Remodeling of Kv4.3 Potassium Channel Gene Expression under the Control of Sex Hormones*

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Kv4.3 channels are important molecular components of transient K+ currents (Ito currents) in brain and heart. They are involved in setting the frequency of neuronal firing and heart pacing. Altered Kv4.3 channel expression has been demonstrated under pathological conditions like heart failure indicating their critical role in heart function. Thyroid hormone studies suggest that their expression in the heart may be hormonally regulated. To explore the possibility that sex hormones control Kv4.3 expression, we investigated whether its expression changes in the pregnant uterus. This organ represents a unique model to study Ito currents, because it possesses this type of K+ current and undergoes dramatic changes in function and excitability during pregnancy. We cloned Kv4.3 channel from myometrium and found that its protein and transcript expression is greatly diminished during pregnancy. Experiments in ovariectomized rats demonstrate that estrogen is one mechanism responsible for the dramatic reduction in Kv4.3 expression and function prior to parturition. Furthermore, the reduction of plasma membrane Kv4.3 protein is accompanied by a perinuclear localization suggesting that cell trafficking is also controlled by sex hormones. Thus, estrogen remodels the expression of Kv4.3 in myometrium by directly diminishing its transcription and, indirectly, by altering Kv4.3 delivery to the plasma membrane.

Kv4.3 channel activity gives rise to transient Ca2+-independent outward K+ currents (Ito currents) that play a key role in pacing electrical activity of neurons and cardiac myocytes. In neurons, they shape the action potential and favor firing frequency (1). In the heart, Kv4.3 channels participate in setting the plateau potential and overall action potential duration as demonstrated in in vivo gene transfer experiments (2). Consistent with this role, pathological conditions such as chronic human atrial fibrillation (3) and human heart failure (4) exhibit a reduced expression of this class of channels. However, in other systems like smooth muscle their role is less clear. Kv4.3 transcripts have been detected in mesenteric artery (5), aortic, vas deferens, stomach, and urinary bladder (6) smooth muscles, but correlation with their function in intact tissues is still missing.

Ito (A-type) currents in smooth muscle were first reported in uterine smooth muscle (7) and later have been reported in renal (8) and pulmonary (9) vascular beds and in colonic smooth muscle (10). In the heart, Ito currents can be formed, depending on the species, by either Kv4.3 and/or Kv4.2 channels (2, 11–15). Given the facts that in myocardial dysfunction the Kv4 genes are down-regulated and their protein and mRNA distribution seems specific for different heart regions, transcriptional regulation may be a significant factor that controls potassium channel molecular remodeling (3, 4, 12, 13, 16). Furthermore, the transcription seems to be under hormonal control, because the reduction in Kv4.3 and Kv4.2 mRNA levels after myocardial infarction can be restored by the administration of thyroid hormone (17). Thus, it is very likely that in smooth muscle Kv4 genes are also under transcriptional regulation during pathological and physiological conditions. In keeping with this view, we now show that in myometrium the Kv4.3 gene may be the predominant molecular correlate of Ito currents and that its expression varies during pregnancy, with the rise of estrogen being one important factor that determines the Kv4.3 down-regulation at the end of pregnancy.

EXPERIMENTAL PROCEDURES

Animals—Animal protocols were approved by the UCLA School of Medicine Animal Committee. Sprague-Dawley rats were used at non-pregnant stage (diestrus/estrum) and various times of pregnancy (days 7–8, 10–11, 13–15, 17–18, 19–22) or 6–12 h postpartum (n = 3–5 in each group). Ovariectomized (ovx) rats were purchased from Charles River. Prior to tissue collection, rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). Xenopus oocytes were obtained from adult female Xenopus laevis (Xenopus One, Ltd.).

Hormonal Treatment—Ovx rats were injected subcutaneously twice a day. One group of rats was injected for 4 days with 3 mg/kg progesterone (P4) or 50 μg/kg 17β estradiol (E2). The second group, E2-primed rats, was initially injected for 2 days with 50 μg/kg E2 and afterward for 4 days with 3 mg/kg P4. As control, the same injection protocol was performed with the vehicle (2 ml/kg 1:10 ethanol:sesame oil). Seven to eight rats were used in each group. E2 and P4 stock solutions were in 100% ethanol at a concentration of 4 mg/ml E2 and 30 mg/ml P4. Rats were sacrificed 12 h after the last injection. Tissue and blood samples were obtained at the same time. Plasma levels of P4 and E2 were determined using radioimmunoassay by Dr. John K. H. Lu, Departments OB & GYN-Endo/Neurobiology at UCLA.

