

## Review

# A role of stretch-activated potassium currents in the regulation of uterine smooth muscle contraction

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Rates of premature birth are alarming and threaten societies and healthcare systems worldwide. Premature labor results in premature birth in over 50% of cases. Preterm birth accounts for three-quarters of infant morbidity and mortality. Children that survive birth before 34 weeks gestation often face life-long disability. Current treatments for preterm labor are wanting. No treatment has been found to be generally effective and none are systematically evaluated beyond 48 h. New approaches to the treatment of preterm labor are desperately needed. Recent studies from our laboratory suggest that the uterine muscle is a unique compartment with regulation of uterine relaxation unlike that of other smooth muscles. Here we discuss recent evidence that the mechanically activated 2-pore potassium channel, TREK-1, may contribute to contraction-relaxation signaling in uterine smooth muscle and that TREK-1 gene variants associated with human labor and preterm labor may lead to a better understanding of preterm labor and its possible prevention.

**Keywords:** stretch-activated potassium currents; preterm labor; uterine muscle; nitric oxide; arachidonic acid; pregnancy; TREK-1

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## Introduction

Preterm labor and delivery of an under-developed fetus affect approximately 13 million births worldwide each year<sup>[1]</sup>. The number of babies that die annually due solely to their prematurity ranges from 20 000 (4%) in the US to 336 000 of the 1.2 million newborn deaths (28%) in Sub-Saharan Africa<sup>[2]</sup>. Those that survive their prematurity have numerous chronic health disabilities that constitute a major human tragedy, are enormously costly to societies and cripple the third world development. Advanced medical care makes it possible for premature fetuses (some as early as 22–24 weeks) to survive, but at huge cost often resulting in life-long disability for survivors. Prematurity, whether due to infection or occurring spontaneously, threatens global health and must be addressed.

Despite decades of interest, the majority of cases of preterm labor are unexplained, and there is currently no effective medication to prevent uterine contractions at the time of labor as evidenced by a lack of an FDA approved indication in the US for any treatment for preterm labor (PTL). Challenging also is the absence of a useful animal model in which to propose studies for PTL since there is no animal that experiences this uniquely human problem. The impact of delivery of a pre-

mature fetus on society is very high; the Institute of Medicine estimates that this costs the American economy \$26.2 billion annually (in 2006 dollars).

In the last several years it has become clear that uterine smooth muscle mechanisms of relaxation differ significantly from those of other human smooth muscles. In this review we explore the nature of contractile regulation of myometrial smooth muscle and describe a channel target that we posit may be of significant interest as a therapeutic target for tocolysis.

## The onset of labor

The signal(s) that initiate labor at term in women are unknown. This is an embarrassing truth when viewed in the context of what we have learned in a relatively short time about diseases such as AIDS. Our current ability to treat HIV-AIDS with highly active antiretroviral therapy has drastically changed HIV infection from an acute, very deadly disease, to a chronic, long-lasting, manageable disease<sup>[3]</sup>. Such truth offers lessons; among the truths is that targeted research funding can yield results rapidly and the embarrassment surely is that we still don't understand our own reproductive physiology very well.

Theories of the proximate cause of labor have varied over time as we have learned more about what is not, rather than what is responsible for the regulation of uterine contractions.

