Regulation of the Ca\textsuperscript{2+}-Sensitive Domains of the Maxi-K Channel in the Mouse Myometrium During Gestation

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Running Title: Regulation of maxi-K channels in uterus
INTRODUCTION

Uterine smooth muscle cells contain a diversity of ion channels that regulate membrane excitability (1-3). The large-conductance Ca^{2+}-activated K^+ channel (maxi-K) has been shown to play a significant role in modulating uterine function (4-6). Pharmacological inhibition of the maxi-K channel by the specific channel blocker iberiotoxin depolarizes the smooth muscle cell and increases myometrial contractile activity in both rat and human (7), while NS1619, a compound that promotes maxi-K channel opening, has a potent relaxant effect on pregnant human myometrium (4). Beta-adrenergic agents and other uterine relaxants that stimulate the protein kinase A (PKA) cascade have different effects on maxi-K channels (5,8). Maxi-K channels reconstituted from nonpregnant rat and human myometrium are stimulated by PKA, while channels isolated from midpregnant rats are inhibited by PKA (5), suggesting that this channel may be differentially regulated during gestation. Recent data from rat myometrium indicates that maxi-K channel mRNA and protein expression is modulated during gestation with protein levels decreasing significantly towards the end of pregnancy (9). This decrease would lessen the repolarizing capacity of the maxi-K channel protein and may represent one mechanism to facilitate uterine contraction. However, other studies have reported that maxi-K channels in late pregnancy have a diminished Ca^{2+} sensitivity (10) and contribute less to the overall K^+ current as compared to myometrial cells isolated from non-pregnant rats. These results were obtained without a change in channel density, suggesting that other mechanisms may be involved in opposing uterine contractile activity. While these results may indicate the presence of a novel channel type, or altered association of the maxi-K channel with its accessory β-subunits (11), differential expression of alternatively spliced versions of the maxi-K channel that vary in their voltage and Ca^{2+} sensitivity may also contribute to this attenuation.
The molecular diversity of the maxi-K channel stems from alternative splicing of transcripts from a single gene encoded by the slo locus on human chromosome 10 (12-14). To date, at least nine maxi-K channel isoforms from four splice sites in the cytoplasmic C-terminal domain have been found in human brain tissue. Heterologous expression of these isoforms shows differences in their voltage and Ca\(^{2+}\) sensitivities, suggesting a potential role of the maxi-K channel in regulating neuronal excitability (15). Alternative splicing of the maxi-K channel has been reported in the chicken cochlea with differential expression of the variants along the basilar papilla (16). To date, the reason for multiple splice variants within a single tissue remains unknown.

Six sites within the mouse maxi-K channel transcript have been reported to be alternatively spliced (13,17). Splice site 1 is located at the N-terminal region of the maxi-K channel protein near the binding site of the accessory \(\beta\)-subunit of the mammalian homologs, however the function of this variant is unknown (18). Sites 2 through 6 are located within the C-terminal “tail” region of the channel (19). Sites 3 and 4 are known to alter the Ca\(^{2+}\) sensitivity of the maxi-K channel. The presence of the “SRKR” insert at splice site 3 decreases the maxi-K channel’s sensitivity to Ca\(^{2+}\) (15), while the 174 bp insert at splice site 4 increases the Ca\(^{2+}\) sensitivity of the channel and shifts the activation curve 20 mV in the hyperpolarizing direction (20). During gestation, myometrial cell permeability to Ca\(^{2+}\) increases, while membrane potential increases to more depolarized potentials at term (21). Therefore, alternative splicing of regions of a channel transcript that are sensitive to both Ca\(^{2+}\) and voltage may be an essential pathway for modulating uterine excitability. To better understand the mechanisms regulating uterine excitability and contractility during pregnancy, we investigated the expression of
isoforms that modulate Ca$^{2+}$ sensitivity of the maxi-K channel in the mouse myometrium during gestation.

We report differential expression of alternatively spliced transcripts of the maxi-K channel that modulates Ca$^{2+}$ sensitivity in the mouse myometrium during gestation. The maxi-K channel protein present in mouse myometrial membranes differs between non-pregnant and pregnant mice both in their expression and in their function. Our data demonstrates that four of the six splice sites previously described are alternatively spliced in the mouse myometrium. The expression of the insertless transcript at splice site 3 is upregulated at late gestation and decreases at postpartum, similar to whole maxi-K channel regulation. Although observed by PCR, the “SRKR” splice variant at site 3, which decreases the channel’s sensitivity to Ca$^{2+}$, is not detected in the myometrium by RNase protection assays. The 174 bp insert at splice site 4, an insert that increases the channel’s sensitivity to voltage and Ca$^{2+}$, is detected at the same level of expression in the myometrium at all five stages of gestation, suggesting that this is a constitutively expressed isoform of the channel. However, the insertless form of splice site 4, which encodes an isoform of the maxi-K channel that is less sensitive to Ca$^{2+}$ and voltage, is present towards late gestation. Our results suggest that multiple isoforms of the maxi-K channel are present and are regulated differentially during gestation with the prevalence of an isoform that is less sensitive to Ca$^{2+}$ and voltage closer to term. These results suggest that alternative splicing of the maxi-K channel during gestation is a likely mechanism to modulate uterine excitability.
EXPERIMENTAL PROCEDURES

Mouse Breeding

Adult C57/BL6J mice were mated at 8-10 weeks of age. Impregnation was assessed by the presence of a vaginal plug and that day was designated day 1 of gestation with term at day 19. Mice were euthanized by CO2 exposure at one of five stages of gestation (non-pregnant (NP), days 7, 14, 19, and postpartum (PP) day 1 or 2). Postpartum day 1 or 2 tissue was harvested from mice that delivered on day 19. For all experiments, the uteri were excised and the endometrium was stripped rapidly. For electrophysiological analysis, cells were isolated at this point. For the remaining experiments, the tissues were flash-frozen in liquid nitrogen.

