Hormonal Regulation of the Contractile Response Induced by Okadaic Acid in the Rat Uterus

MAR TRUJILLO, LUZ CANDENAS, CRISTINA G. CINTADO, JOSEFINA MAGRANER, JAVIER FERNANDEZ, JULIO D. MARTÍN, and FRANCISCO M. PINTO

Institute of Chemical Research, Scientific Research Center Isla de La Cartuja, Sevilla, Spain (M.T., C.G.C., J.M., J.D.M., F.M.P.); and Institute of Bio-Organic Research, University of la Laguna, Tenerife, Spain (J.F.)

Received August 1, 2000; accepted October 31, 2000 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The contractile effect of okadaic acid (OA), a highly selective inhibitor of protein serine/threonine phosphatases, was analyzed in the rat uterus during the estrous cycle and during the course of pregnancy. Contractile effects were related to circulating levels of estrogen and progesterone and to mRNA levels of myosin light chain kinase (MLCK) and of myosin light chain protein phosphatase catalytic (PP1-δ) and larger regulatory subunit (MYPT). Both in nonpregnant and pregnant uteri, OA (20 μM) induced a transient contraction, which after plateauing, slowly decreased. In the nonpregnant uterus, the amplitude of this contraction varied at different stages of the estrous cycle, being higher at proestrus and lower at diestrus. In the pregnant uterus, the contraction to OA increased significantly during the course of pregnancy, reaching a maximum in day 21 pregnant rats, and declined after delivery. Whatever the day of pregnancy, the amplitude of the contraction to OA was not significantly modified when obtained in Ca²⁺-free solution. The magnitude of the OA-induced contraction in spontaneously cycling and pregnant rats was positively correlated to the ratio of estrogen/progesterone serum levels. Reverse transcription-polymerase chain reaction assays on myometrial tissue demonstrated that mRNA expression of PP1-δ and MYPT was higher at early (day 3) than at late (day 21) pregnancy. MLCK mRNA levels were similar in day 3 and day 21 pregnant rats. These data suggest that changes in the expression and activity of myosin phosphatase may contribute to modulating the level of uterine contractile force during the estrous cycle, pregnancy, and labor.

It has long been recognized that the link between excitation at the plasma membrane and smooth muscle contraction is the rise in the intracellular free Ca²⁺ level, [Ca²⁺]i, (Somlyo and Somlyo, 1994; Horowitz et al., 1996). As a consequence of its elevated concentration, Ca²⁺ binds to calmodulin, leading to its activation and the subsequent interaction with a number of target proteins, including MLCK (Adelstein and Klee, 1981; Horowitz et al., 1996). Phosphorylation of the 20-kDa myosin light chain (LC20) by MLCK is considered the final and essential step to initiate contraction (Mackenzie et al., 1990; Somlyo and Somlyo, 1994). Relaxation occurs when myosin is dephosphorylated by MLCP. A multisubunit myosin phosphatase has been isolated from different tissues (Chen et al., 1994; Shimizu et al., 1994; Shirazi et al., 1994). The holoenzyme is trimeric and consists of the δ-isofrom of type 1 protein serine/threonine phosphatase (PP1-δ) and two regulatory subunits. The myosin phosphatase target (MYPT) is a 110- to 130-kDa protein that binds to both PP1-δ and phosphorylated myosin (Chen et al., 1994; Shimizu et al., 1994; Hartshorne and Hirano, 1999). The second regulatory protein (M20) has an apparent molecular mass of 20 to 21 kDa and also appears to bind to MYPT (Hartshorne and Hirano, 1999).

Protein phosphatase inhibitors have been crucial for recent advances in our understanding of smooth muscle contractility (Bialojan et al., 1988; Gong et al., 1992). Among them, OA, a polyether derivative responsible for diarrhetic shellfish poisoning, was the first known inhibitor of protein serine/threonine phosphatases of the PPP family (Takai et al., 1987; Cohen et al., 1990) and these enzymes appear to be the only cellular target of the toxin (Cohen et al., 1990; Schönhthal, 1998). The effects of OA are usually attributed to inhibition of PP1 and PP2A, which are thought to be the dominant phosphatases in vivo and account for approximately 90% of cellular PSP activity (Cohen et al., 1990; Schönhthal, 1998). Experiments on smooth muscle have shown that OA and

