Myometrial maxi-K channel β1 subunit modulation during pregnancy and after 17β-estradiol stimulation

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Received 25 April 2002; revised 14 June 2002; accepted 14 June 2002

First published online 2 July 2002

Edited by Ned Mantei

Abstract Myometrial maxi-K channels are modulated by β subunits. We aimed to determine whether β subunits are modulated to affect uterine excitability during gestation. RNase protection analyses revealed that mouse β1 subunit transcripts are regulated during gestation with peak expression at day 14 of pregnancy. Immunohistochemical analysis indicates an increase of this subunit during gestation. Upregulation of the β1 transcript occurs with 4-day exposure to 17β-estradiol but not progesterone, and acute estradiol exposure has no effect on β1 transcript expression. These findings verify that β1 subunit transcript is regulated in mouse myometrium during gestation and estrogens may contribute to this increase.

Key words: Mouse; Uterus; Maxi-K; β Subunit; Gestation; Estrogen; Progesterone

1. Introduction

Large-conductance calcium- and voltage-activated potassium channels (maxi-K channels) play a prominent role in uterine contractility [1] by providing a potent repolarizing current that is essential for uterine quiescence. RNase protection assays (RPAs) and immunoblotting studies have shown that myometrial maxi-K channel α subunit expression is modulated in mouse during gestation with expression levels increasing significantly at late gestation and decreasing post-partum [2]. However, using electrophysiological methods Benkusky et al. found a lower current density in 19-day pregnant mice [2]. Other studies by Wang et al., in late gestation rats, detected either no change in maxi-K current density in some cells, a change in the proportional amount the maxi-K current contributed to total current, or total absence of maxi-K in other cells [3], suggesting that the maxi-K channel current contributes less to the repolarizing current in uterine smooth muscle cells. One explanation for these findings is altered regulation of the maxi-K channel accessory β subunit, which enhances the channel's sensitivity to voltage and Ca^{2+} [4,5]. While studies of the maxi-K channel α subunit indicate that this transcript is regulated by hormones involved in stress and pregnancy [2,6], recent evidence suggests that activation of the maxi-K channel by 17β-estradiol occurs when the hormone binds to the channel's accessory β1 subunit [7]. Since estrogen levels increase at late gestation in the mouse [8], an increase in the β1 subunit could contribute to the potent repolarizing buffering current needed from late gestation until parturition commences. We provide evidence that the maxi-K channel β1 subunit transcript and protein is modulated during gestation in the mouse myometrium. Upregulation of the maxi-K channel β1 subunit transcript expression occurs with 4-day exposure to 17β-estradiol in both ovariectomized and non-ovariectomized mice and is unaffected by progesterone.

2. Materials and methods

2.1. Mouse breeding and 17β-estradiol and progesterone stimulation

Adult C57BL/6J mice were mated at 8–10 weeks of age as previously described [2]. The uteri were excised and flash-frozen in liquid nitrogen.

For estradiol experiments, female C57BL/6J mice 8–10 weeks of age were divided into two groups. The non-stimulated group received injections of vegetable oil (vehicle) and the estrogen-stimulated group received 8.5 μg injections of 17β-estradiol per day (Sigma, St. Louis, MO, USA) for 4 days based on a previously published protocol in rats [1]. The same protocol was administered to ovariectomized mice from Harlan Laboratories (Indianapolis, IN, USA). For acute stimulation studies, ovariectomized mice were injected with similar 17β-estradiol concentrations and uteri excised 4 h after injection [9,10].

For progesterone experiments, the non-stimulated group received injections of vegetable oil, twice daily for 4 days. The progesterone-stimulated group received a single injection of 8.5 μg 17β-estradiol, to upregulate progesterone receptors, followed by injections of 0.2 mg of progesterone (Sigma, St. Louis, MO, USA) for 4 days, twice daily [11,12]. All injections were administered subcutaneously. Mice were euthanized by CO_2 exposure either 24 h after (4-day exposure) or 4 h (acute) after the last injection and the uteri flash-frozen.

