Hormonal control of protein expression and mRNA levels of the MaxiK channel α subunit in myometrium

Min Song[a], Ning Zhu[a], Riccardo Olcese[a], Bruna Barila[a], Ligia Toro[a,c,d], Enrico Stefani[a,b,d,*]

[a]Department of Anesthesiology, UCLA School of Medicine, BH-509A CHS, Box 957115, Los Angeles, CA 90095-1778, USA
[b]Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1778, USA
[c]Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095-1778, USA
[d]Brain Research Institute, UCLA School of Medicine, Los Angeles, CA 90095-1778, USA

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Abstract Large conductance voltage-dependent and Ca^{2+}-modulated K^+ channels play a crucial role in myometrium contractility. Western blots and immunocytochemistry of rat uterine sections or isolated cells show that MaxiK channel protein signals drastically decrease towards the end of pregnancy. Consistent with a transcriptional regulation of channel expression, mRNA levels quantified with the ribonuclease protection assay correlated well with MaxiK protein levels. As a control, Na^+/K^+-ATPase protein and RNA levels do not significantly change at different stages of pregnancy. The low numbers of MaxiK channels at the end of pregnancy may facilitate uterine contraction needed for parturition.

Key words: K channel; Protein expression; Myometrium; Hormone; mRNA

1. Introduction

Voltage-dependent and Ca^{2+}-activated K^+ (MaxiK) channels are highly expressed in non-pregnant human [2] and rat uterine smooth muscle [3,4], and they play a key role in the maintenance of uterine tone by regulating membrane potential and intracellular Ca^{2+} [1]. Moreover, blockade of this class of channels with iberiotoxin (IbTx) [5,6] induces or increases the frequency of contractions of uterine muscle [1] suggesting that at least a proportion of MaxiK channels are open under physiological conditions opposing contractile activity. Recently, Wang et al. [7] reported that MaxiK channels in 'late-pregnant' rats (days 17–21) have a diminished apparent Ca^{2+} sensitivity, retain typical pharmacological properties and appear to contribute less to the total K^+ current when compared to non-pregnant myocytes; however, a decrease in channel density was not apparent. These observations suggest a diminution of 'typical' MaxiK channels and/or differential expression of an isoform(s) or auxiliary subunits with distinct characteristics during pregnancy. In this work, we demonstrate that the MaxiK channel α subunit *protein* dramatically diminishes in rats reaching term (days 20–22), and that mRNA levels correlate with these changes.

2. Materials and methods

2.1. Cell preparation and electrophysiology

Freshly isolated single smooth muscle cells were prepared from the longitudinal layer of pregnant and non-pregnant Sprague-Dawley rat uteri as previously described [3]. Experiments were performed at room temperature with the patch-clamp technique in the whole-cell configuration. Patch pipettes (5–8 MΩ) were filled with the intracellular solution (mM): 140 K-methanesulfonate, 2 $MgCl_2$, 0.1 $CaCl_2$, 0.146 EGTA, 10 HEPES, 2 Na_2ATP, titrated to pH 7.4 with methanesulfonic acid. The bath solution contained (mM) 137 Na-methanesulfonate, 5.9 K-methanesulfonate, 1.2 $MgCl_2$, 0.8 $CaCl_2$, 10 HEPES, 10 glucose, titrated to pH 7.4 with NaOH. Data were acquired and analyzed with customized software.

2.2. MaxiK channel α subunit antibody

The polyclonal antibody is directed against the sequence VNDTNVQFLDQDDD that corresponds to residues 883–896 of the human MaxiK channel α subunit (*hSlo*), GenBank accession number U11058 [8]. This sequence is conserved in all analyzed species, including rat.

2.3. Protein lysates of transfected COS-M6 cells

COS-M6 cells (SV40 transformed African green monkey kidney cells) were transfected with *hSlo* DNA as previously described [9]. After culturing for 48–72 h, cells were lysed (0.4 ml lysis buffer/10^6 cells) at 4°C for 1 h. The lysis buffer contained (mM): 20 HEPES-KOH, 1 EDTA, 250 sucrose at pH 7.4 supplemented with 17 mM CHAPS and 0.1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 4°C for 10 min at 10 000 rpm and stored at −70°C [10].

