Myometrial expression of small conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels depresses phasic uterine contraction

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Contraction of the uterus is primarily dependent on the activity of L-type Ca\textsuperscript{2+} channels, particularly at term (10). Spontaneous depolarization of the uterine smooth muscle cell membrane potential leads to coordinated opening of voltage-gated Ca\textsuperscript{2+} channels. The resulting Ca\textsuperscript{2+} action potentials spread through the myometrium via gap junctions, eliciting phasic contractions (29, 31). The sarcoplasmic reticulum may modify these uterine contractions both by mobilizing additional Ca\textsuperscript{2+} with agonist stimulation and by buffering cytosolic Ca\textsuperscript{2+} levels via sequestration (16). The generation of coordinated forceful contractions in the wall of the uterus is a hallmark of active labor. K\textsuperscript{+} channels critically regulate smooth muscle contractility by opposing membrane potential depolarization and hence Ca\textsuperscript{2+} elevation. Certain K\textsuperscript{+} channels, including ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels (18, 25) and large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels (1, 2, 8, 26), have been implicated in the control of uterine excitability in both pregnant and nonpregnant states, but their direct impact on uterine function remains unclear. Recent findings indicate that a specific class of small conductance Ca\textsuperscript{2+} activated K\textsuperscript{+} (SK) channels, particularly the SK3 isoform, plays a key role in the regulation of vasculature tone (5, 28) as well as phasic contractions of the gastrointestinal tract (23) and bladder (14). Due to a constitutive association with calmodulin, SK3 channels are highly sensitive to changes in cytosolic Ca\textsuperscript{2+} levels (3, 30) and are thus capable of exerting abrupt negative feedback regulation of intracellular Ca\textsuperscript{2+}. Although SK3 channels have been identified in the human uterus (17), their specific functional role remains largely unexplored. Interestingly, previous findings (4) revealed that in genetically altered mice that conditionally overexpress SK3 (SK3\textsuperscript{T/T}), parturition is severely compromised, often resulting in the death of the dam and pups. This incomplete or interrupted labor could be prevented by selectively suppressing SK3 expression. We hypothesized that the expression level of SK3 in the myometrium is a key determinant of uterine contractility. Here, we assess the direct impact of differential SK3 channel expression on uterine function.

MATERIALS AND METHODS

Animals and tissue preparation. Nonpregnant wild-type (WT) c57BL/6 mice and nonpregnant SK3\textsuperscript{T/T} were used in this study. The animals were treated and the experiments were conducted in accordance with the guidelines of the University of South Alabama Institutional Animal Care and Use Committee and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of Health’s Guide for the Humane Treatment of Experimental Animals. For SK3T/T mice, conditional expression of SK3 was achieved through the insertion of a doxycycline (Dox)-sensitive gene switch. Briefly, a regulatory cassette was inserted encoding a binary tetracycline/doxycycline-inhibitable transactivator protein acting as an intermediate between the promoter and gene expression. Dietary administration of 0.5 mg/ml doxycycline (and 2% sucrose) in the drinking water for at least 6 days suppresses SK3 expression (SK3T/T+Dox). Thus, in tissues that express SK3, levels are relatively high in the absence of Dox and low in the presence of Dox. The mice were euthanized by an intraperitoneal injection of pentobarbital (100 µg/g, 0.05 ml) and subsequent decapitation. The abdominal cavity was opened and the uterus excised.

Western blot analysis. Excised uterus was frozen in liquid nitrogen and pulverized. Tissue was then homogenized (Polytron homogenizer) in ice-cold lysis buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 2 mM EDTA, 5 mM EGTA, 100 mM NaF, 0.5% alkylaryl polyether alcohol (Triton X-100), protease inhibitors (1:100 dilution of protease inhibitor cocktail set III, Calbiochem), and 1% solutions of leupeptin and phenylmethylsulfonyl fluoride. Samples were then centrifuged at 13,000 g for 15 min. Total protein in the supernatant was determined by the Lowry method (Bio-Rad Laboratories). Samples were subjected to SDS-polyacrylamide gel (6%) electrophoresis (20 µg of protein per lane). Samples from WT, SK3T/T, and SK3T/T+Dox uteri were always run in parallel to ensure uniform conditions. The proteins were then transferred to nitrocellulose membranes and blocked with 5% BSA. The membranes were then incubated with primary rabbit SK3 antibody (1:1,000; Alomone Labs) and subsequently exposed to secondary horseradish peroxidase-linked anti-rabbit antibody (Cell Signaling Technology). Some samples were preexposed to a SK3-specific blocking peptide (1:1,000, Alomone Labs) before primary incubation to assess specific binding. SK3 protein was detected by chemiluminescence. Densitometry was performed on resulting blots using Sigmagel software.