2.2. RT-PCR

Oligonucleotide primer pairs flanking the coding region of the maxi-K channel β subunits (β1–β4) were created. Mouse brain and myometrial RNA were extracted and separated as described previously [2]. Human brain RNA was purchased (Clontech, Palo Alto, CA, USA). The RNA was used for RT-PCR of the β subunits (Stratagene Prostar kit, La Jolla, CA, USA). The detected products were purified and sequenced.

2.3. Generation of antisense RNA probes and RPAs

A biotin-labeled antisense probe corresponding to nt 1–195 of the β1 subunit was synthesized using Ambion's Maxiscript in vitro transcription kit. The mouse cyclophilin loading control construct was a 103 nt fragment (Ambion). The RPAs were performed as previously described [2] using 25 μg of total RNA electrophoresed through 7.5% polyacrylamide/8 M urea denaturing gels at 250 V for 4 h. The blots were detected using chemiluminescence. Optical densities of the protected fragments were measured using LabWorks 3.0 (Ultra-Violet Products, Inc.) and normalized to the most intense signal or the loading controls. Mean normalized values are plotted ± S.E.M.

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0014-5793/02/$22.00
PII: S0014-5793(02)03011-9

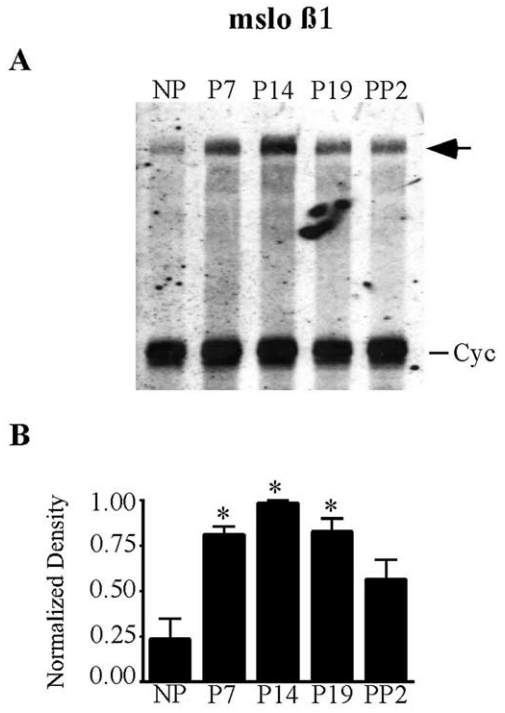

Fig. 1. Determination of transcript expression of the maxi-K channel β1 transcript during gestation. 25 μg of total RNA isolated from mouse uteri at five gestational stages was hybridized with 1 ng of a biotin-labeled antisense RNA probe of the β1 subunit. A: A representative blot shows the β1 transcript (arrow) peaks at mid-gestation (P14) and decreases at post-partum day 2 (PP2). A probe against cyclophilin (Cyc) was used as a loading control. B: A densitometric summary shows the transcript expression level of the β1 subunit during gestation. Optical densities of the protected fragments were measured and normalized to the most intense band ($n=4$). The asterisk denotes statistical significance of differences from the non-pregnant (NP) controls.

2.4. Immunoblotting and immunohistochemistry

For immunoblotting and immunofluorescent experiments, the mouse maxi-K channel β1 subunit polyclonal antibody (Affinity Bioreagents) was used at a 1:500 dilution. Western blot analysis was performed as described previously [2]. For immunofluorescence experiments, uteri isolated from mice were fixed in 4% paraformaldehyde and embedded in OCT. Cryopreserved tissues were sectioned in 10 nm sections, blocked in buffer containing 5% heat-inactivated fetal bovine serum and 0.3% bovine serum albumin and incubated with the maxi-K channel β1 subunit (Affinity Bioreagents) for 2 h at room temperature. Sections were subsequently incubated with biotin-conjugated donkey anti-rabbit IgG (1:1000) for 30 min at room temperature (Jackson ImmunoResearch) and streptavidin-conjugate Cy5 (1 mg/ml) for 20 min at room temperature (Jackson ImmunoResearch). Myometrial smooth muscle tissue was identified by Cy2 conjugate of mouse monoclonal anti-α-smooth muscle actin (Sigma, 1:500). Images were visualized and recorded using a laser confocal microscope and quantified with image analysis software (Zeiss). Control experiments were performed using a commercial competing peptide (100 μg/ml; Affinity Bioreagents) and by immunocytochemistry of cells transfected with a control or maxi-K channel β1 adenoviral construct, both containing green fluorescent protein (GFP) as a reporter gene. Immunofluorescence intensity of the maxi-K channel β1 signal was measured in fields of similar size at different stages of gestation. Results are expressed as average pixel intensity.