2.4. Myometrium membrane preparation

The uterus from each rat was divided into two parts, one for immunostaining and the other for Western blots. After scraping off the endometrium, the myometrium was homogenized in (mM): 20 HEPES-KOH, 1 EDTA, 250 sucrose at pH 7.4 supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The homogenate was centrifuged at $1000\times g$ for 10 min and the supernatant was centrifuged at $100\,000\times g$ for 30 min to obtain crude membranes. Membranes were suspended in 250 mM sucrose, 10 mM HEPES-KOH at pH 7.4 and supplemented with 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 42 mM DTT, and stored at −70°C. Protein content was determined using the Bradford method (Pierce Chemical).

2.5. Western blots

COS-M6 proteins (10 μg) or myometrial membrane proteins (30 μg) were separated by 6% SDS-polyacrylamide gels under reducing conditions and electrotransferred to nitrocellulose paper. Blots were blocked with Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 0.1% Tween, at pH 7.4) containing 5% non-fat dry milk for 1 h at room temperature. Blots were incubated with 1:600 affinity-purified anti-MaxiK channel $\alpha_{(883-896)}$ subunit polyclonal antibody or 1:8000 monoclonal antibody against the Na^+/K^+-ATPase (Upstate Biotechnology) in 1% non-fat milk/TBS

*Corresponding author. Fax: (1) (310) 825-6649.
E-mail: estefani@ucla.edu

Abbreviations: MaxiK, large conductance voltage-dependent and calcium-activated K^+ channel; *hSlo*, human MaxiK; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; IbTx, iberiotoxin

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for 12 h at 4°C, washed with TBS three times for 10 min each, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000) for 1 h. After washing, blots were treated for 1 min with Chemiluminescence Reagent *Plus* (NEN) and autoradiographed on Kodak BioMax film.

As control, COS-M6 cells transfected with the MaxiK channel α subunit hSlo displayed a band with a molecular mass of ~120 kDa as expected for the MaxiK α subunit. Non-transfected COS-M6 cells had no signals. Membranes from rat myometrium showed two bands at an approximately equivalent position that were identified as being MaxiK channel protein. The MaxiK signals in MaxiK transfected COS-M6 cells and myometrium membranes were absent when the polyclonal antibody was preadsorbed with the antigenic peptide (100 mg peptide/mg antibody). Bands corresponding to the immunoreactive hSlo protein (estimated M.W. ~120 kDa) were quantified using the Bio-Rad GS670 Imaging Densitometer. Results are expressed as densitometric units.

2.6. Immunocytochemistry

Fresh uteri were fixed by immersion in fixative (4% paraformaldehyde, 2% picric acid in 0.1 M phosphate-buffered saline (PBS), pH 7.4) for 2 h. Transverse cryostat sections (10 μm) were incubated for 12 h at 4°C with a 1:100 dilution of anti-$\alpha_{(883-896)}$ affinity-purified antibody in 1% normal goat serum and 0.2% Triton X-100 in PBS (0.01 M, Sigma). After washing, sections were incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Vector Laboratory Inc.). In the control sections, the MaxiK antibody was blocked by adding an excess amount of the corresponding antigenic peptide (100 μg/ml). Immunofluorescence was measured in 5–7 randomly selected visual fields from each tissue section or isolated cells using Image-Pro plus software (Media Cybernetics). Results are expressed as average pixel intensity. A protocol similar to that described above was used for immunostaining isolated single smooth muscle cells, except that in this case cells were fixed for 15 min at room temperature.

2.7. Rat MaxiK channel α subunit (rSlo) and rat Na/K-ATPase α_1 subunit (rNa/K-ATPase) RNA probes and ribonuclease protection assay (RPA)

Antisense RNA probes were obtained for $rSlo_{2778-3014}$ (residues 2778–3014 according to GenBank accession number U55995) and $rNa/K\text{-}ATPase_{2027-2159}$ (residues 2027–2159 according to GenBank accession number M28647). $rSlo_{2778-3014}$ segment is specific for MaxiK channel, is highly conserved among species and splice inserts in this region have not been reported [11].

