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Changes in Rat Myometrial Plasma Membrane Protein Kinase A are Confined to Parturition

Chun-Ying Ku, PhD¹, Dilyara A. Murtazina, PhD¹, Yoon-Sun Kim, DVM, PhD¹, Robert E. Garfield, PhD², and Barbara M. Sanborn, PhD^{1,*}

¹ Colorado State University, Dept. of Biomedical Sciences, Fort Collins, CO

² St. Joseph's Hospital and Medical Center, Dept. of Ob/Gyn, Phoenix, AZ

Abstract

We have previously shown that pregnant rat myometrial plasma membrane-associated cAMP-dependent protein kinase A (PKA) decreases prior to delivery, coincident with a decline in the inhibitory effect of cAMP on contractant-stimulated parameters. We now find that rat myometrial membrane-associated PKA concentrations in early to mid-pregnancy are equivalent to those in cycling rats. Following the decline associated with parturition, membrane PKA recovers within 1–2 days postpartum. Treatment with the antiprogesterin onapristone caused a decrease in myometrial membrane PKA-catalytic and regulatory subunits compared to untreated controls by 12 h. This coincided temporally with recently reported increases in electrical and contractile activity. In unilaterally pregnant rats, the decline in plasma membrane PKA was observed in both nonpregnant and pregnant horns but was more rapid in the pregnant horns. These data indicate that the myometrial plasma membrane PKA pattern before and during most of pregnancy is not consistent with progesterone exerting a primary influence on PKA membrane localization. Rather, the fall in membrane PKA associated with parturition may contribute to or be influenced by the increased contractile and electrical activity of labor that is a consequence of the loss of progesterone influence and is not absolutely dependent on the presence of fetuses.

Keywords

myometrium; protein kinase A; parturition; pregnancy; progesterone; onapristone

INTRODUCTION

The myometrium undergoes a number of changes in the process of adapting to pregnancy, the presence of the growing conceptus(es), delivery and involution^{1–5}. Unlike vascular smooth muscle, which responds to stretch with an increase in contraction and maintenance of tone, the myometrium of most species is relative quiescent during gestation despite the increased stretch associated with the growing fetus(es). This is partially attributable to the enhanced expression of pathways favoring relaxation and of the agents that stimulate those pathways, such as cAMP and NO/cGMP pathways^{4–6}. As parturition approaches, a number of biochemical changes occur that result in a shift in balance from relaxant to contractant pathways^{2–5}. There is an up-regulation of contractant hormone receptors that activate Gαq-linked phospholipase C and increase inositol trisphosphate (IP₃) formation. IP₃ binds to its receptor on the endoplasmic reticulum and releases Ca²⁺ from intracellular stores, thus promoting contraction and

* Address correspondence to: Barbara M. Sanborn, Ph.D., Department of Biomedical Sciences, ARBL, Campus Delivery 1683, Colorado State University, Fort Collins, CO 80523, Phone: (970)-491-4263, Fax: (970)-491-7569, Barbara.Sanborn@colostate.edu.

stimulates Ca^{2+} entry from the extracellular environment as a result of signal-regulated ion channel mechanisms. Other proteins associated with labor such as cyclooxygenase 2 and connexin 43, important in generation of contractile prostaglandins and for gap junction formation that enhances coordinated contractions, respectively, are up-regulated at the end of pregnancy in response to stretch and/or the decline in the influence of progesterone^{1,7-9}.

Both cAMP and cGMP inhibit uterine contractions at mid-pregnancy but their effectiveness decreases near term^{6,10}. In part, this is due to a decline in the prevalence of the pathways for their synthesis and upregulation of degradation pathways. Nonetheless, even the ability of nonhydrolyzable cAMP analogs to inhibit $\text{G}\alpha_q$ -stimulated IP_3 formation, which would theoretically bypass these mechanisms, declines between day 19 and day 21 of gestation in the rat¹¹. This decline is associated with a decrease in plasma membrane PKA, and the effects of cAMP can be inhibited by an AKAP (A kinase anchoring protein) interaction inhibitor^{11,12}. A similar, although less dramatic, decline in partially purified membrane PKA occurs in laboring versus nonlaboring term human myometrium¹³. Another study reports higher amounts of $\text{RII}\alpha$ subunit protein in cell homogenates and the $40,000\times$ g particulate fraction (total cellular membranes), and higher PKA II catalytic activity in nonlaboring human myometrium than in comparable fractions from laboring or nonpregnant human myometrium¹⁴.