ABBREVIATIONS: [Ca²⁺]i, cytosolic Ca²⁺ concentration; MLCK, myosin light chain kinase; LC20, 20-kDa myosin light chain; MLCP, myosin light chain protein phosphatase; PP1-δ, protein phosphatase type 1 δ-isofrom; MYPT, large myosin phosphatase target subunit; OA, okadaic acid; PSP, protein serine/threonine phosphatases; E2, 17β-estradiol; P4, progesterone; RT-PCR, reverse transcription-polymerase chain reaction; PSS, physiological salt solution; ACh, acetylcholine; bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
other PSP inhibitors caused a contraction that was highly resistant to the absence of extracellular Ca\(^{2+}\) (Obara et al., 1989; Gong et al., 1992) and was accompanied by little or no increase in [Ca\(^{2+}\)]. (Ozaki et al., 1987; Hirano et al., 1989). OA induced phosphorylation of LC\(_{20}\) and inhibited dephosphorylation of phosphorylated LC\(_{20}\) in all smooth muscles that have been studied (Bialojan et al., 1988; Erdödi et al., 1988; Obara et al., 1989; Gong et al., 1992). In parallel, experiments in vascular and visceral smooth muscles demonstrated that agonists that activate G protein-coupled membrane receptors were able to develop a higher level of force at a given intracellular level of [Ca\(^{2+}\)]\(_i\), than simple membrane depolarization (Somlyo and Somlyo, 1994). These data have been essential to observe that Ca\(^{2+}\)/force relationships are not invariable but depend on the balance between MLCK and MLCPP activities. Therefore, mechanisms that interfere with the activities of the phosphorylating (MLCK) and dephosphorylating (MLCPP) enzymes are able to modulate the level of force and LC\(_{20}\) phosphorylation achieved at a constant [Ca\(^{2+}\)]\(_i\) (Kubota et al., 1992; Gong et al., 1995).

Pregnancy is associated with important changes in electrical and metabolic properties of uterine smooth muscle (Lefebvre et al., 1992; Wray, 1993; Garfield, 1994; Mershon et al., 1994; Tezuka et al., 1995; Pinto et al., 2000). In spite of recent advances in our understanding of the regulation and physiological functions of contractile proteins in smooth muscle, relatively little information exists on the mechanisms of adaptation of uterine smooth muscle during pregnancy. There are conflicting reports suggesting that mechanical activity may (Izumi, 1985) or may not change (Munns and Pennefather, 1998). In the estrogen (E\(_2\))-primed rat uterus, we have previously shown that OA induces a contraction that is independent of neurotransmitter release and membrane receptor activation (Candenas et al., 1992, 1994) and depends on a direct interaction with MLCPP (Arteche et al., 1997). The contraction is only slightly reduced in Ca\(^{2+}\)-free medium (Candenas et al., 1994) and is not accompanied by changes in [Ca\(^{2+}\)]\(_i\) (Arteche et al., 1997). In the present study, we examined the contractile response elicited by OA in the rat uterus during pregnancy and in the nonpregnant rat uterus at different stages of the estrous cycle and under different hormonal conditions. Serum levels of E\(_2\) and P\(_4\) were measured by radioimmunoassay, in an attempt to correlate ovarian hormone levels with OA functional activity. In addition, mRNA expression levels of PP1-δ, MYPT, and MLCK were analyzed by RT-PCR on uteri from early (day 3) and late (day 21) pregnant rats.

### Materials and Methods

**Animals and Tissue Preparation.** All experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Three-month-old virgin female Wistar rats were purchased from Charles River Laboratories (Criffa, Spain). Animals were maintained in an air conditioned room at 22°C under controlled lighting (12-h light/dark cycle) and provided with food and water ad libitum. Vaginal smears were checked daily and rats with at least two consecutive 4-day estrous cycles were used. Uteri were obtained from rats at different stages of the estrous cycle. Some animals were pretreated with 17β-estradiol benzoate (E\(_2\); 20, 200, or 3000 μg · kg\(^{-1}\); Sigma, St. Louis, MO). The compounds were dissolved in olive oil and injected (i.p., final volume 1 ml · kg\(^{-1}\)) 24 h before the experiment. Pregnancy was produced by

---

**Fig. 1.** Concentration-response curves for OA-induced contraction in the nonpregnant rat uterus at the estrus and diestrus stages of the estrous cycle and in animals treated with E\(_2\) (200 μg · kg\(^{-1}\)). Data are means ± S.E.M. of three to eight experiments, expressed as a percentage of the contraction to ACh (1 mM).