1 The abbreviations used are: ovx, ovariectomized; FTIC, fluororescin isothiocyanate; TBS, Tris-buffered saline; MES, methanesulfonate; P4, progesterone; E2, 17β estradiol; HP, holding potential; nt, nucleotide(s); RT-PCR, reverse transcription-polymerase chain reaction; RPA, RNase protection assay; r, rat.
Electrophysiology—One day before injection, oocytes were defolliculated with collagenase treatment (2 mg/ml for 40 min at room temperature). Oocytes were maintained at 19 °C in Barth solution supplemented with 50 μg/ml gentamicin. Kv4.3 currents were recorded 2 days after cRNA injection (10 ng per oocyte) using the cut-open oocyte vaseline seal method. The patch electrodes (3 M potassium acetate, 206 units/ml collagenase type 4, 9.2 units/ml elastase, and 1.5 mg/ml trypsin inhibitor (Worthington Biochemicals)) were filled with 2 M NaCl, 140 mM Na-MES, 2 CaCl2, 2 MgCl2, 10 mM HEPES, 10 mM phenylmethylsulfonyl fluoride, 160 units/ml collagenase, 2 mg/ml bovine serum albumin for 1 h. After washing, the blots were incubated in sub- jected to molecular analysis using RT-PCR. Gene-specific primers for the Kv4.3 and Kv4.2 were: 30 cycles at 94 °C, 60 °C, and 72 °C for 1 min, 1.3 mg/ml collagenase F, 2120 units/ml hyaluronidase (Sigma Chemical Co.), 2 mg/ml calpain I and II inhibitors, 100 mg/ml benzamidine, and 0.2 mM aprotinin. cDNA was confirmed by the lack of detectable genomic DNA using oligo-dT primer, followed by a 20-μl reverse transcription reaction with 20 units of avian myeloblastosis virus reverse transcriptase. Conditions were: 30 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Gene-specific primers for the Kv4.1, Kv4.2, and the various isoforms of the Kv4.3 channel are shown in Table 1. As controls, we used the reaction mixture without the cDNA or plasmid DNA. The quality of cDNA was confirmed by the lack of detectable genomic DNA using primers flanking intronic regions (β-actin, Kv4.3 long and medium, Kv4.2 and Kv4.1, see Table 1). We used real time PCR to quantify the relative abundance of each gene (iCycler iQ Real Time PCR, Bio-Rad) with SYBR green I (stock solution >10,000 diluted at 1,800,000) as the fluorescent probe (Molecular Probes) and Platinum Quantitative PCR SuperMix-udg (Life Technologies, Inc.) (20). Calibration curves were constructed using known concentrations of pre-amplified Kv4.1, Kv4.2, and Kv4.3 segments (amplicons) subcloned into the pcRCl2.1 vector (In- vitrogen). Reaction conditions were: 5 min at 95 °C to activate the "hot start" platinum Taq DNA polymerase, followed by 40 cycles at 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s.

Cloning of the Full-length Myometrium Kv4.3 K Channel—The rat uterus was used as the source for the cloned full-length Kv4.3 gene. The rat uterus was dissected as described above and incubated for 1 h at 37 °C with 3.5 units Expand High Fidelity DNA polymerase in a 100-μl reaction using Taq DNA polymerase, followed by 40 cycles at 95 °C, 60 °C, and 72 °C for 1 min, 20 μl reverse transcription reaction with 20 units of avian myeloblastosis virus reverse transcriptase. The PCR was performed in a 50-μl reaction using Taq and Pwo DNA polymerases (Roche Molecular Biochemicals) following cDNA synthesis. Incubation conditions were: 94 °C for 1 min, 60 °C for 1 min, and 68 °C for 3 min. The PCR product with the expected 2000-nucleotide size was first analyzed with restriction enzymes; and, its identity was confirmed by complete sequencing. The PCR product was purified and subcloned into either pBSthai vector (21) or into pcdNAS.

RNase Protection Assay—Probe DNAs were obtained using RT-PCR amplification and gene-specific primers for rKv4.3 and rNa1, K− ATTase α1 subunit (Table 1). Myometrial cDNA (10 μl) was amplified with 3.5 units Expand High Fidelity DNA polymerase in a 100-μl reaction for 35 cycles. The amplification products of the correct size were subcloned into EcoRI- and BamHI-digested pBluescript II KS vector. Radiolabeled antisense riboprobes, which contained bases of the vector (294 nt for rKv4.3 and 202 nt for rNa1, K− ATTase) were synthesized from HindIII-linearized plasmid constructs with T7 RNA polynucleotide transcriptase and [32P]UTP. RNase protection assays of rat total RNA isolated from non-pregnant myometrium and at different stages of pregnancy were performed using an RPA III kit (Ambion). Each sample, 40 μg of total RNA was hybridized with 3.5 × 10⁶ cpm of the gel-purified RNA probes for 16 h at 37 °C, followed by a 100-μl reaction with 160 units/ml collagenase, 2 mg/ml bovine serum albumin for 1 h. After washing, the gel was exposed to a storage phosphor screen. The bands were quantified using a Molecular Dynamics PhosphorImager 445 SI. Radioactive counts were quantified in identical areas, and values are expressed as a percentage of the total volume of all samples for a given clone.

