screen thoroughly for

Table 2. Fetal weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>RU</th>
<th>U0 + RU</th>
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<tbody>
<tr>
<td>Fetal wt, g</td>
<td>2.37±0.05 (38)</td>
<td>2.46±0.07 (16)</td>
<td>2.46±0.04 (31)</td>
</tr>
<tr>
<td>Fetal no./rat</td>
<td>12</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Gestation, days</td>
<td>20</td>
<td>20</td>
<td>20</td>
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Values are means ± SE of no. of rats in parentheses.
toxic effects and do not intend to imply that this particular MEK inhibitor could be directly used therapeutically. In the present study, U-0126-treated animals were monitored between days 18 and 20 of pregnancy; thus most developmental changes in the fetus were nearing completion. Another study has demonstrated inhibitory effects of PD-98059 and U-0126 on the development of kidneys cultured in vitro from 15-day embryos (22). In the present study, we did not investigate whether such effects could be demonstrated in our animals, but this raises the possibility that additional effects of U-0126 on other organs may exist.

Another finding of the present study is that RU-486-induced labor in vivo is associated with a persistent increase in myometrial contractility in vitro, suggesting long-term changes. Although it is known that RU-486 is a progesterone receptor antagonist, its subcellular mechanism of action on uterine contractility has not been known. The effect of RU-486 is unlikely to be a direct action on contractile proteins, because RU-486 is a progesterone nuclear receptor antagonist (19). In contrast to contraction, RU-486 actually relaxed KCl-induced contraction of rat nonpregnant uterine smooth muscle in vitro (38). In the present study, the in vitro persistent increase in contractility caused by in vivo administration of RU-486 could be a consequence of progesterone-related signal transduction or an indirect consequence resulting from labor commencing. We have shown that the subcellular mechanisms of RU-486 on uterine contractility include, but may not be limited to, activation of the ERK/CaD signaling pathway and increased basal LC20 phosphorylation. The upstream signaling events causing these changes remain to be determined, but it is of considerable interest that tissues from rats undergoing spontaneous labor and RU-486-induced preterm labor display identical physiological and biochemical profiles regarding in vitro contractility and the phosphorylation of ERK2, CaD, and LC20.

Numerous factors can influence the complex process of preterm labor, such as genetic factors, multiple gestations, nutritional status, stress, infection, premature rupture of membranes, and some maternal diseases. In the present study, we have focused on the regulation of contractile filament function. The alterations we have reported in basal contractility and basal CaD, ERK, and LC20 phosphorylation will depend on and perhaps will be synergistic with other hormone-induced effects in vivo but, clearly, are only part of the picture.

In summary, we have demonstrated that ERK activation, phosphorylation of CaD at an ERK site, and an increase in basal LC20 phosphorylation at constant [Ca^{2+}], occur during RU-486-induced preterm labor in rats. The MEK inhibitor U-0126 causes a significantly delayed RU-486-induced preterm labor associated with an inhibition of myometrial contractility and ERK and CaD phosphorylation. These results constitute further evidence for a role of the ERK/CaD pathway in the regulation of myometrial contractility and development of parturition and suggest that the ERK/CaD pathway could possibly provide potential target(s) for the development of a novel class of tocolytics.

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