**Fig. 2.** Contractile response induced by OA (20 μM) in uteri from early (day 3, △) and late (day 16, ●) pregnant rats. Data are expressed as a percentage of the contraction to ACh (1 mM) (A); as the tension change induced by OA, in milligrams (B); and as the ratio of milligrams of contraction to milligrams of tissue dry weight (C). Each data are the mean ± S.E.M. of five experiments.
mating proestrus rats with male rats overnight. The day of gestation when sperm was observed in the vaginal lavage was defined as day 0 of gestation. Parturition usually occurs in the evening of day 21 or the morning of day 22. Uteri were obtained from rats on days 1, 3, 6, 11, 16, and 21 of pregnancy or from day 1 postpartum rats, the day of birth being zero postpartum. A group of day 10 pregnant rats was injected (s.c.) with either RU486 (mifepristone, 10 mg·kg⁻¹, kindly provided by Roussel-Uclaf, Romainville, France) or vehicle (1 ml·kg⁻¹ olive oil, control rats) and killed on day 11. Both pregnant and nonpregnant rats were killed by decapitation at 10:00 AM. Trunk blood was collected and the uterine horns were rapidly removed, trimmed of surrounding connective tissue, and opened longitudinally. Uteri from pregnant animals were freed of blood, pups, and placenta. The endometrium was carefully scraped with the aid of a binocular microscope. Tissue samples were excised from the longitudinally smooth muscle layer and quickly frozen in liquid nitrogen and stored at −80°C (RT-PCR studies) or used fresh (functional studies).

**Tissue Bath Experiments.** Strips of longitudinal uterine smooth muscle (8–10 mm in length and 1–2 mm in width) were prepared and mounted in isolated tissue baths containing 4 ml of a physiological salt solution (PSS) of the following composition: 118 mM NaCl, 5.6 mM KCl, 1.9 mM CaCl₂, 0.95 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose. The preparations were bubbled continuously with 95% O₂, 5% CO₂ and warmed to 37°C. Mechanical responses were recorded isometrically by means of force-displacement transducers (Grass FT-03) connected to a LETICA amplifier and an ABB GOERZ SE 130 multichannel recorder. The tissue was immersed in PSS and equilibrated for 45 min (with changes in bath fluid every 15 min) under a resting tension of 0.5 g. After the equilibration period, the preparation was challenged twice by administration of a maximally effective concentration of acetylcholine (ACh, 1 mM). Uterine strips were then allowed to equilibrate for a further 60-min period before addition of OA (5–20 μM, obtained in Instituto de Bio-Organica, Universidad de la Laguna, Tenerife, Spain; Arteche et al., 1997). Only one concentration of OA was applied to each strip since we found in previous experiments that the biphasic response to OA (time for 50% contraction, time to peak tension, and time for 50% relaxation) were calculated by considering the maximal contraction induced by OA as 100%.

**Serum Steroid Levels.** Trunk blood was allowed to clot at 4°C. The clotted blood was centrifuged at 2000g for 15 min. Sera were collected, frozen, and stored at −80°C until analysis. Serum concentrations of E₂ and P₄ were measured by using double-antibody radioimmunoassay kits (DRG Instruments, Marburg, Germany), following instructions by the manufacturers. Both assays used 125I-labeled tracers.

**RNA Isolation.** Total RNA of approximately 20 mg of rat uterine tissue was isolated according to the acid guanidium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) as previously described (Magraner et al., 1998). The RNA samples were treated with fast protein liquid chromatography pure DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden) in DNase buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂) containing 10 units of RNasin (Promega, Madison, WI) to eliminate contaminating genomic DNA. The integrity of the purified RNA was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde gel. The quantity of total RNA was determined by spectrophotometric measurement at 260 nM. RNA samples (10 μg each) were resuspended in diethylpyrocarbonate-treated water and stored at −80°C until use.