- Remodeling of Kv4.3 Channel Expression by Estrogen

Immunocytochemistry—Fresh uteri were fixed by immersion in fixative (4% paraformaldehyde, 2% picric acid in 0.1 M phosphate-buffered saline, pH 7.4) for 2 h. Transverse cryostat sections (10 μm) were incubated for 12 h at 4 °C with either anti-Kv4.3siA-ATG affinity-purified antibody (1:250) or anti-smooth muscle α-actin monoclonal antibody (1:500) raised in rabbit. Goat secondary antibody (1:250) was purchased from Abcam for staining for Kv4.3. Sections were incubated in secondary antibody for 30 min and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:4000) (Amersham Pharmacia Biotech) for 1 h. After washing, the blots were incubated in substrate for enhanced chemiluminescence (ECL) for 1 min and autoradiographed on Kodak BioMax film. The bands were quantified using the Bio-Rad GS670 Imaging Densitometer and software.
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TABLE I  
Gene-specific primers

| Gene        | Experiment                           | GenBank™ accession number | Expected amplicon size\(^a\) | Forward primers                                      | Reverse primers                                         |
|-------------|--------------------------------------|---------------------------|------------------------------|------------------------------------------------------|---------------------------------------------------------|
| Kv4.3       | Full-length cloning of Kv4.3 long form | AF334791                  | 1994                         | 5'-GCACGATCTGCGCCAC CAGAGGCCAGGATTGAGCAGGC-3 \(^b\) ATCGGCCGGAAGAGCT TAGGACC-3 \(^c\), nt 1-21 | 5'-GGTCATGATTGCCACCAATCGGAGCTG-3 \(^c\) TCGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.1\(^d\) | Detection of Kv4.1                   | U89873                    | 293                          | 5'-CGCTAAACGTATCAAGC TACGGAGCTGCGCAAGAGGAGGAGGAC-3 \(^c\), nt 1479-1498 | 5'-GGTCATGATTGCCACCAATCGGAGCTG-3 \(^c\) TCGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.2\(^e\) | Detection of Kv4.2                   | S64320                    | 283                          | 5'-CCCAAGCTGAATGTC ATGGAGATCTAGAGAAGAGGAGGAGGAGGAGG-3 \(^c\), nt 1498-1498 | 5'-GGTCATGATTGCCACCAATCGGAGCTG-3 \(^c\) TCGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.3\(^f\) | Detection of Kv4.3 long form         | AF334791                  | 296                          | 5'-CCCAAGCTGAATGTC ATGGAGATCTAGAGAAGAGGAGGAGGAGGAGG-3 \(^c\), nt 1498-1498 | 5'-GGTCATGATTGCCACCAATCGGAGCTG-3 \(^c\) TCGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.3\(^g\) | Detection of Kv4.3 long and medium form | AF334791                  | 342 (long); 285 (medium)     | 5'-GCACGATGTAATCGTCGTC ATCGGCTGCGC-3 \(^c\), nt 1397-1416 | 5'-GGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.3       | RPA probe for Kv4.3 short            | L48619                    | 329                          | 5'-GCACGATGTAATCGTCGTC ATCGGCTGCGC-3 \(^c\), nt 1397-1416 | 5'-GGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| Kv4.3\(^h\) | RPA probe for Kv4.3                 | U42975                    | 250                          | 5'-GCACGATGTAATCGTCGTC ATCGGCTGCGC-3 \(^c\), nt 1397-1416 | 5'-GGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| β-Actin\(^i\) | Detection of β-actin                | V01217                    | 494                          | 5'-GCACGATGTAATCGTCGTC ATCGGCTGCGC-3 \(^c\), nt 1397-1416 | 5'-GGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |
| rNa\(^+\)/K\(^+\)-ATPase | RPA probe for rNa\(^+\)/K\(^+\) -ATPase | M28647                    | 157                          | 5'-GCACGATGTAATCGTCGTC ATCGGCTGCGC-3 \(^c\), nt 1397-1416 | 5'-GGGCTGAGGAGGAGGG-3 \(^c\), nt 1228-1427 |

\(^a\) Unless otherwise stated, expected sizes for amplicon cDNAs are given.  
\(^b\) nt, nucleotide numbers; underlined nucleotides, restriction enzyme sites to facilitate subcloning.  
\(^c\) Primers could distinguish cDNA from genomic DNA (gDNA) serving as internal control (36).

Statistics—Data were expressed as means ± S.E. Comparisons between two groups were analyzed by Student’s t test. A probability level less than 0.05 was considered statistically significant.

RESULTS

Detection of Kv4 K\(^+\) Channel Isoforms in Rat Myometrium—Non-pregnant (7) and late (18 day) pregnant (22, 23) myometrial cells possess a fast, 4-aminopyridine-sensitive, transient outward current (Ito), but its molecular identity, functional role, and possible regulation during the course of pregnancy is still unknown. As mentioned, in other systems this current seems to be due to the activation of Kv4.3 and/or Kv4.2 channels. Transcripts of Kv4.3 have been detected in various smooth muscles (6); however, uterine smooth muscle has not been examined. Therefore, we initially screened with qualitative RT-PCR non-pregnant myometrium cDNA for Kv4.1, Kv4.2, and Kv4.3 transcripts using gene-specific primers (see Table I). Fig. 1A shows that transcripts of the three members of the Kv4 K\(^+\) channel family are present and that the Kv4.3 gene transcripts seem significantly more abundant in non-pregnant myometrium.