Cell Isolation for Electrophysiological Analyses

Following excision, the myometrium was cut into 2 mm pieces and incubated in a series of three different solutions to isolate cells for electrophysiological analysis. All solutions were comprised of a standard dissociation solution containing (in mM): 145 NaCl, 4 KCl, 1 MgCl2, 0.05 CaCl2, 10 HEPES, and 10 glucose (pH to 7.4 with 1 M NaOH) as previously described (22). First, myometrial pieces were placed in 1 mg BSA/1 ml dissociation solution for ~10 min at room temperature. Next, the strips were incubated in 1.5 mg papain + 1 mg DTT/1 ml dissociation solution for 20 minutes at 37°C. The tissue was then transferred to 1 mg collagenase + 1 mg trypsin inhibitor + 0.25 mg elastase/1 ml dissociation solution for 10-20 minutes depending on release of single cells from the tissue. If cells were not released after 10 minutes at 37°C, the tissue was transferred to fresh solution. The solution was pipetted gently to release cells from the tissue. The cell suspension was diluted with dissociation solution and placed on ice until electrophysiological measurements were performed.
Electrophysiological Analyses

A drop of myometrial cell suspension was placed in a perfusion chamber on the stage of an inverted Olympus IX-70 microscope. After cells adhered to the underlying glass coverslip for ~10-20 minutes, the chamber was superfused with an external solution containing (in mM): 135 NaCl, 4.7 KCl, 1 MgCl2, 5 HEPES, 10 glucose, 1 EGTA, and 2 CaCl2, pH 7.4 to give a final free Ca2+ concentration of 1 mM. Heat-polished patch pipettes with tip resistances of 3-5 MΩ were filled with solution containing (in mM): 130 K+ glutamate, 1 MgCl2, 0.1 EGTA, and 10 HEPES. High resistance seals (2-30 GΩ) were obtained for whole-cell recording. Membrane potential was clamped, series resistance was 75-80% compensated, and current was measured by an Axopatch 200-B (Axon Instruments) amplifier. Signals were filtered with a cutoff frequency of 5 kHz. Data acquisition was controlled by commercial pClamp 6.0.3 software (Axon Instruments), and data were digitized using a Digidata 1200 interface (Axon Instruments) and stored on a hard disk for later analysis. Iberiotoxin (100-200 nM, Sigma, St. Louis, MO) was used to confirm the presence of maxi-K channel current. All experiments were done at room temperature.

Membrane area was estimated by integrating capacitive currents generated by a 5 mV pulse after cancellation of the patch-pipette capacitance. Using this method, the capacitance measurements of the myometrial cells were 7.4 ± 0.9 pF for non-pregnant myocytes and 44.3 ± 7.0 pF for term pregnant myocytes. Mean sustained K+ current amplitudes were calculated using the Clampfit 6.0.4. program and plotted in pA/pF to normalize for differences in cell size. Results are plotted as means ± SEM. Significance of differences were evaluated by the Bonferroni multiple comparisons test. Differences were considered to be significant at p ≤ 0.05.
Western Blot Analyses

Mouse uteri were homogenized on ice in buffer containing (in mM): 250 sucrose, 50 MOPS, 0.1 PMSF, 2 EDTA, and 2 EGTA, pH 7.4, plus a mini-Complete® protease inhibitor cocktail tablet (Boehringer Mannheim). The homogenates were centrifuged at 1000 x g and 14,000 x g, and the resulting supernatant was centrifuged at 100,000 x g to pellet cell membranes. Following membrane purification, the pellets were resuspended in phosphate buffered saline (PBS), quantified using a Biorad Protein Assay kit, and stored at –20°C. Thirty µg of mouse myometrial membranes were fractionated by SDS-PAGE and transferred to nitrocellulose. To ensure equal loading of protein, blots were stained with Ponceau S. Blots were then blocked with Tris-buffered saline + Tween-20 (TBS-T: 20 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.05% Tween-20) containing 10% non-fat dry milk overnight at 4°C. The blots were immunoblotted with two different polyclonal antibodies targeted against the C-terminal region of the maxi-K channel α subunit, (aa 913-926, gift from H-G. Knaus) and (aa 1098-1196, Alomone Labs, Jerusalem, Israel), in TBS-T for 2 hours at room temperature. Following incubation with a primary antibody, the blots were washed two times with TBS-T for 5 minutes each, and incubated with horseradish peroxidase (HRP)–conjugated secondary antibody in TBS-T for one hour at room temperature. The blots were then washed 5 times for 5 minutes per wash with TBS-T, and maxi-K channels detected with the ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Maxi-K channel protein synthesized using the TNT-coupled reticulocyte lysate system (Promega) was used as a positive control. The protein translated in the absence of the maxi-K channel DNA was used as a negative control. To determine the specificity of the polyclonal antibody, multiple peptide block experiments were performed using 5 µg of antigenic peptide preadsorbed with 1 µg antibody (aa 1098-1196).
RNA Isolation and Generation of Constructs