While we know from a previous study that antiprogestins trigger a gradual fall in pregnant rat myometrial plasma membrane PKA and that progesterone prolongs both pregnancy and plasma membrane PKA in this species¹⁵, many questions remained as to the dynamics and control of this process. In this study, we determine the pattern of expression of membrane-associated rat myometrial PKA before and during pregnancy and delivery, the relationship between the change in myometrial membrane-bound PKA following exposure to an antiprogestin at mid-pregnancy and the onset of uterine electrical and contractile activity, and the effect of the presence of fetuses on the decline in myometrial membrane-bound PKA in unilaterally pregnant rats.

MATERIALS AND METHODS

Animals

Adult female and male Sprague Dawley rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The day when the vaginal plug was observed was designated by the supplier as day 0 of gestation. Labor/delivery was defined as the presence of 1 or more pups. Rats from this supplier delivered between day 21 and day 23 of gestation. All pregnancy samples were collected in the early morning.

Animals were housed individually under standard environmental conditions (12L:12D cycle). The experiments were conducted in accordance with institutional practices in an AAALAC-accredited facility. The stage of the estrus cycle of female rats was determined by examination of cellular types in vaginal smears¹⁶. Where indicated, pregnant animals were injected subcutaneously on day 16 of pregnancy with 3 mg onapristone (Schering AG, Berlin, Germany) in oil. Animals were sacrificed by CO_2 inhalation according to AVMA Guidelines on Euthanasia. Each uterine horn was quickly excised. In pregnant rats, the placentae and fetuses in each horn were rapidly removed and the fetuses subjected to isofluorane inhalation. In all cases, the endometrium was removed by gentle scraping with a scalpel.

Unilateral Uterine Horn Ligation

Female rats were anesthetized and the abdominal area shaved. A ventral midline incision was made under sterile conditions cranial to the pubis. One uterine horn was ligated near the cervical end with 5-0 Ethilon sterile nylon sutures, taking care not to affect vasculature. The incision

was closed with sterile 4-0 Dexon II absorbable suture in the abdominal wall and with wound clips in the skin. Animals were treated with buprenorphine for 3 days and allowed to recover from surgery for at least 1 week before mating. Myometrial samples were collected from both uterine horns on gestational days 16, 19 and 21, and myometrial plasma membrane was isolated as described below. No pregnancies were observed in the ligated horns of any animals subjected to unilateral uterine horn ligation.

Membrane Preparation

Myometrial plasma membranes from individual animals were prepared by discontinuous sucrose density centrifugation from crude membrane pellets as described previously¹⁷. Membranes were resuspended and stored at -80°C in sample buffer (10 mM Tris-HCl, pH 7.2, 250 mM sucrose, 1 mM EGTA, 1 X protease inhibitor cocktail (1.04 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM N-(trans-Epoxy succinyl)-L-leucine 4- guanidinobutylamide, Sigma-Aldrich, St. Louis, MO). Protein concentration was measured using the Bradford reagent (BioRad, Hercules, CA).

Western Blot Analysis

Myometrial plasma membrane prepared from individual animals (10 μg protein) was subjected to SDS-PAGE in 4–15% gels (BioRad, Hercules, CA). Protein was transferred to nitrocellulose membranes (Whatman Schleicher and Schuell, Florham Park, NJ) at 100 V for 1.25 hours at 4°C . The membranes were blocked in 5% milk in phosphate buffered saline containing Tween-20 (2.68 mM KCl, 1.47 mM KH_2PO_4 , 136.9 mM NaCl, 8.1 mM Na_2HPO_4 , 0.1% Tween-20, pH 7.2) for 1 hour at room temperature. Primary antibodies were polyclonal rabbit anti PKA α catalytic (sc903) and RII α (sc909) regulatory subunits (1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibody (1:2000) was horseradish peroxidase (HRP) - conjugated donkey anti-rabbit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were detected with Enhanced Chemiluminescence Plus (ECL) reagent (Amersham Biosciences, Piscataway, NJ) and visualized using a Storm imager (Amersham Biosciences, Piscataway, NJ). Densitometric quantitation of immunoreactive bands was accomplished with ImageQuant software analysis. Membranes were stripped and reprobed with anti-G-beta (sc378) or caveolin-1 (sc894)(1:500) antibodies for normalization (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistics

Data are expressed as the mean $\pm$ SEM of samples from 3–5 separate animals. Results were analyzed by one-way analysis of variance and Duncan's modified multiple range or Tukey's test. Differences were regarded as statistically significant at $P < 0.05$.