**RT-PCR.** RT-PCR reactions were carried out as previously described (Pinto et al., 1999). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random hexamers according to Amersham Pharmacia Biotech instructions (First-Strand cDNA Synthesis kit; Amersham Pharmacia Biotech) in a 15-μl volume reaction containing 5 μg of DNase-treated total RNA. The resulting cDNA samples were amplified by polymerase chain reaction (PCR) using a DNA thermal cycler (MJ Research, Watertown, MA) and the following specific primer pairs: 1) PP1-δ isoform, forward 5’-AACCAT-GAGTGTCGATCAC-3’ and reverse 5’-CACCAGCATTGTCAAA-ACTCGCC-3’, correspond to nucleotides 411 to 432 and 861 to 882, respectively, based on the published cDNA sequence of the rat PP1-δ (or β) isoform (Sasaki et al., 1990) and were designed to amplify a PCR product of 472 bp; 2) MYPT, forward 5’-GACTCCTTGCTGG-TCGTC-3’ and reverse 5’-AGGCCCCATTCTCTATCATC-3’, correspond to nucleotides 2632 to 2651 and 2969 to 2989, respectively, of MYPT cDNA from rat aorta (Chen et al., 1994), giving a PCR product of 358 bp; and 3) MLCK, forward 5’-GGAAGACTGCAATCCATGATTC-3’ and reverse 5’-TGCAAGGTGTACCTTGGCG-3’, correspond to nucleotides 625 to 644 and 2651 to 2670, respectively, of MLCK cDNA from rabbit aorta (Chen et al., 1994), giving a PCR product of 358 bp. The PCR reactions were carried out on a Peltier thermal cycle (PTC-100, MJ Research) according to the manu-
ated primers were designed to amplify rat uterine MLCK, based on the cDNA sequence of human MLCK from hippocampus (Potier et al., 1995). Amplification of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcript was used to control the efficiency of RT-PCR among the samples. Sequences of forward and reverse primers for GAPDH were 5′-CTACCCACGGCAAGTTCAAT-3′ and 5′-CTTCTGAGTGGCAGTGATGG-3′, respectively, corresponding to nucleotide position 176 to 195 and 563 to 582, respectively, of the rat GAPDH cDNA sequence (Tso et al., 1985). The expected size of the PCR product was 404 bp.

PCR mixes contained 0.2 μM primers, 1.5 U of Taq polymerase (Amersham Pharmacia Biotech), the buffer supplied, 2.5 mM MgCl₂, 200 μM dNTPs, and cDNA in 25 μl. Each experiment also contained two negative controls, one with the RT reaction containing no added RNA and the other one containing RNA that had not been reverse transcribed. Following heating at 94°C for 2 min, the parameters used for PCR amplification were as follows: denaturation, 10 s at 94°C; annealing, 20 s at 60°C (for PP1-δ, MLCK, and GAPDH) or 20 s at 64°C (for MYPT); and extension, 30 s at 72°C. Cycle numbers were 28 for PP1-δ and GAPDH and 30 for MLCK and MYPT. Number of cycles for each PCR product was chosen from preliminary experiments in which PCR reactions were performed at a serially incremented number of cycles, until linear detection of products. The products of the amplification were separated by gel electrophoresis on 1.7% agarose, stained with ethidium bromide, and visualized and photographed under UV transilluminator (Spectronics Corp., Rochester, NY).

A semiquantitative RT-PCR assay was used to determine the relative concentrations of PP1-δ, MYPT, and MLCK in uteri from rats at early (day 3) and late (day 21) pregnancy (Bove and Koos, 1993; Pinto et al., 1999). After normalization to GAPDH, equal aliquots of the RT solution for the samples to be compared were serially half diluted and then amplified for a fixed number of cycles, to ensure analysis of products in the exponential range of amplification. PCR products of PP1-δ, MYPT, or MLCK and the correspondent molecular size standard were loaded on the same agarose gel in which GAPDH products and the correspondent molecular size standard were loaded, 30 min ahead. mRNA levels for PP1-δ, MYPT, MLCK, and GAPDH were analyzed on each uterine sample and experiments in each day of pregnancy were carried out in five different animals, with each RT-PCR assay being performed at least in triplicate. The level of expression of each PCR product was normalized to GAPDH mRNA levels and the relative amount of the target sequence in samples from day 21 of pregnancy was expressed as a percentage of the value determined in day 3 pregnant rats. The band intensities were scanned by densitometry using a video documentation system and the image analysis software Intelligent Quantifier (BioImage Systems Corp., Ann Arbor, MI). The identity of the PCR products was established by DNA sequence analysis, as previously described (Pinto et al., 1999).