Oligonucleotide primer pairs were designed to flank the six splice sites of the maxi-K channel that have been previously described in the mouse myometrium (13,17). Primer pairs flanking a non-spliced conserved C-terminal region of the channel were used to assess total channel expression. Primers were designed such that the full complement of alternative exons would be detected. Total RNA from mouse myometrium at the previously mentioned gestational stages was extracted using the guanidinium isothiocyanate (GTC) method. Briefly, myometrial tissues were homogenized in solution containing 4 M GTC, 25 mM Na citrate, 0.5% sarkosyl and 0.1 M β-mercaptoethanol. After the addition of 0.4 g CsCl/ml of homogenate, the mixture was centrifuged at 9,200 x g for 20 min. The supernatant was layered onto 1.0 ml of 5.7 M CsCl cushion (5.7 M CsCl, 0.1 M EDTA and 25 mM NaAc) and centrifuged overnight in a SW55 rotor (Beckman Coulter) at 30,000 rpm. The RNA pellet was ethanol precipitated and resuspended in Tris-EDTA, pH 6.5. The RNA was used for RT-PCR of sites 1 through 7 (Stratagene Prostar kit, La Jolla, CA). The generated PCR fragments were subcloned into the pCRII vector (TA Cloning kit, Invitrogen) and sequenced to verify the presence and orientation of the variants. The resulting DNA constructs were used to generate antisense probes for RNase protection analyses.

Southern Blot Analyses

Fragments obtained from RT-PCR of sites 1 through 6 were separated by electrophoresis on 1-4% agarose gels. The gels were denatured for 30 min in 0.5 M NaOH + 1.5 M NaCl and neutralized for 30 min in 1 M Tris- HCl, pH 8.0 + 1.5 M NaCl, before an overnight transfer to
nitrocellulose by capillary action with 20X SSC. The membranes were prehybridized in 20% formamide, 4X SSPE pH 7.3, 5X BFP, 0.05 Na₂PO₄ (pH 7.5) and 0.2% SDS for 6 hrs at 42°C and hybridized overnight at 42°C with the addition of a radiolabeled probe to the appropriate mslo construct 1 through 6 (10⁵ cpm/ml). The blots were washed three times for 30 min at 42°C with 3X SSC + 0.1% SDS, 1X SSC + 0.1% SDS and 0.2X SSC + 0.1% SDS prior to autoradiography. All fragments present following Southern blot analyses were isolated and sequencing attempted.

**RNase Protection Assays**

Mouse uteri were homogenized and total cellular RNA was extracted as previously mentioned and used to generate biotin-labeled antisense probes. Mslo constructs 3, 4, and 7 were linearized on the 5’ side and biotin-labeled antisense probes were synthesized using Ambion’s Maxiscript *in vitro* transcription kit. Probes for RNase protection analysis of splice site 3 contained a 122 nt (insertless) or a 134 nt (insert-containing) fragment. For analysis of splice site 4, either a 119 nt (insertless) or a 174 nt (insert alone) fragment was utilized. Lastly, the mslo construct 7 (non-spliced control) was a 200 nt fragment. The mouse cyclophilin and the human 18S rRNA constructs contained a 103 nt and an 80 nt fragment, respectively and were used as RNA loading controls. Twenty-five µg of total RNA was hybridized overnight at 42°C with 1 ng of probe using the RPA III kit (Ambion). The unprotected single-stranded RNAs were digested with RNase A/T1 diluted 1:100. The products were electrophoresed through a 5-10% polyacrylamide/8M urea denaturing gel at 250V for 5-7 hrs and transferred to Nytran (Schleicher and Schuell) using a semidyry electroblotter. Shorter RNA fragments were electrophoresed in a higher percentage of acrylamide for better resolution. The protected RNA fragments were
visualized using the BrightStar Biodetect nonisotopic detection kit (Ambion) following incubation with the streptavidin-alkaline phosphatase conjugate solution. The developed blots were subsequently detected using chemiluminescence for 2-4 hrs depending on signal intensity. Optical densities of the protected fragments for each gestational stage were measured using LabWorks 3.0 (Ultra-Violet Products, Inc.) and normalized to the heaviest band (Day 19). The mean normalized values were plotted ± SEM.
RESULTS