Ribonucleotide protection assay as described by Gilman et al. [12] (RPA II system, Ambion) was performed using total RNA (0.5–1.0 g) extracted from freshly isolated myometrium of non-pregnant, pregnant (days 8, 13, 17, 21) and post-partum rats using the ToTALLY RNA isolation kit (Ambion). For each sample, 40 μg of total RNA was hybridized with 3.5×10^5 cpm of gel-purified ^{32}P-antisense RNA probe ($rSlo_{2778-3014}$ and $rNa/K\text{-}ATPase_{2027-2159}$) for 16 h at 42°C, and then digested with a mixture of RNase A and RNase T1 at a final dilution of 1:100. Protected fragments were separated on a 5% polyacrylamide-8 M urea gel, afterwards the gel was dried and exposed overnight to a phosphor screen. The [^{32}P]UTP-labeled RNA molecular weight marker was synthesized using the RNA Century Marker Template Set (Ambion). The band intensities were quantified with a PhosphorImager (445 SI, Molecular Dynamics). Identical areas were quantified and values are expressed as a percentage of the total volume of all samples.

2.8. Materials

Affinity-purified HRP-conjugated goat anti-rabbit IgG and nitrocellulose membranes were from Amersham. Tween 20, Triton X-100, collagenase F and H, and papain were from Sigma. Prestained molecular weight standards (range 14.3–200) were from Gibco BRL. IbTx was from Alomone Labs. All other reagents were obtained from commercial sources and were of the highest purity grade commercially available.

2.9. Statistics

Data are expressed as means ± S.E.M. Comparisons between two groups were analyzed by the two-tailed Student's *t*-test. A probability level (P) less than 0.05 was considered statistically significant.

3. Results

3.1. The majority of K^+ currents in non-pregnant myometrial cells are sensitive to the MaxiK channel blocker IbTx

To evaluate what fraction of outward K^+ currents was carried by the activity of MaxiK channels, we measured the degree of blockade of K^+ currents by 100 nM external IbTx. Bath application of IbTx reduced outward K^+ currents, elicited with a pulse to 100 mV, by 85.3% ± 4.3 ($n = 3$). Fig. 1A shows current traces (without subtraction of 'leak' currents) elicited with 150 ms depolarizing steps from −60 to 180 mV in 20 mV increments from a holding potential of −60 mV. Pulses more positive than 20 mV elicited noisy outward currents typical of MaxiK channels due to their large single channel conductance [3,7]. Fig. 1B shows in the same cell the remaining outward K^+ currents after 4 min following blockade with 100 nM IbTx. The graph in Fig. 1C shows the current voltage relationship in control (○) and after IbTx (●). Fig. 1D,E shows the time course of the effect of 100 nM IbTx. K^+ currents were evoked by repetitive pulses (every 2 s) to +100 mV from a holding potential of −60 mV. The decrease in K^+ current began 10–40 s after bath application, likely due to a diffusion delay of the toxin, and reached steady level of blockade within 4 min. Fig. 1D illustrates that as the current is blocked, the current noise is reduced as expected for large

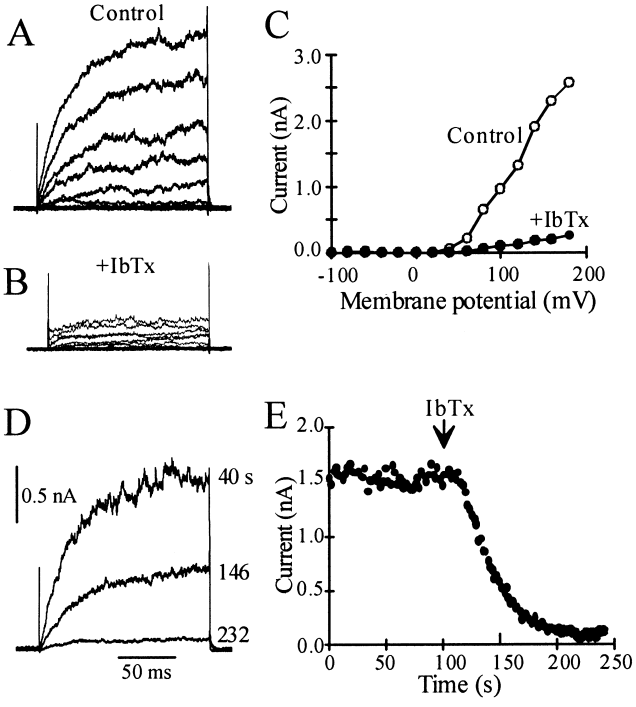

Fig. 1. Iberiotoxin-sensitive K^+ currents in non-pregnant rat myometrium. A: Whole-cell K^+ currents from an acutely dissociated smooth muscle cell. Control currents elicited by test pulses (150 ms duration) to −60 to 180 mV every 20 mV from a holding potential of −70 mV. B: Outward current recorded from the same cell as in A 4 min after application of 100 nM IbTx. Pulse protocol as in A. C: Corresponding *I-V* curves; control (○), in the presence of IbTx (●). D: Current traces to the same step potential (100 mV) acquired at different times (40, 146 and 232 s, respectively) after perfusion of the toxin. Holding potential was −60 mV. E: Time course of K^+ current inhibition after application of 100 nM IbTx (marked by arrow).