2.5. Statistics

Significant differences between groups in the gestational RPAs were determined by one-way analysis of variance followed by Tukey–Kramer multiple comparisons test. Statistical significance of differences between groups in the estrogen-stimulated RPAs were determined by Student's t-test. Differences were considered significant at $P<0.05$.

3. Results

3.1. The maxi-K channel β1 subunit transcript peaks at mid-gestation

PCR analyses detected the presence of maxi-K channel β1 and β2 subunit transcripts in the mouse uterus, however the β3 and β4 subunit transcripts were not detected (data not shown). RPAs were performed to assess whether differential transcript expressions of the β1 and β2 subunits are potential mechanisms for changes in uterine excitability during gestation. Total RNA from the five gestational stages was hybridized with an antisense biotin-labeled RNA probe of the β1 subunit. A representative RPA blot demonstrates that the β1 subunit transcript peaks at mid-gestation (P14) and decreases by day 19 (arrow, Fig. 1A). This trend in transcript regulation differs from the regulation of the maxi-K channel α subunit, which peaks at term gestation (P19) [2]. These data are summarized in Fig. 1B where the densities of the protected fragments of the β1 subunit were measured and normalized to the most intense signal ($n=4$). Quantification confirms that the

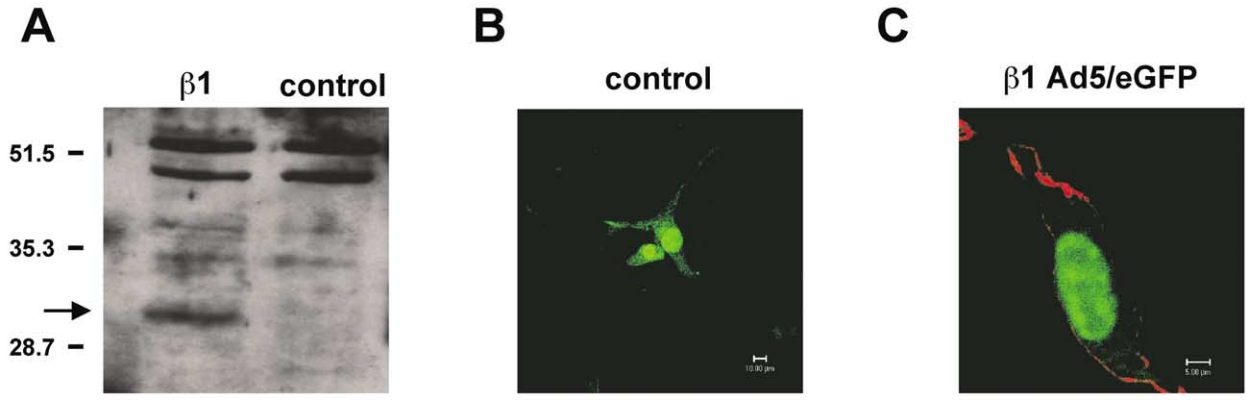

Fig. 2. Maxi-K channel β1 subunit antibody specificity. 25 μg of membrane protein from cells transfected with maxi-K channel β1 and sham-transfected cells were probed with a polyclonal antibody for this protein (A). A band corresponding to ~30 kDa was detected in the β1-transfected cells (β1; arrow). β1 was detected in cells transfected with a β1 expression construct (red fluorescence; C) but not in cells transfected with a control vector (B). Both vectors express GFP (green fluorescence).