Maxi-K channel current density is decreased in term pregnant mice. To determine whether the maxi-K channel current is altered in pregnancy, freshly dissociated myometrial cells from non-pregnant and term-pregnant (day 19) mice were voltage-clamped and maxi-K channel currents measured in the whole-cell configuration. Cells were held at –80 mV and pre-pulsed to +80 mV to eliminate the A-type current previously described in myometrial cells (10). Progressive voltage steps from –100 mV to +80 mV elicited currents that were outwardly rectifying in cells isolated from both non-pregnant and term pregnant myometrium (Fig. 1A). To evaluate the contribution of the maxi-K channel to the total myometrial K+ currents, iberiotoxin (IbTX) was added to the bath solution and current-voltage relationships were measured. As seen from Figure 1B, 200 nM IbTX decreased the myometrial maxi-K channel current. An IbTX concentration of 100 nM reduced K+ current density by >50% within a time frame similar to those reported by Song et al. (9). The density of maxi-K channel current between the two preparations is different (Fig. 1C). Maxi-K current in term-pregnant mice was significantly less than that in non-pregnant mice (at +60 mV, current density was 134.5 ± 37.7 pA/pF for non-pregnant versus 35.8 ± 9.2 pA/pF for term pregnant cells). In addition, the detection threshold for the non-pregnant cells was approximately 0 mV compared to +40 mV in the term pregnant cells.

Maxi-K channel protein expression in mouse myometrium increases during gestation. To determine whether the decrease in maxi-K channel current at term pregnancy is due to a decrease in protein expression, Western blot analyses were performed. Two different antibodies targeted against amino acids 913-926 or amino acids 1098-1196 (Fig. 2, dotted and dashed lines, respectively) of the maxi-K channel α subunit were used for immunoblotting. These antibodies
are targeted against the “tail region” of the channel located in the carboxy-terminal domain. Multiple bands corresponding to the size of the ~125 kDa maxi-K channel protein were detected in all stages of gestation with channel protein expression increasing at the later stages of gestation and decreasing at postpartum day 2 (Fig. 3A, (n=3) and 3B, (n=5)). The lower fragment likely represents a highly reproducible 65 kDa proteolytic fragment of the maxi-K channel α subunit that has been previously described (23). The antibodies also detected an in vitro translated control of the maxi-K channel protein (positive) and did not recognize a reaction translated in the absence of the maxi-K channel cDNA (negative). Equal loading of protein was confirmed by staining the blots with Ponceau S. The pattern of protein expression was shown to be reproducible and consistent by performing experiments multiple times using different membrane samples. To confirm the specificity of the maxi-K channel antibody, a peptide block was performed under similar conditions on the same membrane using the antibody (aa 1098-1196) preadsorbed with 5 µg fusion protein/1 µg antibody (Fig. 3C, n=3). The bands detected at ~125 kDa by immunoblot and the positive control in vitro translated protein were not visible after blocking with the antigenic peptide. The presence of multiple bands corresponding to the predicted size of the maxi-K channel may represent post-translational modification of the maxi-K channel or different mobilities of multiple alternatively spliced maxi-K channel isoforms. Figure 3 indicates that maxi-K channel protein expression increases in mouse uterine smooth muscle throughout gestation and diminishes at postpartum. These results suggest that a decrease in maxi-K channel protein is not a probable explanation for the reduction of current density in term pregnant mice.

*The maxi-K channel is alternatively spliced in the mouse myometrium.* The expression of the diverse maxi-K channel variants is thought to have tissue-specific effects on cellular
excitability, including regulating the electrical resonance of hair cells (16) and modulating neuronal excitability (15). Thus, one way to regulate the level of uterine excitability, and consequently contraction, may be to regulate the voltage or Ca\(^{2+}\) sensitivity of the maxi-K channel, which has potent repolarizing capacity in the myometrium. From electrophysiological and immunoblot analyses in Figures 1 and 3, it is evident that the maxi-K channel is regulated in mouse uterine smooth muscle. One potential mechanism by which smooth muscle cell excitability could be modulated in the myometrium is by expressing multiple maxi-K channel isoforms that differ in their Ca\(^{2+}\) and voltage sensitivities.

Splice-site specific RT-PCR analyses were performed on random-primed total RNA isolated from five gestational stages as previously described. Initial experiments isolated the entire channel cDNA, which corresponded to a sequence previously described (24). The Southern blots in Figure 4 illustrate the PCR fragments generated with primer pairs that flank each splice site (Fig. 2, sites 1-6) and probed with the corresponding sequence. At least two splice variants were detected at sites 1, 3, 4 and 5, while no variants were detected at sites 2 and 6 (Fig. 4). The presence of the upper band in the Southern blot of splice site 1 was inconsistent between blots indicating that this band may be the product of a PCR from a contaminating non-uterine tissue. Southern blot analyses of splice sites 3, 4 and 5 located in the C-terminal “tail” region of the channel detected the presence of 12 bp, 174 bp and 81 bp inserts, respectively. This region of the maxi-K channel has been described as containing sequences that mediate voltage and Ca\(^{2+}\) sensitivity (Fig. 5) (17,20,25,26). All fragments that hybridized the corresponding probes were subsequently subcloned and sequencing attempted to confirm the presence of previously described splice variants. Not all fragments produced sequence results, which may be a result of heteroduplexing of different length PCR products. These results
demonstrate that alternative splicing of the maxi-K channel transcript occurs in the mouse myometrium during gestation, and that alternative splicing of domains of the maxi-K channel that are sensitive to both Ca\(^{2+}\) and voltage may be one mechanism for modulating uterine behavior.