Immunofluorescence. The intact uterus was placed in phosphate-buffered saline solution (PBS; pH 7.4). Very thin longitudinal segments of myometrium (<50 µm thick and ~3 mm in length) were removed from just beneath the serosa. Segments were pinned to the bottom of a silicone-lined dish, fixed in 4% formaldehyde for 15 min at room temperature, and subsequently washed with PBS. The strips were placed on glass slides, incubated for 5 min with 0.05% Chicago blue, permeabilized with 0.2% Triton X+PBS for 10 min and blocked for 1 h at room temperature with 2% BSA/0.2% Triton X+PBS. Following exposure to rabbit anti-SK3 primary antibody (1:500) overnight at 4°C, strips were washed with PBS and exposed to Alexa Fluor 568 anti-rabbit secondary antibody (Molecular Probes; 1:200) at room temperature for 1 h. Strips were exposed to the nuclear stain DAPI (1:2,000) and washed with PBS. The slides were viewed with a PerkinElmer Ultraview RS-3 spinning disk confocal microscope at excitation wavelengths of 488 and 568 nm and emission wavelengths of 510 and 600 nm. SK3-dependent fluorescence was captured under identical conditions for all preparations. For illustrations, SK3-dependent fluorescence was displayed as green and nuclear staining as red.

Organ bath myography. The uterus was placed in physiological saline solution (pH 7.4) containing (in mM) 119 NaCl, 4.7 KCl, 2.3 NaHCO3, 1.2 KH2PO4, 0.026 EDTA, 1.2 MgSO4, 2 CaCl2, and 10.5 glucose. After removal of adipose and connective tissue, two longitudinal strips (~0.5 x 0.2 cm) were excised from each of the uterine horns. Each strip was attached via metal hooks to a force myograph system (Grass Instruments); one end was connected to a force transducer and the other one was held fixed. Mounted strips were submerged in baths of gassed (95% O2-5% CO2) physiological saline solution at 37°C and allowed to equilibrate for ~30 min. Because of the considerable and variable distensibility of the tissue, active length-tension curves were generated for all strips at the beginning of each experiment to determine the optimal length (LO) for contraction. To accomplish this, each strip was first stretched from an unloaded state to a point at which base tension remained stable at 3 g (to date, we have found that LO occurs within this range in all uterine strips tested). This maximal length was divided into five equal increments. Each strip was then reset to its unloaded position, stretched incrementally to the five predetermined positions up to the maximal length, and stimulated with 60 mM KCl at each. Each strip was then set back to the length at which maximal active force development was achieved (LO) and allowed to equilibrate for at least 1 h. Data were recorded via computer using PolyView software. Deflections above baseline were counted as peaks if they reached a value ≥20% of maximal KCl-induced force for that individual strip. Phasic event amplitudes were defined from trough to peak and tone from trough to baseline (i.e., remainder of force after phasic events were subtracted).

Materials. All drugs were purchased from Sigma Aldrich (St. Louis, MO). Primary SK3 antibodies were obtained from Alomone Labs (Jerusalem, Israel). Secondary antibodies and nuclear stain were purchased from Molecular Probes.

Data analysis. Data are expressed as means ± SE. Grouped data were assessed via two-way ANOVA, and subsequent individual comparisons were made via Bonferroni posttest where indicated. Statistical significance is indicated where P < 0.05.