RESULTS

Membrane PKA Profile in Cycling and Pregnant Rats

We have previously shown a decline in plasma membrane PKA regulatory and catalytic subunits between day 16 and day 21 of gestation in pregnant rat myometrium, coincident with a decline in the ability of cAMP to inhibit oxytocin-stimulated phosphatidylinositol turnover¹¹. However, these studies did not determine whether myometrial membrane PKA increased in pregnancy relative to the nonpregnant myometrium or simply declined from basal levels near term and also did not determine what happened to membrane PKA after delivery. To address these questions, we determined myometrial membrane PKA at different stages of the estrus cycle in non-pregnant rats, across pregnancy and several days postpartum.

Figure 1 (left) shows that the concentration of plasma membrane PKA catalytic subunit, expressed relative to day 19 of pregnancy, in cycling rats did not change between proestrus, estrus and diestrus. In pregnant rats, plasma membrane PKA catalytic subunit concentrations were not changed compared to nonpregnant values until day 16, where there was a small increase that was only significant compared to day 5, day 12 and day 19 (Figure 1, right). There was a dramatic decline in plasma membrane PKA after day 16, with day 22 values reaching significance versus nonpregnant and all pre-delivery values. The samples on day 21 included 2 animals who had not yet delivered (mean PKA ratio of 1.02) and one animal who had delivered (0.51). On day 22, one animal had delivered (0.53) and two had not yet delivered (mean of 0.52). Following delivery, plasma membrane PKA increased, reaching pre-term levels by day 23. For purposes of comparison, reported plasma progesterone concentrations during the estrus cycle and rat pregnancy^{18,19} are also plotted on this graph.

Temporal Changes in Membrane-bound PKA in Relation to the Onset of Uterine Electrical and Contractile Activity

Onapristone is an antiprogestin that acts at the level of progesterone receptor and has reduced antiglucocorticoid activity in comparison with RU486^{20,21}. Onapristone triggers premature labor and delivery in instrumented rats, preceded by an increase in electrical and contractile activity²². In an attempt to determine whether the decline in rat myometrial plasma membrane PKA precedes, coincides with or follows the onset of electrical and contractile activity associated with parturition, we treated pregnant rats with 3 mg onapristone on day 16 of gestation and determined membrane PKA 6–25 hours later. Control animals received injection of vehicle. No change in PKA was observed between 6 and 20 hours after injection in this group, consistent with a previous study using RU486¹⁵, where there was no difference between 0 and 24 hours after vehicle injection. No pups were delivered between 0 and 20 hours after injection with onapristone. As shown in Figure 2, onapristone treatment caused a decline in myometrial membrane PKA catalytic subunit (PKA-cat) ($94 \pm 9\%$, $50 \pm 13\%*$ and $43 \pm 10\%*$ of the 6 hour control remaining) at 6, 12 and 25 h, respectively ($n=3$, $*P<0.05$), and a decline in regulatory subunit (PKA-RII) ($83 \pm 14\%$, $57 \pm 7\%*$, $60 \pm 14\%*$ remaining at 6, 12 and 25 hours, respectively ($n=3$, $*P<0.05$)).

The data in Figure 2 are superimposed on the findings of Shi et al²², obtained in pregnant rats in which a microtip catheter connected to a transducer was introduced into the uterine cavity between the membranes and the uterine wall and electromyographic electrodes were implanted in the uterine wall²³. Myometrial electrical (EMG energy) and contractile activity (IUP intensity) activity were recorded. In this study, 3 mg of onapristone was injected subcutaneously on the morning of day 16 (counting the day of the vaginal plug as day 0) and myoelectrical (EMG energy) and contractile activity (IUP intensity) increased dramatically by day 17. Most animals delivered pups between day 17 and day 19²². As can be seen from Figure 2, the decline in plasma membrane PKA in our study coincided temporally with the increase in myometrial electrical and contractile activity observed by Shi et al.

The Relationship of the Presence of Fetuses to the Decline in Rat Myometrial Membrane PKA near Term

The data in Figure 2 are consistent with an influence of progesterone in maintaining the concentration of PKA in the myometrial plasma membrane. If this is the result of a systemic effect of progesterone independent of any influence of the presence of fetuses or the associated stretch, the same effect should be seen in nonpregnant and pregnant horns of unilaterally pregnant animals. To determine if this is the case, we ligated one uterine horn in nonpregnant female rats, mated the animals and determined PKA in myometrial plasma membrane isolated from both uterine horns on day 16, day 19 and day 21 of gestation. Membrane PKA-cat concentrations in the nonpregnant uterine horns gradually declined to $77 \pm 14\%$, and $58 \pm 7\%$

* of the value in day 16 nonpregnant horns on day 19 and day 21 of gestation, respectively (n=3, *P<0.05) (Figure 3). PKA-cat changes in the pregnant horn were more rapid: $103 \pm 13\%$, $38 \pm 1\%$ *, $34 \pm 5\%$ * of day 16 nonpregnant horn values at day 16, day 19 and day 21, respectively (n=3, *P<0.05) (Fig. 3). Membrane PKA concentrations in day 19 pregnant horns were significantly lower than in nonpregnant horns, but by day 21, this difference had disappeared. These data suggest that the decline in myometrial plasma membrane-associated PKA prior to delivery in the rat is due in part to a systemic effect related to the fall in progesterone prior to labor but is also influenced by the presence of the fetuses or associated mechanical factors.