**Statistical Analysis.** Values are expressed as the mean ± S.E.M. Unless otherwise indicated, n represents the number of experiments in n different animals. Statistical significance of differences between two means was assessed by Student’s t test. Multiple means were compared by one-way analysis of variance followed by Tukey’s multiple comparison test (GraphPad Prism 3.0; GraphPad, San Diego, CA). A probability level of $P < 0.05$ was regarded as significant.

**Results**

**Effects of Okadaic Acid on the Mechanical Activity in the Rat Uterus.** OA (5–20 μM) elicited concentration-dependent contraction of the isolated rat uterus. The concentration-response curves obtained in nonpregnant uteri under different hormonal conditions are shown in Fig. 1. OA (≥5 μM) caused a transient contraction of similar time course in both the pregnant (Fig. 2) and the nonpregnant rat uterus (Figs. 1 and 3). The contraction developed slowly and after plateauing, was followed by a gradual decay in tension (Fig. 2). Due to the limited availability of the toxin and its irreversible effects, a single concentration of OA (20 μM) was used in subsequent experiments.

**TABLE 1**

Parameters characterizing the contractile response induced by OA (20 μM) in the rat uterus during the course of pregnancy, in day 1 postpartum and in day 11 pregnant rats pretreated with RU486 (10 mg · kg⁻¹) 24 h before the experiment

<table>
<thead>
<tr>
<th>n</th>
<th>$E_{max}$</th>
<th>Time for 50% Contraction</th>
<th>Time to Peak Tension</th>
<th>Time for 50% Tension Decay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mg dry weight</td>
<td>min</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>Day 1</td>
<td>7</td>
<td>28.7 ± 4.6$^P$</td>
<td>6.2 ± 1.3</td>
<td>10.5 ± 1.4$^*$</td>
</tr>
<tr>
<td>Day 3</td>
<td>5</td>
<td>159.0 ± 9.7$^P$</td>
<td>7.4 ± 1.1</td>
<td>20.5 ± 0.8</td>
</tr>
<tr>
<td>Day 6</td>
<td>6</td>
<td>156.5 ± 14.8$^P$</td>
<td>12.5 ± 1.2</td>
<td>30.0 ± 1.1</td>
</tr>
<tr>
<td>Day 11</td>
<td>6</td>
<td>120.1 ± 47.8$^P$</td>
<td>26.5 ± 4.5$^*$</td>
<td>61.5 ± 11.6$^*$</td>
</tr>
<tr>
<td>Day 16</td>
<td>5</td>
<td>436.9 ± 50.6$^*P$</td>
<td>12.5 ± 1.0</td>
<td>49.7 ± 5.1$^*$</td>
</tr>
<tr>
<td>Day 21</td>
<td>5</td>
<td>967.0 ± 40.4$^*P$</td>
<td>13.4 ± 2.1</td>
<td>47.6 ± 7.4</td>
</tr>
<tr>
<td>Postpartum</td>
<td>6</td>
<td>249.1 ± 6.5$^*$</td>
<td>8.1 ± 1.4</td>
<td>37.0 ± 2.0</td>
</tr>
<tr>
<td>Day 11-RU486</td>
<td>3</td>
<td>217.4 ± 16.1$^*$</td>
<td>20.3 ± 3.1</td>
<td>55.6 ± 2.6$^*$</td>
</tr>
</tbody>
</table>

$n$, number of experiments in $n$ different animals.

$^P < 0.05$, significant differences from values on pregnancy day 3.