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For example, quite logical might be the notion that maternal nervous regulation initiates labor. However, we have known for some time that the uterus becomes essentially denervated during gestation<sup>[4]</sup> and as a result, it is unlikely that any coordinated nervous regulation of the myometrium is centrally orchestrated<sup>[5]</sup>. As a result, views of the origin of onset of labor turned early on to examine endocrine and paracrine mechanism of myometrial regulation. A role for corticotrophin releasing hormone (CRH) posits that the hormone, generated in the placenta, builds in the serum and amniotic fluid as pregnancy progresses<sup>[6]</sup>. The human fetus also has a pituitary-adrenal axis by mid-trimester and CRH, although present in lower concentrations than in the maternal circulation, stimulates adrenocorticotrophic hormone (ACTH) secretion from the fetal pituitary. ACTH is also synthesized in the placenta under the influence of CRH<sup>[7]</sup>. Fetal ACTH stimulates production of the androgenic steroids dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS). Cortisol secretion is induced by ACTH and an increase in fetal cortisol production in late pregnancy is responsible for fetal organ maturation including lung maturation<sup>[8]</sup>. DHEA and DHEAS production in the fetus is thought to lead to initiation of parturition through their metabolism by the placenta to estrogen<sup>[9]</sup>, which acts on the myometrium, cervix and fetal membranes. While estrogen is thought to up-regulate the expression of myometrial genes associated with contraction<sup>[10]</sup>, it is progesterone that is thought to modulate parturition timing through its withdrawal. In a number of animal models, progesterone disappearance from the circulation is associated with onset<sup>[11]</sup>. However, maternal progesterone levels in the blood stream do not fall at the time of onset in humans and so research has focused on the notion of changes in progesterone locally in the uterine compartment not reflected in the maternal circulation such that progesterone withdrawal could occur due to local progesterone metabolism<sup>[12]</sup> or receptor-based heterologous regulation of steroid hormone receptors that leads to a functional withdrawal of the action of progesterone as recently reviewed by Mitchell and Taggart<sup>[13]</sup>. Whatever the fact of progesterone withdrawal, it is not at all clear that administering progesterone exogenously will serve as an effective treatment of preterm labor<sup>[14]</sup>.

### Quiescence is key

Relaxation of the myometrium is required for pregnancy to proceed in the face of ever increasing tension on uterine muscle as the fetus grows<sup>[15, 16]</sup>. Approaches to understanding PTL have examined the notion that there is an inappropriate activation of contractile influences that initiate labor too soon, as well as a failure of relaxation influences on the myometrium<sup>[17-19]</sup>. While both may operate, we assert that successful therapeutic intervention will come from probing relaxation mechanisms. This assertion is supported by the finding that the first drug designed specifically for preterm labor, Tractocile® (atosiban) that blocks the actions of oxytocin (OT), is not superior to conventional approaches to the treatment of preterm labor<sup>[20, 21]</sup>, albeit it may pose fewer side

effects and is only evaluated at 48 h. This agent is not yet FDA approved in the US. Examination of its development reveals the unimaginative model of an OT receptor blocker to block the actions of OT<sup>[22]</sup>. In humans however, labor is not just the result of stimulation of the myometrium by oxytocin<sup>[23]</sup>.

### NO-mediated relaxation

Nitric oxide (NO) is particularly effective in relaxing the myometrium and in fact has a lower inhibitory concentration 50% ( $K_i$ ) in human than guinea pig or non-human primate myometrium<sup>[24]</sup>. We have examined the heretical notion that relaxation of the myometrium by NO is independent of cGMP elevation as a result of soluble guanylyl cyclase. Although this notion was initially rejected by many on the basis that the dogma established by the 1998 Nobel for Physiology or Medicine<sup>[25]</sup> demanded that NO signals through cGMP in smooth muscle, it is now clearly established from our work and that of others that NO-mediated relaxation of uterine muscle is independent of global cGMP elevation no matter whether this is studied in animal<sup>[26]</sup>, primate<sup>[27]</sup> or human<sup>[28]</sup>.

Cyclic GMP is not without effect however. When the uterus is relaxed by uroquanylin, cGMP elevation secondary to activation of the guanylyl cyclase activity of the uroquanylin receptor, particulate guanylyl cyclase type C does relax the uterus. The presence of a uroquanylin-particulate cyclase-cGMP relaxation pathway in myometrium taken together with the failure of soluble cGMP elevation to relax the muscle leads to the inevitable conclusion that cGMP is compartmented in the myometrium<sup>[29]</sup>.

The importance of the fact that an effect of NO to relax the uterus is independent of global cGMP accumulation is two-fold. First, data supporting the cGMP-independence of nitro-agent mediated relaxation of uterine muscle that were first introduced by the pioneering work of Jack Diamond<sup>[30-32]</sup> and later by our lab as cited above, establish more dramatically than any other type of study we are aware of that the uterus, particularly the human uterus, is regulated in a disparate manner compared to other smooth muscles<sup>[19, 29]</sup>.

The notion that the uterine smooth muscle biochemistry of relaxation signaling is fundamentally different than vascular or gastrointestinal muscle means that there is hope for discovery of therapeutic targets in the myometrium that are absent or disparately regulated in other smooth muscles and thus, can permit a reasoned line of investigation to find uterine-specific tocolytics. Whatever is learned must impact the final mediator of muscle contraction, calcium availability in the uterine myocyte and while beyond the scope of this perspective, the reader is guided to a recent expert review<sup>[33]</sup>.