To quantify the relative abundance of the Kv4.1, Kv4.2, and Kv4.3 transcripts we performed real time PCR using the same gene-specific primers used for RT-PCR. We initially tested if the primers for each gene had the same efficiency. To this end, we obtained the slopes of the standard curves (threshold cycle \(\text{versus} \) log [amplicon]) that gave values of 3.3, 3.0, and 3.0, respectively. These results indicate that the reaction efficiencies were similar for the three pairs of selected primers, validating our determinations. In addition, the adequacy of the fluorescent measurements with SYBR green I was confirmed by the fact that at the end of each reaction (40 cycles) a single product of the appropriate size was observed by agarose gel electrophoresis, and that the melting curve for each product had a single well defined peak (20). The real time PCR results (Fig. 1B) were similar to those obtained by RT-PCR and demonstrate that the predominant Kv4 transcripts in uterus are of the Kv4.3 class. Thus, we focused our studies on the Kv4.3 K\(^+\) channel.

FIG. 1. Kv4.3 transcripts are more abundant than Kv4.1 and Kv4.2 in non-pregnant myometrium. A, RT-PCRs were performed using non-pregnant myometrium poly(A) RNA and gene-specific primers for Kv4.1, Kv4.2, and Kv4.3. Lane 1, DNA marker (M); lane 2, water (W); lane 3, Kv4.3; lane 4, Kv4.2; and lane 5, Kv4.1. The amplified cDNAs had the expected size, Kv4.1 (293 nt), Kv4.2 (283 nt), and Kv4.3 (296 nt). Each sample is pooled mRNA (approximately 0.4 μg) from at least three different rats. Primers used are in Table I for Kv4.3, primers to recognize the long form were used. Identities were confirmed by sequencing. Amplified products were separated on 2% agarose gels and visualized with ethidium bromide staining. Similar results were obtained in four experiments. B, relative expression of Kv4.3, Kv4.2, and Kv4.1 genes in non-pregnant myometrium mRNA assessed by real-time PCR. Kv4.3 transcripts are about twice as abundant as Kv4.2 and Kv4.1. Bars represent duplicate experiments using mRNA isolated from uteri of three rats. Inset: the fluorescence change as a function of cycle number. Threshold cycle was lower for Kv4.3 channels, demonstrating its higher expression.

Non-pregnant Myometrium Predominantly Possesses the Long Form of the Kv4.3 K\(^+\) Channel—The Kv4.3 K\(^+\) channel subfamily has three splice variants known as long, medium (19), and short (24). The long form is identical to the medium form but with a splice insert of 19 amino acids between amino acid...
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Expression of Kv4.3 K+ Channel Is Reduced during Pregnancy—To test the hypothesis that Kv4.3 channel expression undergoes dynamic changes during physiological conditions and under the control of sex hormones, we planned the following strategy: 1) examine changes in protein and mRNA levels during the course of normal pregnancy using immunocytochemistry, Western blots, and RNase protection assay (RPA); 2) investigate if estradiol or progesterone can mimic the physiological changes that occur prior to parturition; and 3) to measure Ito currents in single myometrial cells in ovarioverted rats under hormonal treatment.

Immunocytochemistry experiments revealed that, during the course of pregnancy, Kv4.3 channel expression is dramatically suppressed (Fig. 3). Panels A–D are confocal images obtained in non-pregnant and pre-term uteri. The images show that myometrium was strongly immunolabeled with the Kv4.3451–467 antibody in non-pregnant uterus (Fig. 3A). In contrast, pre-labor (21-day) uterus showed a tremendous decrease in the Kv4.3 signal intensity, and a punctuated expression pattern was revealed (Fig. 3B). High resolution images showed that in non-pregnant rats, the Kv4.3 immunolabeling was mainly located in the surface membrane of smooth muscle cells (Fig. 3C), whereas prior to delivery Kv4.3 signals almost disappeared from the plasma membrane and were highly concentrated in the perinuclear region (Fig. 3D). It is interesting to note that in most non-pregnant uteri, Kv4.3 was preferentially expressed in the circular layer. The label intensity ratio (circular/longitudinal layers) was 1.5 ± 0.02 (n = 20).

Time course experiments demonstrated that the onset of Kv4.3 down-regulation was established since the early stages of pregnancy (days 7–8) (Fig. 3E) and expression continued to diminish until 6–12 h postpartum. As controls, no significant changes were observed at equivalent stages of gestation when smooth muscle α-actin was labeled, and no labeling was observed when the tissue sections were immunostained in the presence of the Kv4.3451–467 antigenic peptide (10 μg/ml) (not shown).