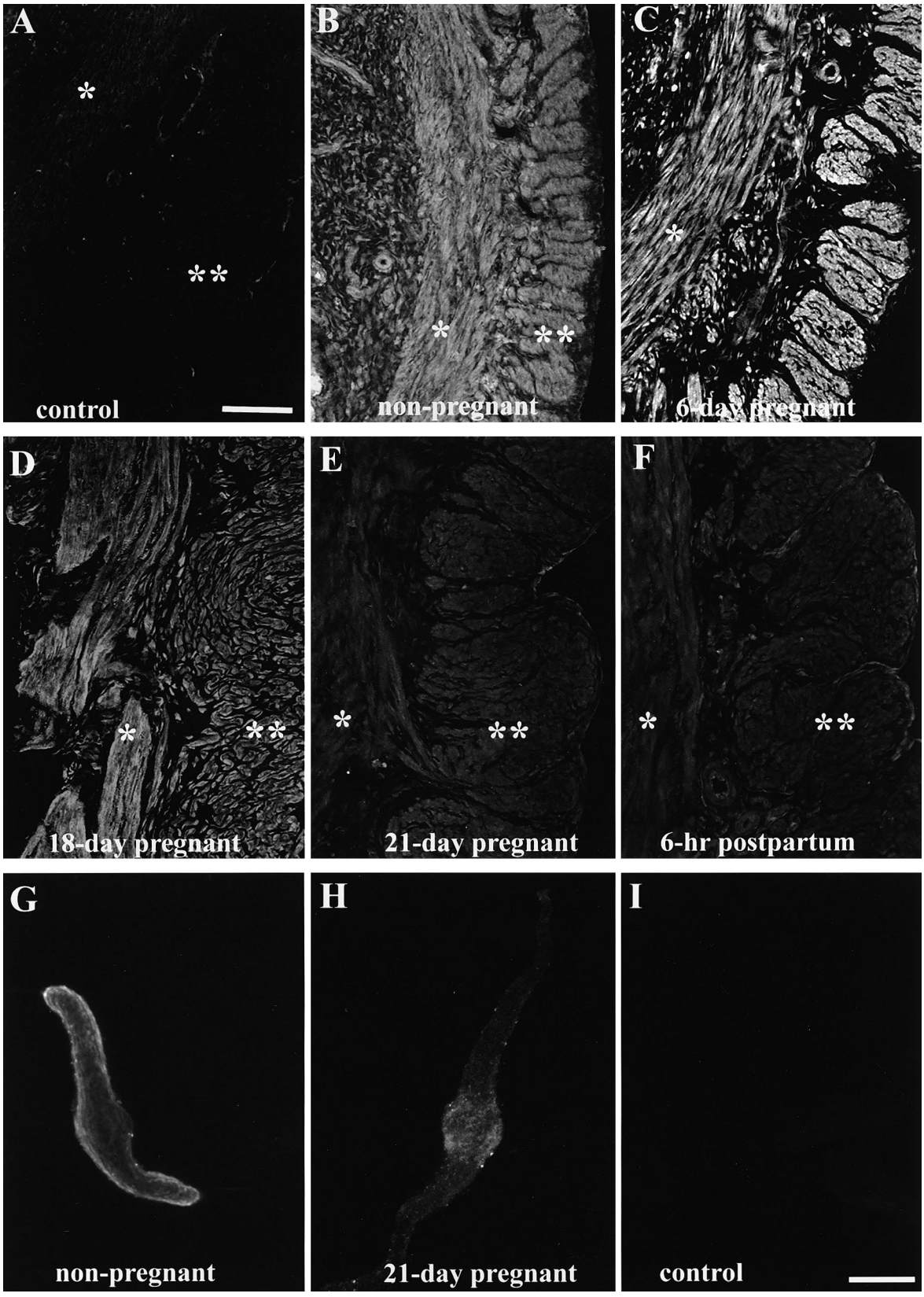

Fig. 2. MaxiK channel immunolabeling in uterine sections and freshly isolated smooth muscle cells. Rat myometrium was labeled using a polyclonal anti-MaxiK channel α subunit antibody. Circular (*) and longitudinal (**) smooth muscle layers. A: Control tissue section showing signal block; the polyclonal antibody was preadsorbed with an excess of antigenic peptide. B: Non-pregnant uterus. C: Uterine section from day 6 of gestation. D: Uterine section from day 18 of gestation. E: Uterine section close to term (day 21 of gestation) shows a remarkable reduction in expression of the MaxiK channel α subunit. F: Low expression is maintained a few hours post-partum (6 h). G: Single uterine smooth muscle cell from a non-pregnant rat. H: Single myocyte from a pregnant rat close to term (day 21 of gestation). I: No staining was detected when the antibody was pre-adsorbed with the corresponding antigen (control). Bar indicates 100 μm for A–F and 10 μm for G–I.