The second point highlighted by the unique nature of NO-mediated relaxation of uterine muscle is that since NO relaxes uterine smooth muscle, NO must act in a fashion other than through activation of the soluble guanylyl cyclase. Thus, NO must directly nitrosylate or indirectly modify one or more proteins in the myometrium that regulate relaxation. Our interest that a stretch-activated potassium channel (K2P) may play a role in normal myometrial relaxation is peaked by the persua-

sive model that gestational growth means continual myometrial stretch along with the possibility that K2P channels, or their expression is(are) a target for NO or consequences of NO signaling.

The notion that NO is generated in myometrium and responsible for regulation of uterine quiescence has been controversial<sup>[19]</sup> and yet high levels of myometrial expression of nitric oxide synthase(s) correlated with gestation until the time of labor need not be the only source of NO to affect the myometrium. Indeed, recent studies have examined the presence of NO-synthase (NOS) in uterine tissues including myometrium<sup>[34]</sup> and actions of NO in reproductive tissues suggest its critical role in normal reproduction<sup>[35]</sup>. A source for myometrial NO may not come from smooth muscle generation directly, but rather from the ability of NO generated in the uterine membranes or the vasculature to bind free cysteine forming cysteine-NO (Cys-NO) or hemoglobin-NO and be transported to the muscle where it may be released as nitrosyl ion (NO<sup>+</sup>) and bind to reduced glutathione (GSH) to generate GSNO<sup>[36]</sup>. Such small molecule S-nitrosothiols (SNO) offer a scientifically more satisfying way to view the preservation of the signaling actions on NO since now the functional nitrosylation of proteins by SNO may be coupled to the redox state of the cell coupling extracellular and intracellular mechanisms mediating contraction-relaxation.

Nitric oxide as Cys-NO can travel through the plasma membrane in this way as a second messenger and hence transduce stimuli and initiate responses in adjacent cells. Of the known NO-mediated reactions with biological materials, nitrosylation is the major form of protein modification under physiological conditions and constitutes an important signal transduction mechanism. S-Nitrosylation is the covalent attachment of NO to the sulfur moiety of cysteine. This chemical reaction constitutes a redox sensitive post-translational, reversible modification of protein in response to stimuli outside the cell<sup>[37]</sup>. Rather than free diffusion of NO from a NOS, S-nitrosylation allows for the compartmentation of the source of NO and the site of nitrosylation (Cysteine). The formation of SNOs is a critical pathway in the signaling of NO between cellular compartments since it maintains the availability of NO generated in one cell to act in another preserving the action of NO in time and space<sup>[38]</sup>. Small molecular weight SNOs such as Cys-NO and GSNO constitute the major determinants of subsequent transnitrosylation of target proteins to alter cellular response<sup>[39]</sup> and cell-cell transfer of NO.

Once accumulated in a target cell such as the uterine myocyte, GSNO may provide a source of both NO for protein transnitrosylation as well as glutathione for glutathionylation via the action of glutathione-S transferase reactions<sup>[40]</sup>. Thus regulation of protein targets in myocytes such as membrane channels in a reversible fashion to activate or inactivate current may have profound influence on uterine quiescence. It is possible that actions of NO or glutathione may accumulate as a consequence of vascular NO generation and redox reactions in uterine myocytes that then manage contractile force. The notion that pregnancy physiology and preterm birth may in

part involve an inflammatory-like state<sup>[41]</sup> fits with this notion and provides a testable platform for these ideas.