_Ca\(^{2+}\)-sensitive domains of the maxi-K channel are differentially expressed during gestation._ The maxi-K channel transcript contains multiple sites for alternative splicing that can yield a diversity of channel isoforms (Fig. 4). To determine whether differential expression of the maxi-K channel isoforms, which mediate the sensitivity of the channel to Ca\(^{2+}\) and voltage, may be a mechanism underlying the attenuation of maxi-K channel current in uterine smooth muscle at term gestation, RNase protection analyses were performed. The experiments were performed on splice variants at sites 3 and 4 because these sites are known to alter maxi-K channel current.

Total RNA from the five gestational stages was hybridized with antisense biotin-labeled RNA probes corresponding to sequences representing insertless and insert-containing variants at sites 3 and 4 of the maxi-K channel, and a conserved non-spliced region in the C-terminus (Fig. 2, site 7) to determine whole channel regulation (Fig. 6). The results of the RNase protection analyses demonstrate that total maxi-K transcript expression concurs with protein levels seen by immunoblotting; maxi-K channel message increases dramatically during gestation and decreases at postpartum day 2 (Fig. 6A). The expression of the insertless form of the transcript at splice site 3 was similar to that of the whole maxi-K channel with an upregulation at mid to late gestation and a decrease at postpartum day 2 (Fig. 6B, left panel). Although the 12 bp insert encoding “SRKR” at splice site 3, which decreases the channel’s Ca\(^{2+}\) sensitivity, was detected by PCR (Fig. 4), it was not detected by RNase protection analyses (Fig. 6B, right panel). Since
all probes used in the RPA experiments recognized *in vitro* transcribed RNA controls containing their sequence (data not shown), this is likely not a result of the probe’s inability to bind the sequence, but rather the PCR from a contaminating non-uterine tissue. The 174 bp insert detected at splice site 4, which increases the voltage and Ca\(^{2+}\) sensitivity of the maxi-K channel, is present at all stages of gestation in the mouse myometrium and possibly represents a constitutively expressed isoform of the maxi-K channel (Fig. 6C, right panel). However, the expression of the insertless transcript at splice site 4, which would decrease the maxi-K channel's sensitivity to both voltage and Ca\(^{2+}\), is upregulated at mid- to late-term gestation (days 14 and 19) and is back to baseline levels at postpartum (Fig. 6C, left panel). The protein encoded by this isoform may explain the differences in maxi-K channel current levels observed between these two gestational states. These experiments were performed several times with RNA from different mice and the results were consistent between experiments. Densitometric analyses confirm that the maxi-K channel as a whole (Fig. 7A) and the insertless form of splice site 3 (Fig. 7B) are upregulated during gestation and decrease at postpartum. While the insertless form of splice site 4 is also upregulated during late gestation and decreases postpartum (Fig. 7C, left panel), the 174 bp transcript remains constant throughout gestation (Fig. 7C, right panel). These results indicate that alternative splicing of the maxi-K channel transcript is a likely mechanism for modulating uterine excitability during gestation.
DISCUSSION

Previous reports have described the contribution of the maxi-K channel to uterine contractility (4,7). Due to the ability of this channel to potently buffer cell depolarization and modulate smooth muscle relaxation, this channel presumably plays an essential role in maintaining the quiescence of the uterus during pregnancy. Because intracellular Ca$^{2+}$ increases and myometrial cells become more depolarized during pregnancy (21), we would expect the maxi-K channel to have a high voltage and Ca$^{2+}$ sensitivity during gestation to maintain K$^{+}$ efflux and promote uterine smooth muscle quiescence. However, at the onset of labor this channel would be expected to decrease its voltage and Ca$^{2+}$ sensitivity, thereby promoting sustained contractions of the uterus during labor. To better understand the mechanism of maxi-K channel regulation of uterine excitability and contractility during pregnancy, we studied the expression of the maxi-K channel in mouse uterine smooth muscle at multiple stages of gestation.

The majority of K$^{+}$ channel current in mouse myometrial cells is due to maxi-K channels as demonstrated by iberiotoxin block (Fig. 1A and 1B). Our data demonstrate a significant reduction in K$^{+}$ current density in term mouse myometrium compared to non-pregnant myometrium (Fig. 1C) consistent with electrophysiological data previously reported in rats (10,27). The mechanism causing this decrease in current levels is not known. Although these results could also be obtained due to a shift in the conductance-voltage relationship, steps to more positive potentials decreased the seal integrity and this could not be determined. Recent studies by Song et al. (9) suggest that maxi-K protein levels decrease in rats at term pregnancy, which may explain this diminution in current density. However, our results were not consistent with their findings. In mouse uterine smooth muscle tissue the maxi-K channel protein is present
at all stages of gestation, is upregulated at term prior to the onset of labor, and decreases at postpartum (Fig. 3). The discrepancy between these data and those of Song is difficult to explain however it may be the result of species differences, or the time at which term pregnant tissue was extracted. Functional channel loss may occur immediately prior to labor to aid in the process of parturition. However, this cannot explain the loss of channel protein noted in mid-gestation in these studies. Studies by Wang et al. (10) have indicated that maxi-K channels lose their functional importance in rat myometrial cells by multiple factors, including a reduction in density, a positive shift in the voltage-activation relationship, and a lowered sensitivity to Ca$^{2+}$. One mechanism for these functional changes may be the expression of isoforms that attenuate their sensitivity to Ca$^{2+}$ and voltage and thereby facilitate changes in cellular excitability. This may be due to differential alternative splicing of the maxi-K channel transcript to elicit multiple isoforms during gestation. Studies in neuronal and cochlear tissues suggest that changes in cellular excitability may be a result of the presence of multiple isoforms of the maxi-K channel (15,16). In addition, evidence that the maxi-K channel transcript can be hormonally induced to alternatively splice (28) makes the study of this channel in the myometrium during gestation even more interesting. Indications that alternative splicing may occur during gestation are suggested by studies by Perez et al. (5) who reported a difference in the modulation by protein kinase A of maxi-K channels isolated from non-pregnant and pregnant rats. Because phosphorylation sites can be introduced into the channel protein, these data give credence to the idea that alternative splicing of the maxi-K channel transcript in the mouse myometrium is regulated during gestation.