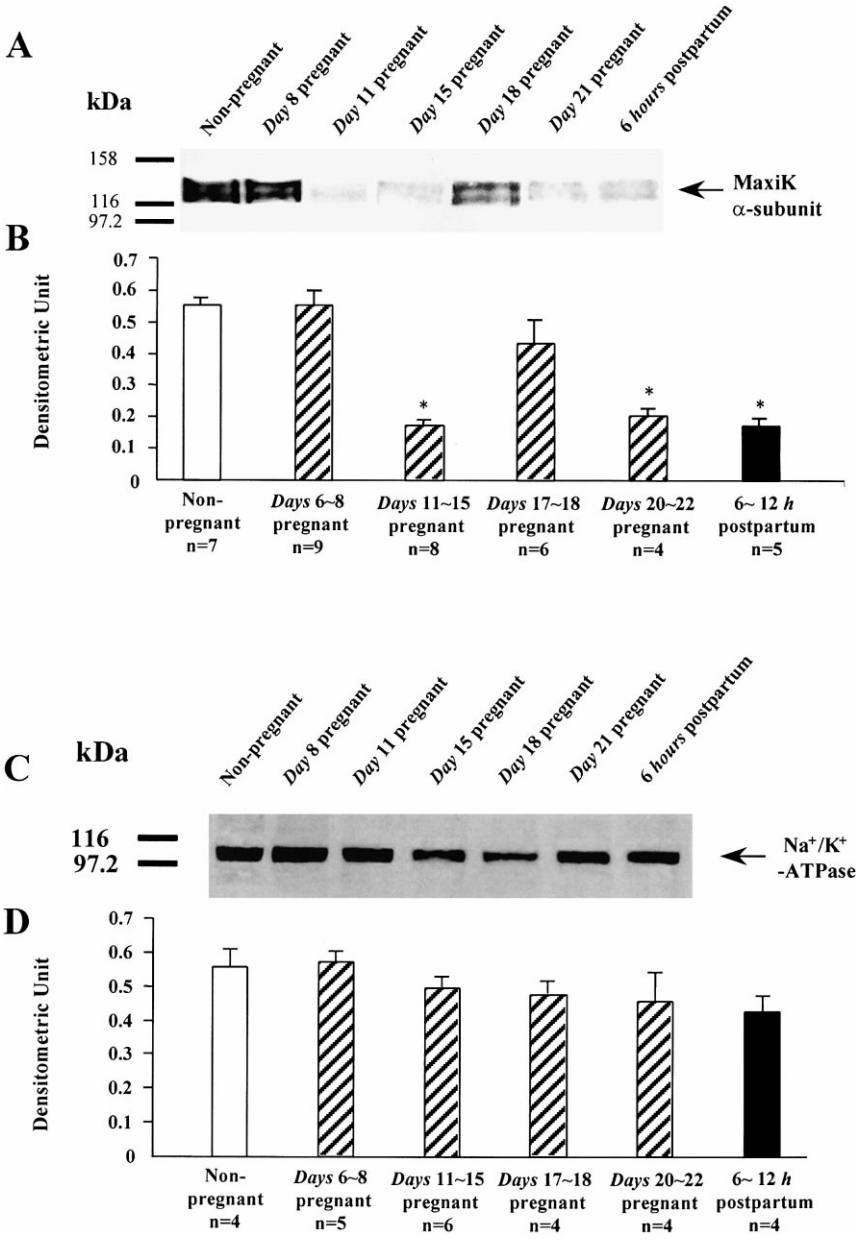

Fig. 3. Western blots of MaxiK channel during pregnancy. A: Western blots of MaxiK channel α subunit in non-pregnant and pregnant rat myometrium. Equal amounts of membrane proteins (30 μg) were separated by 6% SDS-polyacrylamide gels under reducing conditions and electrophoretically transferred to nitrocellulose paper. B: Quantification of several blots as in A. The polyclonal anti-MaxiK channel α subunit antibody was the same as in Fig. 2. Results are expressed in arbitrary densitometric units (DU). Number of experiments are given in the text. $^*P < 0.05$ compared to non-pregnant rats. C: Western blotting of the Na^+/K^+-ATPase in rat myometrium under the same gestational stages as in A. A commercial monoclonal anti-Na^+/K^+-ATPase antibody was used. D: Quantification in densitometric units shows practically no changes during pregnancy. Numbers of experiments were: NP ($n=4$); days 6–8 ($n=5$); days 11–15 ($n=6$); days 17–18 ($n=4$); days 20–22 ($n=4$); 6–12 h PP ($n=4$). NP, non-pregnant; PP, post-partum.

conductance channels like MaxiK channels. We can conclude that in non-pregnant myometrium most of the outward K^+ current is IbTx-sensitive indicating that it is due to the activity of MaxiK channels sensitive to IbTX [2–4]. MaxiK channels are also present in uterine smooth muscle during pregnancy (days 18–19 in rats) [7,13]. In this work we have used combined immunocytochemical and biochemical approaches that examine intact tissue or large cellular populations to determine whether MaxiK channel protein expression changes as a function of pregnancy.

3.2. Expression of the MaxiK channel α subunit varies as a function of pregnancy