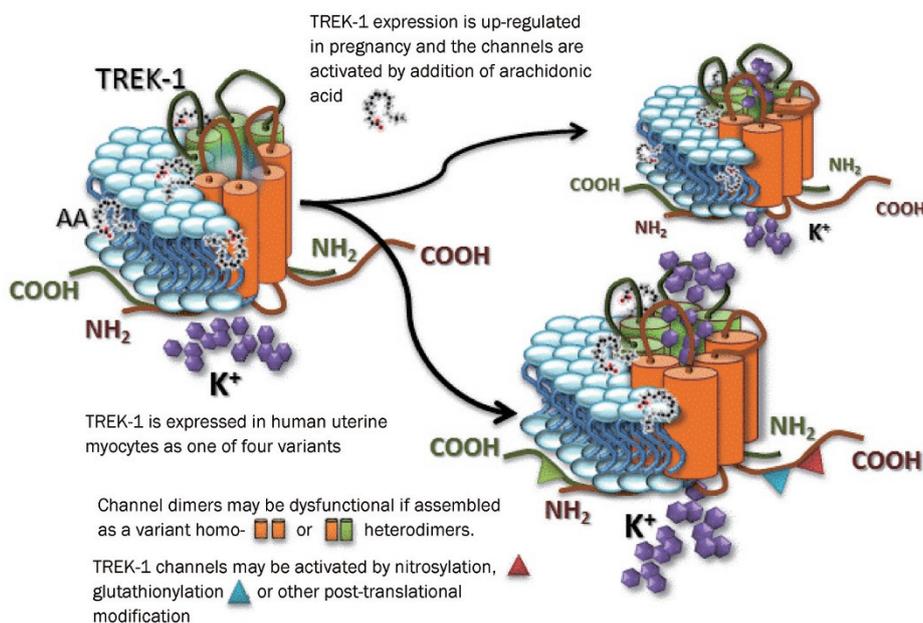
Given then that NO nitrosylates one or more regulatory proteins in myometrial smooth muscle to produce relaxation, we developed a method for showing S-nitrosylation in whole tissue lysates. This method has allowed us to elucidate the human myometrial smooth muscle nitroproteome and to test the effect of these post-secondary modifications on relaxation. Our method, which we are calling Nitro-DIGE, selectively labels S-nitrosylated proteins in a cell extract using spectrally resolvable Alexa Fluor maleimide dyes. Labeled extracts are then analyzed using 2-dimensional difference gel electrophoresis (2D DIGE). With this powerful technique we can identify differences in levels of S-nitrosylation between term and preterm myometrium. These identified proteins are regulatory in nature and preliminary results have identified candidates such as heat shock protein beta-1, desmin, alpha actinin which have been shown to regulate contraction in smooth muscle tissue. Importantly, we have found nitrosylation of the K2P channel TREK-1 and confirmed the identity of this protein by LC-MS/MS. Current work is directed at both hypothesis-directed examination of the function of nitrosylated TREK channels and the smooth muscle thin filament contraction-relaxation pathway, as well as identifying prominent proteins that are selectively nitrosylated by pregnancy progression and/or in response to experimental NO-stimulation of tissues in functional assays where relaxation/contraction can be correlated with protein nitrosylation.

#### TRAAK-family channels

Two-pore-domain K<sup>+</sup> (K2P) channel subunits are made up of four transmembrane segments and two pore-forming domains that are arranged in tandem and function as either homo- or heterodimeric channels (Figure 1). This structural motif is associated with unique gating properties that would well serve quiescence in myometrium, including background channel activity (so-called leak current) and sensitivity to membrane stretch. Moreover, TRAAK-family K2P channels are modulated by cellular lipids and pharmacological agents, including polyunsaturated fatty acids such as arachidonic acid and volatile general anesthetics<sup>[42]</sup>. Acidity and heat have also been suggested to activate the channel<sup>[43, 44]</sup>, while antipsychotics such as fluphenazine have been shown to block TREK-1 but not the related TRAAK channels<sup>[45]</sup>.

#### Arachidonic acid and acidic pH in pregnancy myometrium

Blood flow is temporarily compromised during forceful uterine contractions during labor<sup>[46]</sup>. This transient ischemia is associated with intracellular acidosis and has been proffered as a mechanism for contractile regulation in myometrium<sup>[47]</sup>. Myocyte signaling domains may be acidic on the basis of their construction *per se*, independent of relative ischemia<sup>[48]</sup>. Relative acidification at the myocyte membrane activating TREK-1 during gestation would fit with hyperpolarization of the membrane and decreased uterine activity. An abundance of