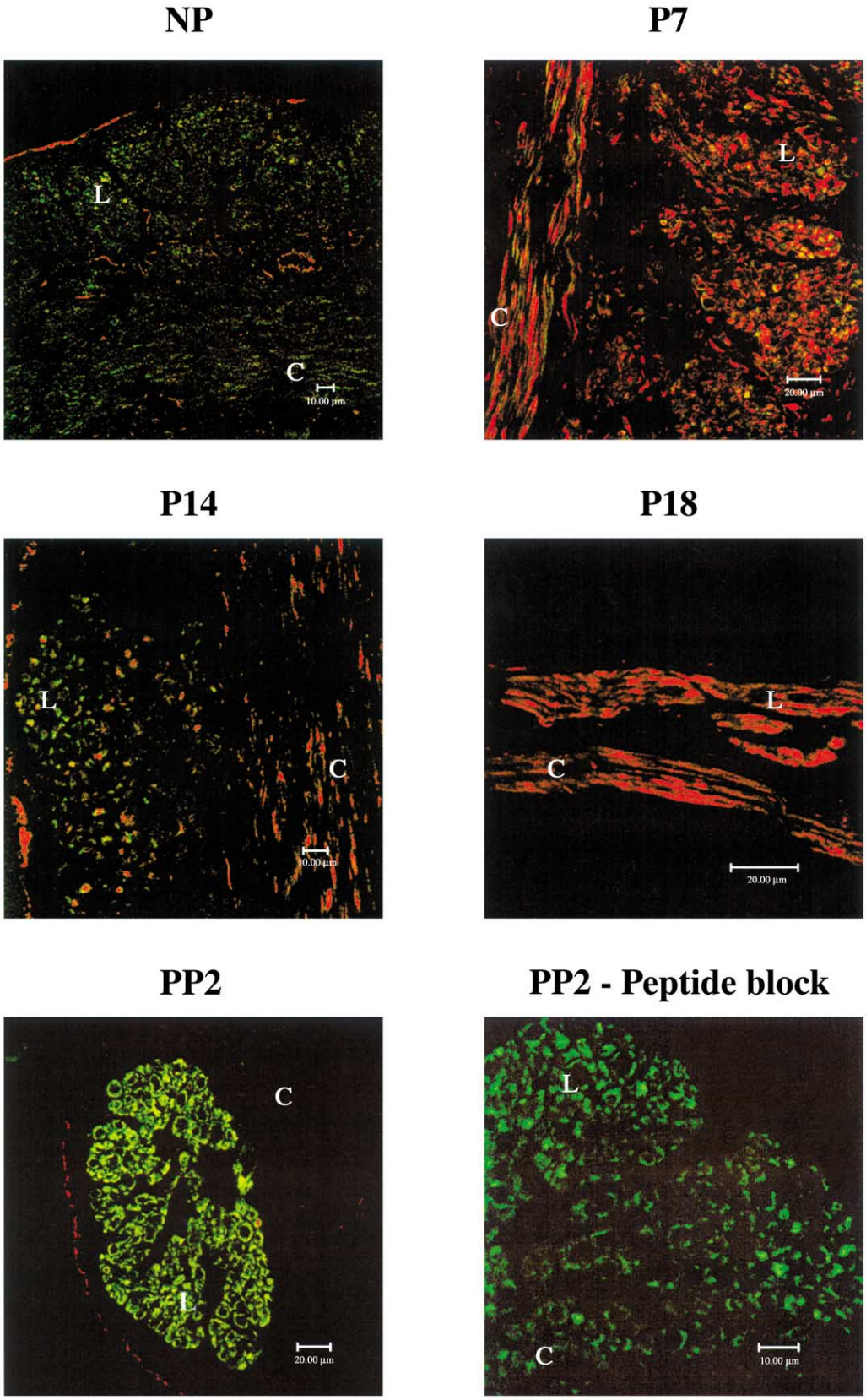

Fig. 3. Immunohistochemical analyses demonstrate β1 subunit protein localization and regulation in mouse myometrium during pregnancy. Cryosections of mouse uteri isolated at various stages of pregnancy were incubated with rabbit polyclonal β1 antibody and donkey anti-rabbit Cy5 IgG (red). Myometrial smooth muscle tissue was identified by α-smooth muscle actin fluorescence (green). Maxi-K channel β1 subunit expression appears to increase from the non-pregnant state to higher levels mid-gestation. Expressions in both the longitudinal smooth muscle (L) and circular smooth muscle layer (C) are similar. The bars represent 20 μm. Addition of the competing peptide block detected no β1 (red fluorescence).