Tissue sections from non-pregnant, pregnant (days 6–8, 11–15, 17–18, 20–22) and post-partum (6–12 h) rats were examined using the affinity purified anti-peptide polyclonal antibody, anti-$\alpha_{(883-896)}$. The specificity of the MaxiK channel antibody has been assessed using transfected or untransfected COS-M6 cells as previously reported [9] or by preadsorbing anti-$\alpha_{(883-896)}$ with excess antigenic peptide, a maneuver that practically abolishes the fluorescent signal, as shown in Fig.

2A,I, for non-pregnant myometrium. This control protocol was performed in all the experiments. Fig. 2B–F illustrates confocal images of uterine cross-sections immunostained with MaxiK anti-$\alpha_{(883-896)}$ antibody (smooth muscle circular layer, *; smooth muscle longitudinal layer, **). For comparative purposes the same staining and photograph protocols were used. The high signal levels in non-pregnant smooth muscle cells are also high in early (day 6) and 'late' (day 18) pregnancy, but are drastically reduced close to term (day 21) and 6 h post-partum. The drastic reduction in MaxiK channel α subunit signals in the whole tissue was also observed in freshly dissociated cells; compare Fig. 2G (cell from a non-pregnant rat) and Fig. 2H (cell from a 21-day pregnant rat). Quantification of signal intensity in isolated cells showed that signals at term were reduced to about 40% of the values obtained from non-pregnant cells. The quantification of signals in uterine sections as those illustrated in Fig. 2B–F, showed a drastic reduction just before (days 20–22: 22 ± 4 PI, $n = 8$) and shortly after (6–12 h: 17 ± 3 PI, $n = 8$) partum when compared with non-pregnant myometrium (55 ± 8 PI, $n = 8$).