Western Blot Analysis of Kv4.3 Shows a Marked Decrease in Channel Expression as a Function of Pregnancy—Western blot analysis of myometrial membranes produced similar results as immunocytochemistry measurements (Fig. 3 versus Fig. 4) and demonstrated that Kv4.3 protein expression is greatly suppressed prior to labor. Fig. 4A shows that the same Kv4.3451–467 antibody recognized a protein of the expected molecular size of ~72 kDa in non-pregnant (NP), at different stages of pregnancy (7 days pregnant (7DP) to 21 days pregnant (21DP)), and post-partum (PP) myometrium. This signal is specific for all gestational stages, because the protein-antibody interaction was fully inhibited when the antibody was pre-adsorbed with...
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FIG. 3. Kv4.3 channel immunolabeling of uterine tissue sections. Non-pregnant and pregnant myometrium was immunostained using a polyclonal anti-Kv4.3 antibody. As a control, no staining was detected when the anti-Kv4.3 antibody was preadsorbed with the corresponding antigen (not shown). A and B, non-pregnant and pregnant myometrium at low magnification. Note a prominent reduction in Kv4.3 channel expression prior to parturition (21-day pregnant) and a punctuated pattern. C and D, same as for A and B, but at higher magnification. Note the high staining of Kv4.3 channels in non-pregnant myometrium (C, single arrow) when compared with pregnant myometrium (D). In addition, in the latter, Kv4.3 channels accumulate in the perinuclear region (double arrow). P, perimtrium; L, longitudinal layer of myometrium; C, circular layer of myometrium; E, endometrium. Tissue sections were of 10 mm. Single confocal sections of 1-mm optical depth. E, quantification of Kv4.3 using conventional fluorescence microscopy. Similar results were obtained if confocal images were analyzed: NP = 73 ± 17 pixel intensity, pregnant 20–22 days = 22 ± 0.2 pixel intensity using confocal versus NP = 63 ± 6 pixel intensity, pregnant 20–22 days = 24 ± 6 using conventional fluorescence. Each point represents the mean values of three to six rats. *

the corresponding antigen (10 μg/ml) (Fig. 4B). For comparison, Fig. 4C shows the signals obtained in the same blot using a Na+/K+ ATPase α1 monoclonal antibody. In contrast to Kv4.3, the Na+/K+ ATPase α1 remained fairly constant throughout gestation with a small but significant increase at days 10–11. Fig. 4D plots the normalized mean values of several Western blot experiments, and demonstrates that, similar to tissue labeling experiments (Fig. 3), down-regulation of Kv4.3 starts to be evident since the early stages (7–8 days) of pregnancy and that expression is greatly reduced near term (20–22 days). Note that at day 18 of pregnancy, there is a slight increase in protein expression that coincides with an increase of mRNA at this stage (see Fig. 5). This slight increase in Kv4.3 membrane expression was not detected with immunocytochemistry and may be due to a lower technique resolution. Nevertheless, both immunocytochemistry and Western blot analysis demonstrate that a strong Kv4.3 remodeling occurs during pregnancy with a sharp reduction in plasma membrane expression near parturition.

Kv4.3 mRNA Levels Also Diminish During Pregnancy—Poten...
is clear, however, that Kv4.3 transcript and protein expression are both greatly diminished at the end of pregnancy. Thus, one of the mechanisms for Kv4.3 down-regulation during pregnancy seems to be at the transcriptional level.

Kv4.3 Channel Down-regulation at the End of Pregnancy Can Be Mimicked by Estrogen—During pregnancy, the levels of sexual hormones change drastically. In general, estrogen favors the expression of contraction associated proteins; whereas progesterone has the opposite role (25). In the rat, the onset of parturition follows a sharp decrease in progesterone levels, whereas estrogen levels remain high (26, 27). Thus, the decreased “relaxing force” of progesterone at the end of pregnancy switches the uterus to an estrogen prevalent state where the synthesis of contraction-associated proteins is dramatically enhanced and/or the synthesis of relaxing-associated proteins is greatly reduced. To determine if this assumption applies to the Kv4.3 expression observed at the end of pregnancy, we performed experiments using ovx rats treated with progesterone (P4) or 17β estradiol (E2) to mimic the plasma concentrations of the sex hormones. Using immunocytochemistry, Western blot analysis, and RPA we found that Kv4.3 protein, mRNA, and RNA are dramatically reduced at the end of pregnancy and that this reduction can be mimicked in E2-primed but not in P4-primed rats. Furthermore, we show using RT-PCR that myometrium possesses the long form of the Kv4.3 transcript and protein expression are both greatly diminished at the end of pregnancy. Thus, one of the mechanisms for Kv4.3 down-regulation during pregnancy seems to be at the transcriptional level.

Fig. 5. Transcriptional regulation of Kv4.3 channel expression during pregnancy. A, RPA of total RNA (40 μg) isolated from non-pregnant and pregnant rat myometrium at different stages. The first lane shows the RNA markers (sizes in numbers), and the last lane shows the undigested full-length gene-specific probes. The protected fragments (marked with a (lane) are 229 nt for Kv4.3 (nt 86–311) and 133 nt for the Na+/K+ ATPase (nt 2027–2159). As control, we used yeast RNA (lane 9). All conditions were maintained constant. Products were electrophoresed on a 5% polyacrylamide-8 M urea gel and analyzed with a PhosphorImager. B and C, mRNA quantification for rKv4.3 channel and rNa+/K+ ATPase α3 subunit. Experiments were done as in A. Three different preparations of total RNA for each stage were examined, except for non-pregnant myometrium, which includes six RNA preparations. Note that the RNA level of Kv4.3 channel is already decreased at day 8 of pregnancy and is sharply reduced before delivery (21DPF). For comparison, the RNA levels of the rNa+/K+ ATPase α3 subunit are shown. The rNa+/K+ ATPase mRNA levels increase when compared with non-pregnant myometrium but are maintained without significant changes during pregnancy. *, p < 0.05 compared with non-pregnant tissue. **, p < 0.05 compared with non-pregnant and 8, 13, and 21 DP and PP.