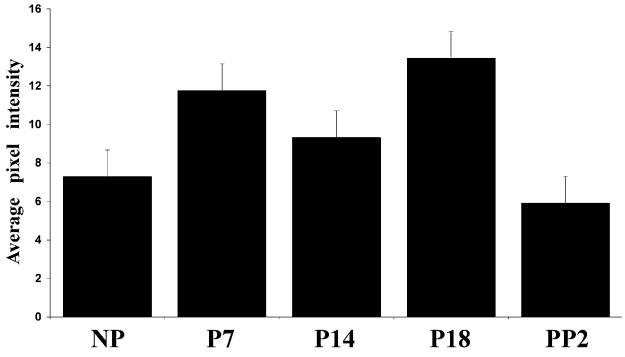

Fig. 4. Quantification of maxi-K channel β1 subunit expression in mouse myometrium during gestation. The bar graph represents the average pixel intensities from three separate immunohistochemical experiments. Maxi-K channel β1 subunit expression is increased in P7, P14, and P18 mice as compared to non-pregnant and post-partum day 2 stages.

transcript level of the β1 subunit is significantly increased throughout gestation (P7–P19) and decreases by post-partum day 2.

Although the β2 subunit transcript was detected by RT-PCR, it was not detected by RPA indicating that the β2 subunit is not present at high levels in the mouse myometrium during gestation or amplification occurred from contaminating non-myometrial tissue.

3.2. *The maxi-K channel β1 subunit protein is upregulated during mid-gestation*

Immunohistochemical experiments were performed to detect both β1 subunit protein expression levels and localization. The antibody used reacted specifically with the β1 subunit in Western blots (Fig. 2A) and in cells transfected with a β1 subunit expressing construct (Fig. 2B,C). As seen in Fig. 3, longitudinal smooth muscle (L) and circular smooth muscle (C) layers both express similar levels of the β1 subunit (red) at different gestational stages. Similar to the transcript measurements, the β1 protein expression is increased in pregnancy days 7, 14, and 18 as compared to non-pregnant and post-partum day 2 stages. α-Actin levels (green) change during gestation, however, the signal from β1 protein was exceedingly high compared to α-actin, and α-actin could be detected at all stages. Inclusion of the competing peptide abolished β1 fluorescence in myometrial tissue (PP2 peptide). The immunohistochemical results suggest that maxi-K channel β1 subunit protein is present in the mouse myometrium and is upregulated during pregnancy as compared to non-pregnant mice uteri.

Quantification of maxi-K channel β1 using fluorescence microscopy demonstrates that this protein is upregulated during pregnancy (Fig. 4). There is a slight decrease at P14 compared to P7 and P18, which was not predicted by transcript levels ($n = 3$).

3.3. *The maxi-K channel β1 subunit transcript is upregulated by 17β-estradiol*

We sought to determine whether estrogen could contribute to increases in transcript expression of the β1 subunit seen during gestation. RPAs were performed on RNA from myometrium of mice that were stimulated 4 days with 17β-estradiol (ES) or the vehicle control (NS). Representative data shown in Fig. 5A indicate that the transcript level of the β1 subunit increases upon 4-day stimulation of 17β-estradiol. In the lower panel the densities of the protected fragments of the β1 subunit were measured and normalized to the corresponding loading control ($n = 5$). Stimulation by 17β-estradiol produced a significant increase in the β1 transcript in the myometrium. These results are not due to endogenous ovarian