3.3. Western blot analysis of MaxiK channel α subunit signals during gestation

Western blot analysis of uterine membranes showed a duplet at ca. 120 kDa corresponding to the hSlo protein (Fig. 3A). Fig. 3A shows examples of signals at different stages of pregnancy; note the high level of MaxiK channel expression in non-pregnant and early pregnancy (day 8), and the drastic reduction at mid-pregnancy (days 11 and 15), close to term (day 21) and post-partum (6 h after delivery). Fig. 3B shows the quantification of Western blots using several membrane preparations. The signals of MaxiK channels were strong in non-pregnant myometrium (0.55 ± 0.02 DU, $n = 7$) and early pregnancy (days 6–8) (0.55 ± 0.05 DU, $n = 9$). As gestation progressed, there was a decrease in channel expression at mid-pregnancy (days 11–15) (0.17 ± 0.02 DU; $n = 8$), but it peaked again (although not as high as in early pregnancy) at days 17–18 (0.43 ± 0.08 DU, $n = 6$). Near or at term (days 20–22) (0.21 ± 0.03 DU, $n = 4$) and after delivery (6–12 h) (0.18 ± 0.02 DU, $n = 5$) the MaxiK channel protein was dramatically reduced to less than half of the values obtained in non-pregnant and early-pregnant tissue. Thus, the reduction of MaxiK channel expression at term was observed both in Western blots and immunocytochemical determinations.

To investigate whether the relative changes of MaxiK expression are specific, we measured the expression levels of the Na^+/K^+-ATPase (Fig. 3C,D). The expression levels were not significantly modified in uterine membrane preparations at equivalent gestational stages.

3.4. Ribonuclease protection assay

To investigate if the changes in the MaxiK channel α subunit protein during pregnancy correlate with the levels of its mRNA, we performed a ribonuclease protection assay [14] for MaxiK α subunit and Na^+/K^+-ATPase α_1 subunit [12]. Fig. 4 shows bands of the protected mRNA fragments (MaxiK 237 nt and Na^+/K^+-ATPase 202 nt) obtained when total RNA (40 μg) of non-pregnant and pregnant myometrium at different stages were used. Fig. 4A is the quantification of several experiments and demonstrates a close correlation with the protein levels shown in Fig. 3. As was the case for protein expression, MaxiK channel α subunit mRNA levels were strong

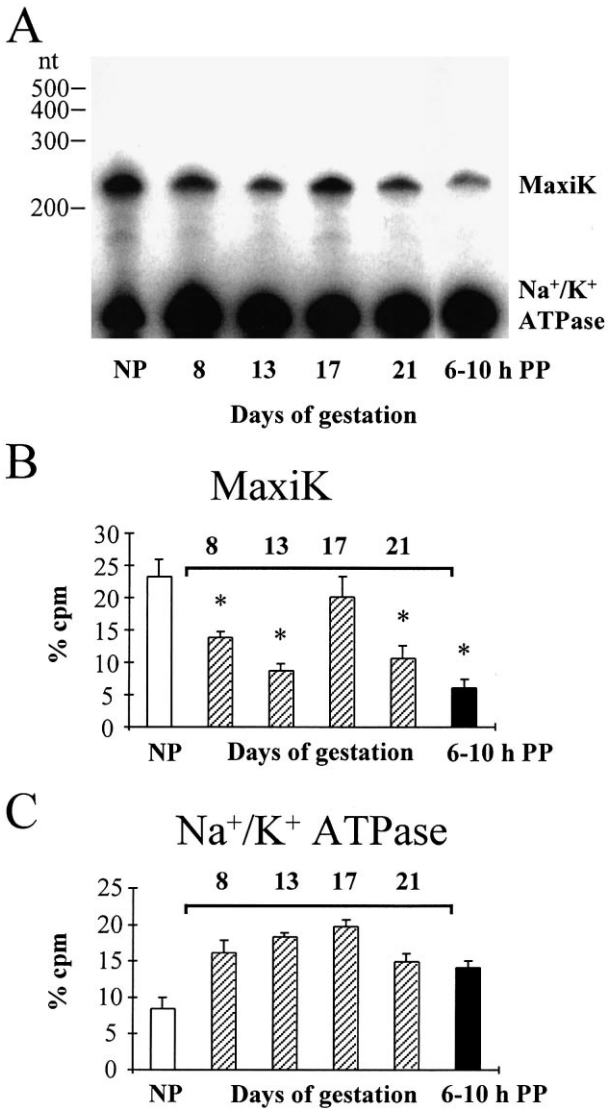

Fig. 4. Transcriptional regulation of MaxiK channel expression during pregnancy. Selective reduction during pregnancy of MaxiK channel α subunit RNA when compared with α_1 subunit Na^+/K^+-ATPase RNA. RPA of total RNA (40 μg) isolated from non-pregnant rats and at different stages of pregnancy using the MaxiK channel α subunit ($rSlo_{2778-3014}$ segment RNA) and the α_1 Na^+/K^+-ATPase subunit (rat Na^+/K^+-ATPase $_{2027-2159}$ segment RNA) as probes. A: RPA of total RNA (40 μg) isolated from non-pregnant rats and at different stages of pregnancy using the MaxiK channel α subunit ($rSlo_{2778-3014}$ segment antisense RNA) as a probe. Control, yeast RNA; all other conditions were maintained constant. B,C: Bar plot of phosphor images. Three different preparations of total RNA for each stage were examined, except for non-pregnant myometrium, which includes six RNA preparations. *$P < 0.05$ compared to non-pregnant rats.