DISCUSSION

The main finding of this work is that Kv4.3 channels are modulated by sex hormones. Using immunocytochemistry, Western blot analysis, and RPA we found that Kv4.3 protein, functions, and mRNA are dramatically reduced at the end of pregnancy and that this reduction can be mimicked in E2-primed but not in P4-primed rats. Furthermore, we show using RT-PCR that myometrium possesses the long form of the Kv4.3 channel, which is predominant when compared with the Kv4.1 and Kv4.2 genes. Functional expression of the myometrium Kv4.3 long form generated outward transient currents that resembled those of native myometrial tissue (7, 28).

Kv4.2 and Kv4.3 channels are thought to be part of the
Fig. 6. Estrogen at concentrations similar to those at term pregnancy induces a dramatic reduction of Kv4.3 channel expression. A, Western blot analysis of crude membranes isolated from ovx rats non-treated (oil) or treated with P4, E2, and P4 after E2 priming (see “Experimental Procedures”). The bar plot is the mean of seven to eight individual rats for each treatment. *, p < 0.05 when compared with oil treatment. The action of P4- in E2-primed rats (E2p-P4) is not significant when compared with oil-injected E2-primed rats (E2p-oil). Protein loading was checked directly by quantification with Coomassie Blue staining. B, plasma levels of P4 and E2 under different treatments in the same animals used in panel A.

Fig. 7. Dramatic reduction of functional expression of transient outward (Ito) currents in E2-treated ovx rats. Outward K+ currents recorded under whole cell patch clamp in single smooth muscle cells from myometrium of ovx rats sham-injected with oil (A) and after E2 treatment (B). Cells were maintained at a HP of −90 mV, and pulses in 10-mV increments were delivered to +80 mV in A and to +160 mV in B. Current amplitudes were normalized by cell capacity. Note the practical absence of Ito currents in E2-treated rats (B).

molecular components of Ito currents in the heart (2, 11–15). Transcriptional regulation may be a significant factor that controls molecular remodeling of Kv4 genes, because they have a specific distribution in different heart regions, they are downregulated in myocardial disease, and are up-regulated by thyroid hormone (3, 4, 12, 13, 16, 17). We now show that Kv4.3 may also be the molecular correlate of Ito current in myometrium and that it is transcriptionally regulated by sex hormones. Our experiments indicate that Ito currents from myometrium (7) are likely encoded by the Kv4 gene family, because Ito currents share the same pharmacology (4-aminopyridine sensitivity and tetraethylammonium resistance) and kinetics with Kv4 channels (7, 28). Furthermore, it is likely that the predominant Kv4 gene underlying myometrium Ito currents is the Kv4.3, because qualitative and real-time PCR showed less than half of the amount of Kv4.2 and Kv4.1 transcripts (Fig. 1). The Kv4.3 long isoform is predominant because the Kv4.3 short form is barely detected and the medium form was undetectable using RT-PCR (Fig. 2). However, at present we cannot rule out the possibility that members of the Kv4 family and associated β subunits (29) also contribute to Ito currents in myometrium, as is the case for cardiac tissues (11, 16).

Consistent with the view that Ito currents in myometrium may result from Kv4.3 gene expression is the fact that Kv4.3 protein could be readily detected using site-directed antibodies (Figs. 3 and 4). The fact that, in E2-primed rats, Kv4.3 channel protein is dramatically reduced and that Ito currents are practically undetected, favors the view that the Kv4.3 gene is one of the major molecular constituents of myometrium Ito currents. It is noteworthy that, similar to the differential distribution in the heart regions (12), Kv4.3 in myometrium seems to have a preferential distribution in the circular layer. The physiological impact of this differential distribution in the heart or myometrium is difficult to assess. Nevertheless, because Ito currents modulate smooth muscle rhythmic activity (10) and myometrium contractile characteristics are dramatically changed during pregnancy and near term, it is conceivable that Kv4.3 undergoes molecular characteristics are dramatically changed during pregnancy and under the action of sex hormones, as shown in the present work.