**Figure 1.** TREK-1 concept. Pregnancy results in up-regulation of channel number and provides for activation by stretch (right-hand figures) and arachidonic acid. The presence of channel variants (depicted as 2, 4-transmembrane units of disparate color) could result in assembly of channels that exhibit altered function or are dysfunctional. Altered conductance may be accompanied by disparate activation characteristics. Posttranslational alteration by nitrosylation, glutathionylation and or phosphorylation may confer sites of disparate regulation in preterm labor. Multimodal activation by stretch, heat, depolarization, intracellular acidosis, lipids and volatile anesthetics has been reviewed<sup>[42]</sup>. TREK-1 is tonically inhibited by the actin cytoskeleton opening the possibility that pregnancy releases such inhibition through cytoskeletal reorganization. Protonation of a glutamic acid residue within the C-terminus of TREK-1 increases the affinity of this domain for inner leaflet phospholipids and increases activity. Stimulation of Gq-coupled extracellular receptors can inhibit TREK-1. A variety of possibilities for regulation of the TREK-1 channel in the uterine myocyte suggests that studies of TREK-1 may reveal a therapeutic target for preterm labor.

arachidonic acid in pregnant myometrium<sup>[49]</sup> also would signal TREK-1 activation. Together, these influences may contribute to gestational uterine quiescence.

### TREK-1

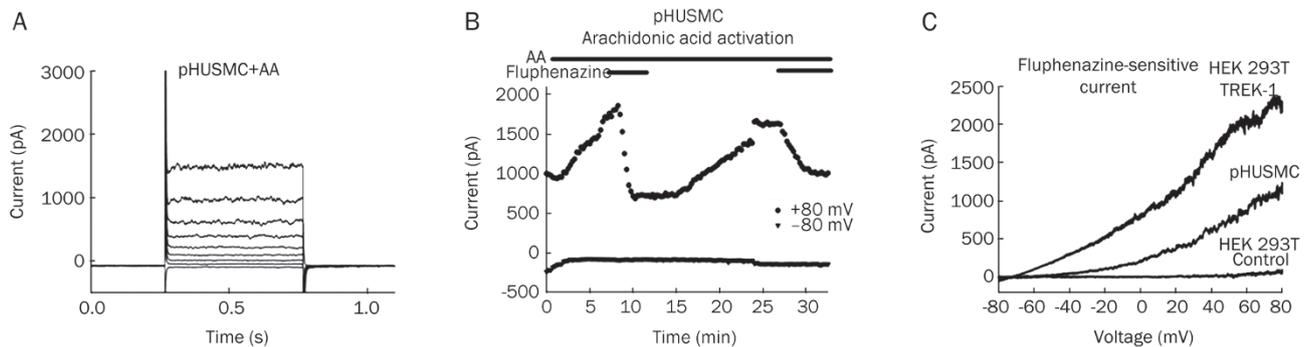
Other than our description of TREK-1 channels in human myometrium in 2005<sup>[50]</sup> and the work of Bai *et al* in the same year<sup>[51]</sup>, very little was known about these channels in the myometrium until recently. Despite early claims that little TREK-1 was expressed in human<sup>[52]</sup> or that no expression could be seen in mouse<sup>[53]</sup> myometrium, we have shown the importance of these channels in human myometrium<sup>[50, 54]</sup>. Recent studies in mice have now also appeared<sup>[55]</sup>. Electrophysiological currents can readily be measured in human uterine myocytes as well as cells overexpressing the human protein (Figure 2). These currents are recorded under conditions that block other potassium channels and are themselves blocked by fluphenazine underscoring their identity as TREK-1 currents. Our ability to determine channel expression and functionally measure these channels in freshly isolated myocytes as well as cells overexpressing cloned channels, permits a thorough examination of the possibility that genetic channel variants underlie cases of preterm labor in women.

Recent *in vivo* studies have shown that TREK-1 has a key role in anesthesia, neuroprotection, pain, depression<sup>[56]</sup>, and

vascular regulation<sup>[57]</sup>. Since it is known in rat heart that TREK-1 splice variants result in two different operating modes of the channel<sup>[58]</sup>, and since channel dysfunction could correlate with early labor, we tested the hypothesis that TREK-1 in human myometrium may also be alternatively spliced. We are aware that other data is available, describing TREK-1 variants associated with failure to respond to antidepressants<sup>[56]</sup> and a variant in rat brain that is thought to code for a channel by alternative translation initiation that is permeable to both Na<sup>+</sup> and K<sup>+</sup> ions<sup>[59]</sup>. Such a channel, if expressed in human myometrium, could readily depolarize the cell based on the Na<sup>+</sup> gradient and contribute to early initiation of labor. Thus, a careful examination of TREK-1 regulation in human myometrium is particularly important.