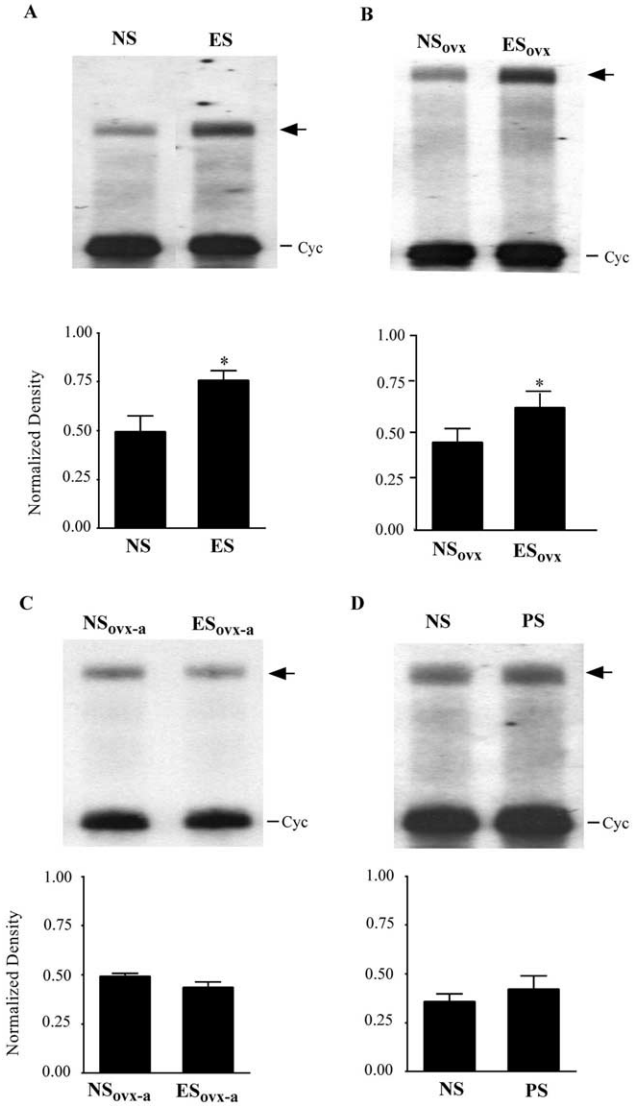

Fig. 5. 17β-Estradiol and progesterone regulation of the maxi-K channel β1 transcript expression in mouse myometrium. RPAs compared transcript levels of the maxi-K channel β1 subunits following 17β-estradiol and progesterone stimulation (arrows). Representative blots (upper panels) and densitometric analyses (lower panels) show that the β1 transcript increases in both non-ovariectomized (A) and ovariectomized (B) mouse myometrium after stimulation for 4 days with 17β-estradiol (ES or ES_{ovx}) as compared to vehicle control (NS or NS_{ovx}). β1 transcript expression does not change significantly with acute 17β-estradiol stimulation (C; ES_{ovx-a}) or 4-day progesterone stimulation (D; PS) as compared to their respective vehicle controls (NS_{ovx-a}, and NS). For all experiments, total mouse uterine RNA was hybridized with 1 ng of a biotin-labeled antisense RNA probe to the β1 subunit. In densitometric analyses cyclophilin (Cyc) was used as the loading control and bands were normalized to cyclophilin ($n = 3$–8). Asterisks show statistically significant differences from the vehicle controls.

hormones as RPAs performed on uteri from ovariectomized mice stimulated with 17β-estradiol (ES_{ovx}) or vehicle control (NS_{ovx}) demonstrated similar results ($n = 8$, Fig. 5B). These data suggest that estrogen is a possible initiator of the increase in the β1 subunit transcript during gestation.

3.4. Maxi-K channel β1 subunit transcript is not regulated by acute exposure to 17β-estradiol or progesterone

Previous studies have shown that 17β-estradiol has an acute effect on the maxi-K channel β1 subunit [7]. To determine whether the increase in the β1 subunit transcript following stimulation by 17β-estradiol is an acute effect, RPAs were performed on uterine RNA from ovariectomized mice stimulated for 4 h with 17β-estradiol (ES_{ovx-a}) or vehicle control (NS_{ovx-a}). Representative data shown in Fig. 5C indicate that the β1 subunit transcript level does not change after 4-h stimulation with 17β-estradiol in ovariectomized mice compared to non-stimulated ovariectomized mice. Densitometric analyses of the blots (Fig. 5C, lower panel) demonstrate that short-term stimulation by 17β-estradiol has no significant effect on the β1 transcript in contrast to the increase seen after longer stimulation ($n = 3$).