Polymerase chain reactions reveal that four of the six splice sites previously described in the mouse maxi-K channel are alternatively spliced in the mouse myometrium (Fig. 4). Multiple
RT-PCR reactions from different mice have indicated that the predominant transcript in the mouse myometrium was one previously described by Pallanck and Ganetzky (24). Other mslo cDNAs have been described in the mouse brain (13), however these were not detected in the myometrium by RT-PCR, suggesting a possible tissue difference. The inserts detected in the mouse myometrium by RT-PCR of splice sites 3, 4 and 5 were sequenced and determined to be previously described variants of the maxi-K channel (13,17). These inserts, located in the Ca$^{2+}$-modulatory regions of the maxi-K channel, which can introduce phosphorylation sites as well as alter voltage and Ca$^{2+}$ sensitivities of the channel, are present during pregnancy. Although RT-PCR analyses show the transcripts of the inserts to be present, they do not provide information regarding their regulation.

To determine the regulation of the maxi-K channel and its voltage- and Ca$^{2+}$-sensitive domains, RNase protection assays were performed. Initial experiments to elucidate the regulation of the total protein message (Fig. 6A) show that transcriptional regulation of the maxi-K channel correlates with the protein expression observed during gestation (Fig. 3). The transcript of a non-spliced conserved region (site 7) of the maxi-K channel increases throughout gestation to term and decreases at postpartum. This pattern of regulation confirms that the maxi-K channel as a whole is upregulated during gestation and decreases at postpartum. While Song reported a decrease in maxi-K channel transcript levels at term (9), this could result from a fast transcript turnover and isolation of RNA closer to the time of parturition than in the studies presented here. While total transcript regulation is representative of protein expression, this is not true of inserts regulating the channel’s voltage and Ca$^{2+}$ sensitivity. The transcript of the insertless form at splice site 3 of the maxi-K channel is upregulated at mid to late gestation and diminishes at postpartum (Fig. 6B, left panel). This transcript likely represents the predominant
sequence of the myometrial maxi-K channel isoform due to its similar regulation to total channel transcript. The 12 bp insert at splice site 3, which decreases the Ca^{2+} sensitivity of the channel, was detected at all stages of gestation by PCR (Fig. 4), but was not detected by RNase protection analyses (Fig. 6B, right panel). Because the presence of this insert decreases the Ca^{2+} sensitivity of the channel, it was postulated that it would be upregulated at term. Since intracellular Ca^{2+} levels increase during pregnancy, the presence of this insert would decrease K^{+} efflux and, subsequently, sustain a contraction. However, the insert is not present at significant levels in the mouse myometrium during gestation and may represent contamination from non-uterine tissue.

The transcript of the insertless form of splice site 4 is also upregulated at late gestation and decreases at postpartum (Fig. 6C, left panel). However, this transcript is not detected until day 14 of gestation in the mouse. The 174 bp insert at this site, which increases the maxi-K channel’s sensitivity to Ca^{2+}, is present at relatively constant levels at all stages of gestation (Fig. 6C, right panel). Given that splicing at other sites is upregulated during gestation, the selection of this splicing configuration would decline dramatically during pregnancy. This difference in the regulation of the transcripts of the insertless and insert-containing forms of the maxi-K channel at splice site 4 implies that different populations of channels are present in the myometrium throughout gestation. The presence of the insertless form of this transcript would induce a 20 mV positive shift in the voltage-activation curve and lessen the Ca^{2+} sensitivity of the channel, which correlates with electrophysiological data from Wang et al. (10). These different populations of variants are a likely mechanism by which the maxi-K channel modulates cellular behavior and thereby provides a mechanism to explain the functional differences between nonpregnant and pregnant myometrium (5).
A potential mechanism by which the maxi-K channel transcript may be regulated is by sex hormones. Recent studies have shown that removal of the pituitary alters the expression of rat slo transcripts in adrenal chromaffin tissue, providing the first evidence that hormones may induce alternative splicing of the maxi-K channel (28). In uterine smooth muscle, the response of the maxi-K channel to PKA-dependent phosphorylation is influenced by changes in the hormonal status of the tissue (5). Since hormonal status can affect the splicing of the maxi-K transcript, hormones such as estrogen and/or progesterone, which fluctuate during pregnancy, may induce alternative splicing of maxi-K channel transcripts. Further studies will provide insight into whether these hormones regulate alternative splicing of the maxi-K channel transcript in mouse uterine smooth muscle. An additional mechanism may be that an unidentified channel similar to the maxi-K channel, but lacking voltage and Ca$^{2+}$ sensitivity, exists at late gestation or after the onset of labor. This has been reported in human myometrial tissue (29), and thus a mouse homolog may also exist. Another factor that may affect the attenuation of current density may be a lack of association between the maxi-K channel and an accessory β-subunit that alters its activation by voltage and Ca$^{2+}$. Regulation of the maxi-K channel during gestation is likely a combination of both transcriptional regulation and post-translational modifications.