$^*P < 0.05$, significant differences from values on pregnancy day 21. No significant differences ($P > 0.05$) were found between responses elicited by OA in day 11 pregnant rats pretreated with olive oil ($n = 3$) or untreated ($n = 3$). Results presented are the mean ± S.E.M. in the six different animals.
In the nonpregnant rat uterus, the amplitude of the contraction to OA (20 μM) varied at different stages of the estrous cycle following the sequence: proestrus > estrus > diestrus ≥ metoestrus (Fig. 3A). In rats treated with 17β-estradiol benzoate (20, 200, or 3000 μg·kg⁻¹), the magnitude of the contraction to OA was directly related to the concentration of E₂ administered (Fig. 3B).

In the pregnant uterus, the magnitude of the contraction to OA (20 μM) was low at early pregnancy; increased significantly during the course of pregnancy, reaching a maximum in day 21 pregnant rats; and declined after delivery (Fig. 4). The characteristics of the OA-induced response are shown in Table 1. Whatever the day of pregnancy, the amplitude of the OA-induced contraction was only slightly lower in Ca²⁺-free, 3 mM EGTA-containing solution than in Ca²⁺-containing medium. As a representative example, Fig. 5 shows the OA-induced contractile response on day 3 and day 16 of pregnancy, in the presence and in the absence of Ca²⁺ (Fig. 3A). In rats treated with 17β-estradiol benzoate (20, 200, or 3000 μg·kg⁻¹), the magnitude of the contraction to OA was directly related to the concentration of E₂ administered (Fig. 3B).

In the pregnant uterus, the contraction to OA (20 μM) was low at early pregnancy; increased significantly during the course of pregnancy, reaching a maximum in day 21 pregnant rats; and declined after delivery (Fig. 4). The characteristics of the OA-induced response are shown in Table 1. Whatever the day of pregnancy, the amplitude of the OA-induced contraction was only slightly lower in Ca²⁺-free, 3 mM EGTA-containing solution than in Ca²⁺-containing medium. As a representative example, Fig. 5 shows the OA-induced contractile response on day 3 and day 16 of pregnancy, in the presence and in the absence of Ca²⁺ (Fig. 3A). In rats treated with 17β-estradiol benzoate (20, 200, or 3000 μg·kg⁻¹), the magnitude of the contraction to OA was directly related to the concentration of E₂ administered (Fig. 3B).

The data presented in this article show that the contractile response induced by okadaic acid in the rat uterus is dramatically altered during the course of pregnancy, being significantly higher in late than in early pregnancy. PP1-δ and MYPT mRNA expression levels were higher on pregnancy day 3 than on pregnancy day 21, whereas MLCK mRNA expression was unchanged. These results show that pregnancy is associated with changes in the sensitivity of the rat uterine contractile machinery to MLCP inhibition. A significant alteration in the magnitude of the OA-induced contraction was also observed in nonpregnant uteri during the estrous cycle. Moreover, the amplitude of the OA-induced contraction in spontaneously cycling, estrogen-treated and

### TABLE 2

<table>
<thead>
<tr>
<th>Animals</th>
<th>n</th>
<th>n'</th>
<th>Serum Estradiol (pg/ml)</th>
<th>Serum Progesterone (ng/ml)</th>
<th>E₂/P₄ Ratio (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proestrus</td>
<td>6</td>
<td>3</td>
<td>55.6 ± 16.9</td>
<td>27.9 ± 6.6</td>
<td>2.10</td>
</tr>
<tr>
<td>Estrus</td>
<td>6</td>
<td>3</td>
<td>18.5 ± 6.5</td>
<td>30.3 ± 5.7</td>
<td>0.61</td>
</tr>
<tr>
<td>Metestrus</td>
<td>6</td>
<td>3</td>
<td>8.4 ± 3.3</td>
<td>38.4 ± 7.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Diestrus</td>
<td>6</td>
<td>3</td>
<td>26.9 ± 9.2</td>
<td>32.9 ± 2.5</td>
<td>0.82</td>
</tr>
<tr>
<td>E₂-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μg/kg</td>
<td>8</td>
<td>4</td>
<td>19.8 ± 1.9</td>
<td>26.8 ± 8.1</td>
<td>0.73</td>
</tr>
<tr>
<td>200 μg/kg</td>
<td>8</td>
<td>4</td>
<td>33.1 ± 8.4</td>
<td>29.0 ± 5.1</td>
<td>1.10</td>
</tr>
<tr>
<td>3000 μg/kg</td>
<td>8</td>
<td>4</td>
<td>182.5 ± 28.3</td>
<td>21.7 ± 6.7</td>
<td>8.41</td>
</tr>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>6</td>
<td>3</td>
<td>16.7 ± 1.8</td>
<td>44.6 ± 4.0</td>
<td>0.37</td>
</tr>
<tr>
<td>Day 21</td>
<td>7</td>
<td>3</td>
<td>86.4 ± 4.0</td>
<td>33.7 ± 5.2</td>
<td>2.56</td>
</tr>
</tbody>
</table>