### TREK-1 variant expression

Because *TREK-1* gene expression is regulated by pregnancy increasing at term and falling at the time of labor<sup>[54]</sup>, it stands to reason that TREK-1 could be important in regulating relaxation until labor and may or may not participate in the intercontractile period during labor as suggested by the continued presence of the protein during the laboring period. If TREK-1 currents activated by the biochemistry of pregnancy (*eg*, progesterone dominance, arachidonic acid production) are important in uterine quiescence, then dysfunctional channels or low-



**Figure 2.** TEA-insensitive  $K^+$  current in isolated uterine myocytes from gravid myometrium. (A) Whole cell currents elicited by voltage steps (-80 to +80 mV) under 10  $\mu\text{mol/L}$  arachidonic acid (AA). (B) Whole cell current activated by 10  $\mu\text{mol/L}$  AA and blocked by 10  $\mu\text{mol/L}$  fluphenazine. (C) Fluphenazine-sensitive, AA-activated whole cell currents (AA-AA+Fluphenazine) elicited by 1 s voltage ramps (reversal potential  $\approx 70\text{mV}$  for HEK 293T (human embryonic kidney) cells over expressing human TREK-1 and isolated pregnant myocytes [pHUSMC] indicates  $K^+$  current).

ered TREK-1 expression could underlie cases of spontaneous PTL. We found a high level of variation between samples in the extreme 5'-end of the mRNA.

We identified TREK-1 splice variants in human myometrium (Figure 3) using PCR. We have now cloned each of these putative channels and are expressing them in HEK cells in order to perform electrophysiological measurement of TREK-1 currents that we have examined in isolated uterine myocytes. The variability of expression differences including samples (patients) that express only one variant of the channel such as several of the preterm laboring samples we have collected, suggests the possibility that functional correlations between TREK-1 channel variants and TREK-1 variant channel function will be possible for human TREK-1. Human brain (hB) was amplified as a positive control and expressed only one of the variants seen ( $\alpha$ , as expected) in one or more myometrial samples. This is exciting because it suggests a tissue-specific regulated expression is likely in myometrium and may have functional significance. We have tentatively named these variant transcripts from myometrium  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  (Figure 3). The possibility that each of these ( $\beta$ ,  $\gamma$ ,  $\delta$ ) corresponds to an expressed channel is being pursued.

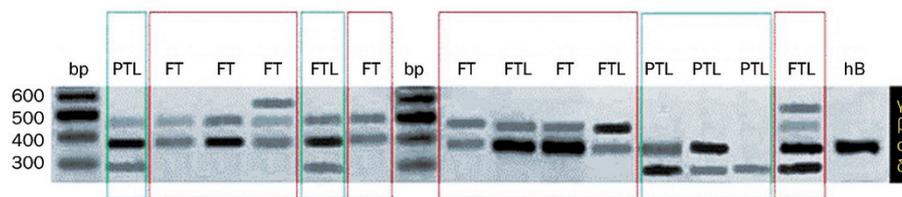
Whatever the final outcome of preterm labor research, there is not a more significant human research imperative than to understand and prevent preterm delivery. If one considers the energy and treasure we expend on studying diseases of the aged only to cripple our healthcare systems with expensive medications and technology for the last years of life, and contrasts this with the relative ignorance we share about the beginning of life, it is possible to imagine we are failing. It is well past time to make preventing premature birth a priority.

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**Figure 3.** Expression of TREK-1 variants in human pregnant myometrium. We used the GenBank accession sequence NT\_021877.18 to generate forward [AGG AAA CAG TAT GGG ACG ATG GCT] and reverse primers [CTG CTC CAA TGC TTT GAA CAC GGT] to examine expression of KCNK2 (K2P) TREK-1 in myometrium. The predicted product was 373 bp. Tissues from women who were in spontaneous preterm labor (PTL); at full term (38–41 weeks) but not in labor (FT); or who were at full term in labor (FTL) were collected under informed consent. A composite gel from several experiments is shown. Human brain cDNA was employed as a positive control (hB) and non-template controls run with water instead of cDNA were included with each experiment and verified the PCR (not shown).  $\alpha$ – $\delta$  are a tentative identification by our lab of unique channel variants in myometrium based on these experiments. These variants have now been cloned and expressed in human embryonic kidney cells for electrophysiological and signal transduction studies.

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