The fact that the overall reduction of protein levels correlated with changes in Kv4.3 mRNA levels suggested that the regulation of Kv4.3 channel protein expression at different stages of pregnancy involves transcription as a mechanism of regulation. The persistent high protein level at day 8, albeit the drastic reduction in mRNA at this time during pregnancy (Figs. 4 versus 5), could be explained by a slow Kv4.3 protein turnover. An intriguing finding is that, at late pregnancy, confocal images show that the Kv4.3 channel protein is preferentially located in perinuclear organelles (Fig. 3). This finding strongly supports the view that, in addition to a direct transcriptional regulation of Kv4.3 protein synthesis, altered intracellular trafficking can be an additional mechanism for Kv4.3 downregulation. In fact, it was recently shown that “chaperon” or “escort” proteins, namely KChAP and KChIP, may favor the expression level of Kv4.3 channels (30, 31). Thus, our results provide evidence for a dual mechanism underlying hormonal control of the number of Kv4.3 channels at the plasma membrane, namely: 1) direct inhibition at the transcriptional level, and 2) indirect inhibition via the reduced or enhanced synthesis of chaperon proteins that favor or inhibit intracellular trafficking, respectively. It is possible that chaperon-like proteins interact with channel proteins and expose or hide exit signals from the intracellular organelles.

In relation to the hypothesis that sex hormones may regulate surface expression of ion channels by altering intracellular trafficking, in rat myometrium we recently showed that decreased channel protein and surface expression of MaxiK channels at the end of pregnancy is accompanied by a perinuclear localization (32). Altered intracellular trafficking is much more accentuated in mouse myometrium where MaxiK protein is efficiently accumulated in perinuclear organelles preventing...
surface expression even though a larger amount of protein is synthesized. This novel mechanism of channel traffic modulation by sex hormones explains the recent report of England et al. (33) showing diminished MaxIK functional surface expression (ionic current measurements), despite an increased overall protein expression (Western blots) at the end of pregnancy. It also highlights the importance of analyzing the subcellular localization of proteins besides functional and total protein measurements, as well as species differences.

Our hormonal studies in ovx rats suggest that the rise of E2 in the last week of pregnancy is one of the mechanisms responsible for the Kv4.3 down-regulation at the end of pregnancy. Physiological plasma concentrations of E2 prior to parturition (~60 pg/ml) in E2-treated ovx rats dramatically reduce the protein level of Kv4.3 in Western blot analysis. In contrast, P4 at concentrations prior to parturition (~5 ng/ml) had no significant effect on Kv4.3 expression when injected alone or co-injected with E2 (not shown) (Fig. 6). However, P4 concentration can be higher at mid-pregnancy reaching values of ~170 ng/ml at day 16 (26). Thus, we cannot rule out a potential regulatory action of P4, which at higher concentrations may have an opposite effect to E2. A closer look at protein and mRNA levels at different stages of pregnancy (Figs. 4 and 5) shows a peak at days 17–18. At this time, P4 plasma concentration practically doubles from 18. At this time, P4 plasma concentrations in non-pregnant myometrium (estrus, metestrus, and diestrus). Thus, the reduction in mRNA levels in the first and second third of pregnancy seem not to be related to plasma levels of E2, and one or more other mechanisms are likely involved (e.g. other hormones or high local concentrations of E2 from the utero-placental system). Also, we cannot exclude the possibility that other ionic currents are modulated by E2/P4 ratios at these early stages in pregnancy and that the significant Kv4.3 protein down-regulation observed in the first 2 weeks of pregnancy (Fig. 4D) has little functional consequences. Nevertheless, the physiological relevance of our studies with ovx rats relates to changes occurring at the end of pregnancy when plasma levels of E2 rise, P4 falls, and Kv4.3 channels are drastically reduced.

The Kv4.3 down-regulation at the end of pregnancy agrees with the reported down-regulation of Ito currents in late pregnant myometrium (23) and with the recent findings that the expression levels of MaxIK channel protein and corresponding mRNA levels dramatically diminish near the end of gestation (32). Because MaxIK channel activity regulates myometrium contractility (34), the lower levels of MaxIK channel protein may facilitate a higher contractile activity. Likewise, a lower density of Kv4.3 channels may prolong the action potentials during delivery resulting in more forceful contractions. In fact, heteropodatoxin 3, a blocker of Kv4 channels, is able to increase basal tone and induce more forceful contractions in non-pregnant myometrium (35).

We conclude that Kv4.3 is under tight hormonal control in myometrium and during pregnancy. Besides transcriptional regulation, sex hormones may affect channel surface expression by modulating intracellular trafficking.