To assess whether the increase in the β1 subunit transcript seen at late gestation was a general effect of hormonal changes, RPAs were performed on uterine RNA from mice treated with progesterone (PS) or vehicle control (NS). Fig. 5D shows a representative blot indicating that β1 subunit transcript does not increase following progesterone stimulation. Densitometric analysis of the blots (bottom panel) in which the densities of the β1 subunit bands were measured and normalized to its loading control ($n = 3$) shows that stimulation by progesterone has no significant effect on the transcript level of the β1 subunit. Song et al. [13] have reported similar results with another myometrial channel transcript, Kv4.3, which is regulated by 17β-estradiol but not by progesterone.

4. Discussion

These experiments provide the first evidence that regulation of the modulatory maxi-K channel β1 subunit occurs during gestation in the mouse myometrium. During gestation, myometrial cell permeability to Ca^{2+} increases, and membrane potential increases to more depolarized potentials at term [14]. Therefore, a maxi-K channel with increased sensitivity to voltage and Ca^{2+} would maintain K^+ efflux promoting uterine quiescence during gestation. Alternatively spliced forms of the maxi-K channel α subunits, which are sensitive to Ca^{2+} and voltage, are regulated during gestation [2]. Another mechanism for altering uterine excitability is by β1 subunit regulation. We have determined that one potential mechanism for the increase in β1 subunit transcript during gestation is via genomic effects of estrogen. Although previous results have shown short-term effects of estrogen on the β1 subunit [7], acute stimulation had no effect on β1 subunit transcript, suggesting that non-genomic effects are also involved in channel regulation. This appears to be estrogen specific as no effect on β1 transcript was observed after progesterone stimulation. In previous studies, doses of 0.5–17 μg of estrogen administered to mice led to serum levels of 17β-estradiol of 45.6–513 pg/ml [15]. These levels are comparable to circulating plasma levels of 17β-estradiol during pregnancy [16], suggesting a physiological role of estrogen on maxi-K channel β1 subunit regulation.

Protein regulation of the β1 subunit may be similar to transcript regulation, however immunohistochemical analyses suggest that β1 protein is slightly reduced at P14 as compared to P7 and P18. This is likely a result of the difficulty in accurately measuring fluorescent signals in a tissue that drastically changes anatomically. Immunohistochemical analyses suggest that the ratio of the maxi-K channel α and β1 subunits would likely change during gestation whereby the α subunit increases throughout gestation peaking at day 19 [2] while the β1 subunit peaks earlier during pregnancy. Recent studies have demonstrated that in brain tissue maxi-K channel α subunits can form functional channels devoid of the β subunit [17], while in vascular smooth muscle it is usually associated with the β1 subunit [18], indicating that altered α and β subunit association can occur. This stoichiometric change may indicate more maxi-K channels which are not associated with β subunits later in pregnancy thereby producing channels that are less sensitive to depolarization and increased $[Ca^{2+}]_i$ caused by myometrial stretch. This decrease in the channel's sensitivity to voltage and Ca^{2+} may be a mechanism to increase the uterine excitability prior to parturition. The β1 subunit transcript is regulated in the mouse myometrium during gestation, in part due to estrogen. The ability of estrogen to regulate this channel after both acute and longer exposure suggests a multifaceted role of this hormone. Further examination of the association of these channel subunit proteins throughout pregnancy will determine whether association of maxi-K channel α and β subunits is altered and stoichiometric differences occur to modulate uterine excitability.

Acknowledgements: The authors thank Mr. Daniel Fergus for critical review of the manuscript. This work was supported by National Institutes of Health Grants HD-37831 and HD-01371 (to S.K.E.) and American Heart Association Postdoctoral Fellowship 0120580Z (to V.P.K.). Reagents for these studies were provided by the Diabetes and Endocrinology Research Center (DK-25295) and the Gene Transfer and Vector Core (DK-54759) at the University of Iowa.

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