n, number of experiments; n', number of animals.
pregnant rats was positively correlated to the ratio of E2/P4 serum levels. These data suggest that, at least in the uterus, ovarian steroids are able to modulate the Ca2+ sensitivity of the contractile machinery.

OA (20 μM) induces a transient contraction of similar time course in uteri from nonpregnant and pregnant rats at all stages of pregnancy. In smooth muscle, the mechanism of action of OA is ascribed to its binding to the catalytic subunit of MLCPP, leading to phosphatase inhibition and then to an increase in the phosphorylation state of LC20 (Bialojan et al., 1988; Obara et al., 1989; Gong et al., 1992). Okadanol, an OA analog that is ineffective as a phosphatase inhibitor (Nishiwaki et al., 1990), failed to contract uterine smooth muscle (Arteche et al., 1997). In the nonpregnant rat uterus, the OA-induced contraction did not involve activation of neurogenic mechanisms or membrane-coupled receptors and was not altered in the presence of various cAMP- and/or cGMP-elevating agents (Candenias et al., 1992). Moreover, the OA-induced response was not dependent on calmodulin and was unaffected by inhibition of protein kinase C, protein kinase A, Ca2+/calmodulin kinase II, phospholipase C, or phospholipase A2 (Arteche et al., 1997). It was also found that the amplitude of the contraction to OA was significantly reduced in the presence of a selective inhibitor of MLCK (Arteche et al., 1997). Taken together, these data suggest that the contractile response induced by OA in the rat uterus is due to a direct interaction with the contractile machinery and appears to be mediated by inhibition of MLCPP. In this connection, it has been shown that OA induces Lc20 phosphorylation and inhibited dephosphorylation of phosphorylated Lc20 in all smooth muscles that have been studied (Bialojan et al., 1988; Erdödi et al., 1988; Obara et al., 1989; Gong et al., 1992).

In the pregnant rat uterus, it has been shown that L-type calcium channel density increases during the course of pregnancy, being maximal at term, just before labor (Mershon et al., 1994; Tezuka et al., 1995). The present study shows that contractions elicited by OA in uteri from pregnant animals at different stages of pregnancy were only slightly lower in amplitude and less sustained in Ca2+-free 3 mM EGTA than in Ca2+-containing solution. These results are similar to those previously obtained in the nonpregnant rat uterus (Candenias et al., 1994). Moreover, OA (20 μM) failed to alter [Ca2+]i values and had no effect on Ca2+ movements in myometrial cells (Arteche et al., 1997) as also occurs in other tissues (Hirano et al., 1989; Obara et al., 1989). This suggests that L-type Ca2+ channels and the subsequent increase in [Ca2+]i, that result from its activation are not responsible for the increase in the amplitude of the response to OA observed during pregnancy.

Fig. 6. Agarose gels showing RT-PCR products for uterine cDNA from day 3 (lanes 1–5) and day 21 (lanes 6–10) pregnant rats. Equal aliquots of the RT solution were serially diluted in a 1:2 ratio and amplified for 28 (PP1-d and GAPDH) or 30 (MYPT and MLCK) cycles with specific primers for each target sequence. Lanes 1 and 6 represent the more diluted samples in each series. The observation of a steadily declining yield of product at each dilution step confirmed that the comparison of the two samples was made in the exponential portion of the amplification curve. The amount of PP1-d, MYPT, or MLCK mRNA was then assessed relative to the amount of the coamplified GAPDH fragment. Data represent results in five different animals per day of pregnancy.