REFERENCES

1. Martina, M., Schultz, J. H., Ehnke, H., Monyer, H., and Jonas, P. (1998) J. Neurosci. 18, 8111–8125
2. Hoppé, U. C., Marban, E., and Johns, D. C. (2000) J. Clin. Invest. 105, 1077–1084
3. Grammer, J. B., Bosch, R. F., Kuhlkap, N., and Seipel, L. (2000) J. Cardiovasc. Electrophysiol. 11, 626–633
4. Kaab, S., Dixon, J., Duc, J., Ashen, D., Nahauer, M., Beuckelmann, D. J., Steinbeck, G., McKinnon, D., and Tomassell, G. F. (1998) Circulation 98, 1383–1393
5. Xu, H., Gu, W., and Nerbome, J. M. (1999) J. Gen. Physiol. 113, 661–678
6. Ohya, S., Tanaka, M., Oku, T., Asai, Y., Watanabe, M., Giles, W. R., and Imai, Y. (1997) FEBS Lett. 420, 47–53
7. Piedras Renteria, E., Stefani, E., and Toro, L. (1991) Am. J. Physiol. 261, 1574–1584
8. Gordenkio, D. V., Clausen, C., and Goligorsky, M. S. (1994) Am. J. Physiol. 266, F325–F341
9. Yuan, X.-J., Tod, M. L., Rubin, L. J., and Blaustein, M. P. (1995) Am. J. Physiol. 268, C259–C270
10. Koh, S. D., Ward, S. M., Dick, G. M., Epperson, A., Bonner, H. P., Sanders, K. M., Horowitz, B., and Kenyon, J. L. (1999) J. Physiol. (Lond.) 515, 457–467
11. Wang, Z., Feng, J., Shi, H., Pond, A., Nerbome, J. M., and Nattel, S. (1999) Circ. Res. 84, 551–561
12. Brahamajothi, M. V., Campbell, D. L., Rasmusson, R. L., Morales, M. J., Trimmer, J. S., Nerbome, J. M., and Straws, H. C. (1999) J. Gen. Physiol. 113, 581–600
13. Dixon, J. E., and McKinnon, D. (1994) Circ. Res. 75, 252–260
14. Dixon, J. E., Shi, W., Wang, H. S., McDonald, C., Yu, H., Wymore, R., Cohen, I. S., and McKinnon, D. (1996) Circ Res. 79, 659–668
15. Barry, D. M., Xu, H., Schuessler, R. B., and Nerbome, J. M. (1998) Circ. Res. 83, 560–567
16. Gibb-Jain, M., Huang, B., Jain, P., and el Sherif, N. (1996) Circ. Res. 79, 669–675
17. Warden, A. D., Kaprielian, R., Yoo, X. M., and Backx, P. H. (2000) Am. J. Physiol. Heart Circ. Physiol. 278, H1105–H1116
18. Stefani, E., Toro, L., Perozo, E., and Bezanilla, F. (1994) Biophys J. 66, 996–1010
19. Sorodi, P., Vega-Saenz, D. M., and Rudy, B. (1996) J. Neurophysiol. 75, 2174–2179
20. Rirke, K. M., Rausmann, R. P., and Wittwer, C. T. (1997) Anal. Biochem. 245, 170–175
21. Shih, T. M., Smith, R. D., Toro, L., and Goldin, A. (1998) Methods Enzymol. 293, 529–556
22. Satoh, H. (1996) Gen. Pharmacol. 27, 445–458
23. Wang, S. Y., Yoshino, M., Sai, J. L., Waku, M., Kao, P. N., and Kao, C. Y. (1998) J. Gen. Physiol. 112, 737–756
24. Tsaur, M. L., Chou, C. C., Shih, Y. H., and Wang, H. L. (1997) FEBS Lett. 400, 215–220
25. Challis, J. R. G., and Lye, S. J. (1994) in The Physiology of Reproduction (Knobil, E., and Neill, J. D., eds) pp. 985–1011, Raven Press, New York
26. LaPolt, S. P., Matt, D. W., Judd, H. L., and Lu, J. K. (1986) Biol. Reprod. 35, 1131–1139
27. Alexandrova, M., and Soloff, M. S. (1980) Endocrinology 106, 730–735
28. Eghbali, M., Olesen, R., Zarei, M., Toro, L., and Stefani, E. (2001) Biophys J. 80, 444a
29. Pongs, O., Leicher, T., Berger, M., Roeger, J., Bahring, R., Wray, D., Giese, K. P., Silva, A. J., and Storm, J. F. (1999) Am. J. Physiol. Cell Physiol. C976–C985
30. Wible, B. A., Kuryshchev, Y. A., and Brown, A. M. (1998) J. Biol. Chem. 273, 11745–11751
31. An, W. F., Bowby, M. R., Betty, M., Cao, J., Ling, H. P., Mendoza, G., Hinson, J. W., Mattson, K. I., Strassle, B. W., Trimmer, J. S., and Rhodes, K. J. (2000) Nature 403, 553–556
32. Song, M., Zhu, N., Olesen, R., Barila, B., Toro, L., and Stefani, E. (1999) FEBS Lett. 460, 427–432
33. Benkuszky, N. A., Fergus, D. J., Zuccherio, T. M., and England, S. K. (2000) J. Biol. Chem. 275, 27712–27719
34. Anser, K., Oberti, C., Perez, P. J., Perez-Reyes, N., McDougall, J. K., Mongs, M., Sanborn, B. M., Stefani, E., and Toro, L. (1993) Am. J. Physiol. 265, C976–C985
35. Sanguinetti, M. C., Johnson, J. H., Hammerland, L. G., Kelbaugh, P. R., Volkman, R. A., Sauceman, N. A., and Mueller, A. L. (1997) Mol. Pharmacol. 51, 491–498
36. Isbrandt, D., Leicher, T., Waldschaetz, R., Zhu, X., Luhmann, U., Michel, U., Sauter, K., and Pongs, O. (2000) Genomics 64, 144–154

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2. M. Song, G. Helguera, M. Eghbali, N. Zhu, M. M. Zarei, R. Olice, L. Toro, and E. Stefani, unpublished results.