DISCUSSION

We had concluded in a previous study that progesterone played a significant role in maintaining the association of PKA with myometrial plasma membrane in the rat¹⁵. Plasma progesterone concentrations rapidly increase 4-fold (to ~250 nM) between day 0 and day 10 of pregnancy, increase to a maximum of 6–7-fold on day 14, remain high through day 18, and then decline precipitously prior to delivery as a consequence of luteolysis^{19,24}. If progesterone were to exert a positive effect on myometrial PKA membrane association via a classical or membrane receptor-mediated mechanism (affinities in the 1–250 nM range for progesterone receptors and progesterone receptor membrane component 1 (PRMC1), respectively^{25,26}), one might expect the concentration of membrane PKA to increase rapidly and follow a similar pattern, or at least plateau at some point. The data presented here show quite a different pattern. Rather than increasing in early pregnancy, membrane PKA was not different from essentially constant values in nonpregnant proestrus, estrus and diestrus rat myometrium. There was a small increase at day 16, but this was significant against only a few of the points. It would take additional experiments to establish whether there is a relationship between plasma progesterone and membrane PKA at that point, but we do not favor that interpretation for the reasons discussed below.

After day 16 of pregnancy, there was a marked decrease in membrane PKA associated with parturition. Membrane PKA rapidly recovered to pre-pregnancy concentrations within 1–2 days postpartum. Taken in total, these data do not support a mechanism whereby progesterone, acting through receptor-mediated mechanisms, increases myometrial membrane-associated PKA during pregnancy in the rat. Rather, they suggest either that progesterone, through receptor-mediated mechanisms, counteracts a process that lowers membrane-associated PKA or that the decline in membrane PKA is a secondary consequence of the chain of events set into motion by the absence of progesterone. For example, the absence of progesterone influence results in an increase in electrical and physical contractile activity that causes changes in myometrial cell and muscle bundle function, including changes in ion flux and intracellular signals. These changes could trigger events that result in the decrease in plasma membrane PKA.

The onapristone study provides additional insight into this question. Onapristone (ZK 98 299) has less antiglucocorticoid activity than RU486, another antiprogesterin^{20,21} and therefore is the preferred antiprogesterin for differentiating the role of progesterone in pregnancy-related events. Myometrial membrane PKA declined following onapristone administration with a time course similar to that previously observed using RU486¹⁵. This time course is remarkably similar to that recorded for the onset of *in vivo* electrical activity and increased uterine pressure indicative of contractile activity²². These data do not definitively indicate if the fall in PKA and the onset of electrical and contractile activity are causally related. Nonetheless, these data using antiprogesterins, coupled with the lack of change in plasma membrane PKA in nonpregnant myometrium and throughout most of pregnancy and our previous finding that progesterone administration to rats in late pregnancy not only prolonged gestation but also prevented the fall

in plasma membrane PKA¹⁵, suggest that the onset of electrical and contractile activity may influence the fall in plasma membrane PKA or vice versa.

The study examining gravid versus nongravid uterine horns provides additional insight into the influence of the presence of fetuses on myometrial membrane PKA. The data indicate that the decline in membrane PKA is not absolutely dependent on the presence of fetuses and/or the accompanying stretch, but the onset of decline was more rapid in the gravid than in the nongravid horn. Therefore, both a systemic influence and a possible enhancement due to local factors are indicated. To our knowledge, no one has measured *in vivo* contractile activity in the nonpregnant and pregnant horns of rats under these considerations, and therefore we do not know anything about the relative strength of the contractile activity in the nonpregnant and pregnant horns *in vivo*. However, we consider it likely that there will be some increase in contractile activity in the nonpregnant horn as progesterone falls at the end of pregnancy. Furthermore, many stretch-related changes in myometrial gene transcription and protein expression have been recorded, in particular for proteins that enhance the contractile nature of the tissue^{3,9}. Increased stretch in the gravid horn could result in increased intracellular calcium as a result of stimulation of stretch-activated ion channels, thus further increasing contractions or stretch-induced signaling. In turn, this could influence the fall in plasma membrane PKA.