Fig. 7. PP1-d, MYPT, and MLCK mRNA levels in uteri from day 3 and day 21 pregnant rats. The relative mRNA level in each tissue was determined as the ratio of PP1-d, MYPT, or MLCK PCR product/GAPDH PCR product measured by densitometry. After normalization to GAPDH, expression of each mRNA in uteri from day 3 pregnant rats was considered as 100. Each column represents the mean ± S.E.M. of 12 to 21 experiments in five different animals. ***, P < 0.001 (significant difference from mRNA levels in pregnancy day 3 rats, paired t test).
Smooth muscle contraction is primarily regulated by the phosphorylation state of LC20, which depends on the balance between MLCK and MLCP activity. We therefore investigated whether MLCK and MLCP mRNA expression varied during the course of pregnancy. Our results show that MLCK mRNA levels were similar in early (day 3) and late (day 21) pregnant rats. On the other hand, PP1-δ mRNA expression levels were 2-fold higher on pregnancy day 3, compared with pregnancy day 21. These data suggest that changes in MLCP activity could explain the alterations in the magnitude of the OA-induced contraction observed during pregnancy.

In the cells, protein phosphatases of the PPP family exist as holoenzymes, with the catalytic subunit associated to one or more regulatory proteins (Hartshorne and Hirano, 1999). The association of catalytic subunits with a variety of regulatory proteins generates a diversity of phosphatase holoenzymes that may be targeted to specific substrates and signaling complexes (Oliver and Shenolikar, 1998). At the present moment, it is unknown whether the formation of these holoenzymes is regulated as a function of subunit abundance or as a consequence of catalytic/regulatory protein modification. In addition to the catalytic subunit, PP1-δ, smooth muscle MLCP is composed of two regulatory subunits (Chen et al., 1994; Hartshorne and Hirano, 1999). The large regulatory subunit MYPT binds to both PP1-δ and phosphorylated myosin and appears to be essential for targeting MLCP to its substrate, i.e., myosin (Shimizu et al., 1994; Hartshorne and Hirano, 1999). The present study shows that MYPT mRNA expression was about 2-fold higher in day 3 than in day 21 pregnant rats. Although these results do not negate the existence of post-translational regulatory mechanisms, the fact that MYPT mRNA expression varied during the course of pregnancy suggests that regulation of expression levels of subunits could be a way by which the cell regulates the formation of a particular PSP holoenzyme.

The magnitude of the contraction to OA was low at early pregnancy and high at late pregnancy. Our data also show (1) in nonpregnant rats treated with E2, the magnitude of the contraction to OA was directly related to the concentration of E2 administered; (2) the contraction to OA was higher in day 11 pregnant rats pretreated with RU486 than in day 11 control animals; and (3) the amplitude of the contraction to OA in spontaneously cycling, E2-treated and pregnant rats was, in most cases, positively correlated to the ratio of E2/P4 serum levels. This strongly suggests that ovarian steroids are able to modulate the Ca2+ sensitivity of the uterine contractile machinery by regulating MLCP activity. The lack of positive correlation under certain hormonal conditions, i.e., the estrus and diestrus stages of the ovarian cycle, could suggest that, in addition to E2 and P4, other still unknown factors may also be involved in the regulation of the sensitivity of the uterine contractile machinery.

The uterus must be maintained in a quiescent state during pregnancy and develop important contractions during labor (Wray, 1993). The present data show that, in addition to the important changes in the expression of different membrane receptors, ion channels, gap junctions, and local hormone levels (Lefebvre et al., 1992; Wray, 1993; Garfield, 1994; Mershon et al., 1994; Pinto et al., 2000), the sensitivity of the uterine contractile proteins to protein phosphatase inhibition varied during the course of pregnancy. This is consistent with the observation that the mechanical response elicited by contractile agents in the myometrium is progressively increased during the course of pregnancy (Izumi, 1985). This could at least partially explain the poor effect of most drugs in the treatment of post- or preterm labor.

In conclusion, the present data show that ovarian steroids regulate the magnitude of the contraction to OA in the rat uterus. Changes in the expression and activity of myosin light chain phosphatase may contribute to modulate the level of uterine contractile force during the estrous cycle, pregnancy, and labor.

Acknowledgment

We are very grateful to Roussel-Uclaf for kind donation of RU486.

References