Within uterine smooth muscle alternative splicing of the maxi-K channel α subunit transcript produces multiple isoforms, some which have altered Ca$^{2+}$ and voltage sensitivities. We report that alternatively spliced regions in the Ca$^{2+}$-sensitive domains of the maxi-K channel are regulated differentially in the mouse myometrium during gestation with the prevalent isoform being less sensitive to Ca$^{2+}$ and voltage closer to term. The presence of different isoforms of the
maxi-K channel, which differ in their voltage and Ca\textsuperscript{2+} sensitivities, makes this channel a logical candidate to promote changes in uterine excitability during pregnancy.

ACKNOWLEDGEMENTS

We would like to thank Victoria P. Korovkina, Amanda J. Holdiman, and Dr. Kathryn Lamping for their assistance and critical review of the manuscript. This work was supported by grants from the National Institutes of Health (HD-37831 to S.K.E.) and the National Science Foundation (IBN 98-19339 to S.K.E.).
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FIGURE LEGENDS

FIGURE 1. **Electrophysiological analysis of myometrial cells from non-pregnant and term-pregnant mice.** Myometrial smooth muscle cells were isolated from non-pregnant and day 19 pregnant mice. Cells were held at -80mV and prepulsed for 200ms to +60 or +80mV to eliminate A-type currents. Membrane potentials were stepped from -100 to +80mV in 20 mV steps. Whole-cell currents were measured in the absence (A) and presence (B) of 200nM IbTX to establish the contribution of maxi-K to the whole cell K⁺ current. Iberiotoxin blocked K⁺ current in myometrial cells isolated from both non-pregnant and term-pregnant mice (data not shown for term-pregnant cells). Whole-cell currents and membrane capacitance were measured from non-pregnant (n=10) and day 19 pregnant (n=8) myometrial smooth muscle cells using the same pulse protocol (C). Mean sustained current of each cell was divided by the capacitance of that cell to determine the current density. Mean current densities from non-pregnant and day 19 mice were plotted ± SEM for each voltage step. Asterisks indicate significant difference (p < 0.05) between groups at a given membrane potential.

FIGURE 2. **Schematic of the alternative splice sites in the mouse uterine maxi-K channel α subunit.** The upper schematic illustrates the location of the six splice sites (1-6) within the mouse maxi-K channel relative to the transmembrane segments of the channel. Site 7 is within a conserved non-spliced region of the C-terminus of the maxi-K channel and is used as a control. The dotted and dashed lines near the S9 and S10 segments indicate the epitopes for maxi-K antibodies amino acids 913-926 and amino acids 1098-1196, respectively. The lower table is a
summary of the primer pairs used for the RT-PCR analysis of each splice site and the expected sizes of the PCR fragments.

FIGURE 3. **Immunoblot analyses of mouse myometrial membranes showing maxi-K channel expression during gestation.** Thirty µg of mouse uterine smooth muscle membranes isolated from the five gestational stages were fractionated by SDS-PAGE and immunblotted with an antibody targeted against amino acids 913-926 (A, n=3) or 1098-1196 (B, n=5) of the maxi-K channel α subunit. Multiple bands of ~125 kDa were detected in all gestational stages indicating the presence of this channel in mouse uterine smooth muscle (arrows). The lower fragment in (A) and (B) likely represents a 65 kDa proteolytic fragment previously described in the maxi-K channel α subunit. The protein expression of the maxi-K channel increases during gestation and decreases at postpartum day 2. An *in vitro* translated maxi-K channel containing the sequence of interest was used as a positive control. The specificity of the antibody was confirmed by preadsorption of the maxi-K (aa 1098-1196) antibody with the antigenic peptide, which abolished the positive control and the bands seen at 125 kDa (C, n=3).

FIGURE 4. **The maxi-K channel transcript is alternatively spliced in the mouse myometrium.** Total RNA from mouse myometrium at the five gestational stages was used for splice site-specific RT-PCR analyses of the α subunit and Southern blotted to detect the presence of potential splice variants. The Southern blots show the PCR fragments that were generated with primer pairs that flank each splice site and hybridized with the corresponding probe. Closed
arrows indicate a PCR fragment that does not contain an insert and open arrows represent an insert-containing PCR fragment. Asterisks (splice sites 1, 4 and 5) represent PCR fragments that did not yield sequence although sequencing was attempted multiple times. At least two splice variants were detected at splice sites 1, 3, 4 and 5, suggesting that alternative splicing of the maxi-K transcript occurs in the mouse myometrium at all stages of gestation. No splice variants were detected using primers flanking splice sites 2 and 6.