in non-pregnant myometrium (23.2 ± 2.76% cpm, $n = 6$). During pregnancy, similar to protein levels, mRNA was low at days 8 (13.8 ± 0.9% cpm, $n = 3$) and 13 (8.7 ± 0.9% cpm, $n = 3$), increased at day 17 (20.1 ± 3.2% cpm, $n = 3$), and was drastically reduced just before (day 21, 10.6 ± 2.0% cpm, $n = 3$) and after delivery (6–10 h post-partum, 6.1 ± 1.2% cpm, $n = 3$). The Na^+/K^+-ATPase RNA level seems to increase in pregnant vs. non-pregnant rat myometrium, and remains without significant variation throughout pregnancy.

4. Discussion

During pregnancy and labor the uterus undergoes dramatic changes in its contractile activity; thus proteins regulating excitability of smooth muscle cells, like gap junctions and ion channels, are logical candidates to go through changes during gestation. In line with this view, gap junctions were first described to increase at the end of gestation [15], which may result in faster propagation of excitation, and hence more forceful uterine contraction during parturition.

We have previously shown that voltage-dependent and Ca^{2+}-activated K^+ (MaxiK, BK, Slo) channels are key regulators of myometrial contractile activity of non-pregnant myometrium [1]. Accordingly, Fig. 1 shows that about 85% of the total potassium current in non-pregnant rats is made by the activity of IbTx-sensitive MaxiK channels. Although MaxiK channels are important for regulating myometrial tone, potential changes in their mRNA and expression levels during the course of gestation have not been examined. In this work, we have addressed this topic using immunocytochemistry of tissue sections, Western blotting and RPA determinations. Our results demonstrate that the expression levels of MaxiK channel protein vary during the course of pregnancy. The expression levels dramatically diminish near or at term (days 20–22), or just after delivery by over 60% in comparison to non-pregnant and early-pregnant tissue (days 6–8). It is interesting that the MaxiK channel protein diminishes at mid-pregnancy (days 11–15), peaks at days 17–18, diminishes again near or at term (days 20–22) and is maintained at low levels after delivery. Because MaxiK channel activity regulates myometrium contractility [1], it can be suggested that under physiological conditions at days 11–15 the uterus develops a higher contractile activity that diminishes at days 17–18 to sustain pregnancy. Towards the end of pregnancy, the lower levels of MaxiK channel protein will facilitate a higher contractile activity.

Parallel results to those obtained from protein levels (Figs. 2 and 3) were obtained from RPA experiments (Fig. 4), suggesting that regulation of MaxiK channel protein expression at different stages of pregnancy involves transcription as a mechanism of regulation. In fact, estrogen increases the mRNA levels of a modulatory subunit of HERG and KvLQT1 K^+ channels, the MinK [16,17]. Since the relative availability of estrogens and progesterone are thought to be important in maintaining uterine quiescence in pregnancy, it is likely that MaxiK channel expression is also under the control of progesterone or estrogens[18].

Recently, Wang et al. [7] examined possible changes in MaxiK channels in non-pregnant and late pregnant rats (days 17–21) using electrophysiological methods. Their results indicate that during late pregnancy the proportion of MaxiK channel current with respect to other K^+ current is diminished or absent in some cells; however, quantification of channel density did not show a decrease in MaxiK currents. This apparent contradiction may be due to the fact that the analysis was performed on a cell population encompassing several stages of gestation, since our experiments show clear differences between uteri at days 17–19 from uteri close to or at term on days 20–22 (Figs. 2 and 3).

We conclude that MaxiK channels in myometrium are under hormonal control and that the downregulation at the end of pregnancy is possibly by transcriptional regulation. This MaxiK channel reduction may enhance myometrial excitability needed for parturition.

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