Cyclic AMP pathways inhibit a number of components of the contractile pathways in myometrium (reviewed in^{4,27,28}). A number of changes in the ability to generate cAMP diminish near the end of pregnancy and play a role in preparing the uterus to respond more efficiently to the signals favoring contractions. These include changes in the concentrations of the receptors linked to Gas, the relative importance of G- proteins stimulating versus inhibiting adenylate cyclase, the isoforms of adenylate cyclase expressed, and the presence of phosphodiesterase isoforms²⁷. As an additional factor, the decline in membrane PKA would contribute to removing the influence of cAMP/PKA in the microdomain associated with the plasma membrane.

Membrane-associated A-Kinase-Anchoring-Proteins (AKAPs) have been reported to move from one cellular location to another in response to receptor signaling and other stimuli^{29,30}. In rat myometrium, the decline in plasma membrane PKA prior to delivery was not accompanied by a loss of the predominant membrane-associated AKAP from the membrane¹¹ but a decline was observed in in-labor compared to not-in-labor late term human myometrial membranes^{13,14}. Some studies have implicated changes in the phosphorylation status of AKAP150 (rat equivalent of AKAP79) or PKA-regII subunit in regulating the AKAP-PKA interaction^{31,32,33}. To date, we have not been able to detect a change in serine/threonine or tyrosine phosphorylation in immunoprecipitated AKAP150 or PKA-regII (unpublished observations), suggesting that other mechanisms may pertain in myometrium.

In summary, we find that myometrial plasma membrane PKA does not increase in early pregnancy compared to cycling rats. PKA increases around day 16 and then markedly declines prior to labor and returns to levels in cycling tissue by 1–2 days postpartum. When triggered by an antiprogesterin, the decline coincides temporally with the onset of electrical and physical activity and preterm delivery of the fetuses. The decline in PKA is more rapid in uterine horns containing fetuses. These data suggest that the fall in membrane PKA associated with delivery may contribute to or be influenced by the increased contractile and electrical activity of labor that is a consequence of a decrease in the influence of progesterone.

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Figure 1.

Expression of membrane PKA-catalytic subunit in cycling and pregnant rat myometrial plasma membranes. Individual measurements were normalized to G-beta protein in the same blot as loading control, and data were expressed relative to day 19 of pregnancy (solid circles). There were no detectable changes in G-beta/ μ g protein over this interval. Data represent mean $\pm$ SEM of data from 2–6 animals. The black bar represents the period over which delivery occurred. Significance by ANOVA analysis of log-transformed data between day 16 and days 19, 21, 22 and 23 are indicated (*P<0.05, **P<0.01, *** P<0.001). The plasma progesterone concentrations during the rat estrus cycle and during rat pregnancy (open triangles), as reported by Smith et al.¹⁸ and by Pepe and Rothchild¹⁹, respectively, are inserted in the figure for comparison.

Figure 2.

A progressive decline in rat myometrial plasma membrane (A) PKA-catalytic (PKA-cat) (diamonds) and (B) regulatory (PKA-RII) subunit (squares) concentrations occurred following administration of the antiprogesteron onapristone (3mg) on day 16 of gestation (indicated by the arrow), where the day the vaginal plug was observed was defined as day 0. Closed symbols represent onapristone treatment and open symbols represent vehicle controls. Individual data points were normalized to caveolin in the same blots as loading control; there were no detectable changes in caveolin/ μ g protein between samples in this study. Data were expressed relative to the 6 hour vehicle control; significant differences ($P < 0.05$) between groups are designated in the text. Superimposed on these data are the changes in contractile activity (IUP

intensity) (open triangles) and electrical activity (EMG energy) (open circles) in instrumented rats injected with the same concentration of onapristone²². The dating for Shi's study has been adjusted to correspond with the convention used by our supplier (day of vaginal plug = day 0). Corresponding Western blots for PKA regulatory and catalytic PKA subunits and caveolin for one set of samples are shown above the respective graphs. The bands for PKA RII (52 kDa) and catalytic (40 kDa) subunits and caveolin (22 kDa) were the expected sizes.

Figure 3. Myometrial rat plasma membrane PKA catalytic (PKA-cat) in the nonpregnant (black bars) and pregnant (grey bars) horns of unilaterally pregnant rats, measured between day 16 and day 21 of pregnancy. Individual data points were normalized to caveolin in the same blot as loading control and expressed relative to the value in the day 16 nonpregnant horn (mean $\pm$ SEM, $n=3$). Significant differences ($P<0.05$) between groups are designated with different lowercase letters. Western blots for one set of samples are shown above the graph.