FIGURE 5. **Summary of the alternative splice variants in the mouse uterine maxi-K channel α subunit.** The upper schematic diagram illustrates the location of the four splice sites (1, 3, 4 and 5) within the mouse maxi-K channel α subunit in which splice variants were detected in the myometrium by RT-PCR. The lower table is a summary of the splice variants’ corresponding amino acid sequences and their Ca\(^{2+}\) and voltage sensitivity effects. The insert at splice site 1 was unsequencable though attempted multiple times. The function of the insert at splice site 5 has not been determined.

FIGURE 6. **Determination of transcript expression of the maxi-K channel isoforms during gestation.** Twenty-five µg of total RNA isolated from mouse uteri at the five gestational stages was hybridized with 1 ng of a biotin-labeled antisense probe containing either the mslo construct 7 (A), 3 (B), or 4 (C). The latter two (B and C) were probed for both the insertless and insert containing sequences. Arrows indicate the presence of the protected fragments of interest. Probes against 18S rRNA or cyclophilin were used as internal loading controls. The RNase
protection analyses demonstrate that the transcript of a non-spliced region of the C-terminus of the maxi-K channel (representing whole channel regulation) increases throughout gestation and decreases at postpartum (A, n=4). The insertless transcript at splice site 3 is upregulated at mid to late gestation and decreases by postpartum, however, the 12 bp insert was not detected by RPA (B, n=5). The insertless transcript at splice site 4 is also upregulated at late gestation and decreases by postpartum while the 174 bp insert is present at relatively constant levels at all stages of gestation (C, n=4).

FIGURE 7. Densitometric summary showing the trends of transcript expression of the maxi-K channel isoforms during gestation. Optical densities of the protected fragments for each isoform from RNase protection analyses of each gestational stage were measured and normalized to the heaviest band. The mean normalized values were plotted ± SEM. The transcript of a non-spliced region of the C-terminus of the maxi-K channel (representing whole channel regulation) increases throughout gestation and decreases at postpartum (A, n=4). The insertless transcript at splice site 3 is upregulated at mid to late gestation and decreases by postpartum (B, n=5), however, the 12 bp insert was not detected by RNase protection analysis (n=5). The insertless transcript at splice site 4 is also upregulated at late gestation and decreases by postpartum (C, left graph, n=4) while the 174 bp insert is present at relatively constant levels at all stages of gestation (C, right graph, n=4).
| Name of Construct | Splice site | Oligonucleotide Primers                                      | nucleotide range | Expected size of PCR fragment (bp) |
|-------------------|------------|-------------------------------------------------------------|------------------|----------------------------------|
| mslo 1            | 1          | 5'-GGG TCG ACT CTT AGA ATG AGC AGC AA 3'-TAT GAT GAG CGC ATC CAT | -7-99            | 106                              |
|                   |            |                                                             |                  | 157                              |
| mslo 2            | 2          | 5'-GGA AAC CGC AGG AAA TAC 3'-CAG GAA GTT AGA GAC ACT         | 1099-1200        | 101                              |
| mslo 3            | 3          | 5'-AAG CTC CGT ATG ATA GCC 3'-GTC ACT TGC GAT GAA            | 1804-1938        | 122                              |
|                   |            |                                                             |                  | 134                              |
| mslo 4            | 4          | 5'-GCC TGT CAT GAT GAC GTC 3'-CCT CAT GCC CCC ATT ACG        | 1972-2091        | 119                              |
|                   |            |                                                             |                  | 293                              |
| mslo 5            | 5          | 5'-CAG CCG TCC ATC ACA ACT 3'-CTC TGT GTC AGG GTC ACT        | 2731-2919        | 108                              |
|                   |            |                                                             |                  | 189                              |
| mslo 6            | 6          | 5'-CAC TCC ATC CCG TCC ACA 3'-GGT CTA GAA TCC GGC TCA TCT GTA AA | 3463-3561        | 98                               |
| mslo 7            | -          | 5'-CCC AGC CAG TGT ACA AAA 3'-ATT TGC TGT GGA CGG GAT        | 3286-3486        | 200                              |
| Splice Site | Amino Acid Sequence of Insert          | Function of Insert                                                                 |
|------------|---------------------------------------|-------------------------------------------------------------------------------------|
| 3          | SRKR                                  | Decrease Ca\(^{2+}\) sensitivity of channel 3-4 fold (15).                         |
| 4          | PKMSIYKRMRRAACCFDCGSRERDCSCMSGRVR     | Increase Ca\(^{2+}\) sensitivity of the channel. Shifts the activation curve 20 mV in the hyperpolarizing direction (20). |
| 5          | AKPGKLPLVSNQEKNSGTHILMITEL           | Unknown                                                                            |
Regulation of the \( \text{Ca}^{2+} \)-sensitive domains of the maxi-K channel in the mouse myometrium during gestation

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*J. Biol. Chem.* published online June 27, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000974200

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