RESULTS

SK3 expression in the mouse uterus. SK3 protein was detected in the mouse uterus via Western blot analysis (Fig. 1A). Compared with that in WT mice, uterine SK3 expression was substantially increased in SK3T/T mice and decreased in SK3T/T mice treated orally with doxycycline (SK3T/T+Dox) for 5 days. Immunofluorescence revealed distinct SK3-positive staining in longitudinal bundles of smooth muscle cells comprising the myometrium, which was substantially reduced in SK3T/T+Dox mice compared with untreated SK3T/T mice (Fig. 1B). No discernable SK3-dependent fluorescence was detected when antibody binding sites were preabsorbed with SK3-specific blocking peptide (Fig. 1B) or primary antibody was omitted (data not shown).

KCl contractions and length-tension relationships. Using organ bath myography, we measured isometric force generation in response to 60 mM KCl in isolated uterine strips. KCl elicited tonic contractions and the magnitude of these contractions increased over a range of initial lengths in all strips tested (Fig. 2A). Maximal force generation was achieved at similar lengths in WT, SK3T/T, and SK3T/T+Dox strips, and the magnitude of the maximal KCl-induced force was not significantly different among the three groups (Fig. 2B). Thus, upregulation and downregulation of SK3 expression levels did not alter the magnitude of uterine contractions in response to direct membrane potential depolarization, indicating that the capacity for active force generation is preserved in the SK3T/T mouse and is indistinguishable from WT under conditions in which modulation of force through K+ efflux is precluded.

Spontaneous uterine contractions. Figure 3 shows spontaneous contractions generated by uterine strips from WT, SK3T/T, and SK3T/T+Dox mice and related summary data. WT strips produced phasic contractions at regular intervals with a mean amplitude of 7.6 ± 1.0 mN and a frequency of 2.9 ± 0.7 per 5 min. Spontaneous contractility was notably depressed and erratic in strips from SK3T/T mice (2.5 ± 0.7 mN at 2.3 ± 0.7 per 5 min) compared with WT. In fact, 50% of the SK3T/T
strips tested failed to generate measurable spontaneous phasic activity compared with 9% of WT strips and 5% of SK3/T/T + Dox strips. In contrast to strips from SK3 T/T mice, strips from SK3T/T + Dox mice exhibited large spontaneous contractions at very regular intervals (10.3 ± 0.1 mN/s, P < 0.01 vs. before apamin) as well as WT strips (2.35 ± 0.20 mN/s, P < 0.01 vs. before apamin) but had no discernable effect on the rate of force development in SK3T/T + Dox strips (2.74 ± 0.20 mN/s).

Next, we assessed the primary mechanism of SK3 modulation of spontaneous uterine contractions. The L-type Ca2+ channel blocker nifedipine (1 μM) completely abolished spontaneous phasic contractions in all uterine strips. As illustrated in Fig. 2.
in Fig. 4, nifedipine not only blocked the small spontaneous contractions in SK3T/T strips but also blocked the large phasic contractions elicited by apamin. Indeed, in the presence of nifedipine, apamin failed to induce or increase force in SK3T/T strips. On the contrary, treatment with the endoplasmic reticulum Ca\(^{2+}\)/H\(^{+}\)-ATPase inhibitor cyclopiazonic acid (CPA; 10 \(\mu\)M) to deplete intracellular Ca\(^{2+}\) stores caused a slight increase in phasic contractile activity in SK3T/T strips but did not significantly impair the contractile influence of apamin (Fig. 5).

**Oxytocin-induced contractions.** Finally, we explored the influence of SK3 channel expression on agonist-induced uterine contractions. Oxytocin-enhanced uterine contraction in a concentration-dependent fashion (10\(^{-10}\)-10\(^{-7}\) M) in all WT, SK3\(^{+/+}\), and SK3\(^{+/+}\)+Dox strips tested. These contractions were complex, consisting of dynamic changes in phasic contraction frequency and amplitude as well as the level of sustained tone at higher concentrations (Fig. 6). In SK3\(^{+/+}\) strips stimulated with a single concentration of oxytocin (0.1 \(\mu\)M) and allowed to reach a steady state, apamin (0.3 \(\mu\)M) effectively increased the frequency and amplitude of transient contractions while slightly decreasing sustained tone (Fig. 7, A and B). This apamin-induced shift to predominantly phasic contraction (from 44\(\pm\)11% to 70\(\pm\)12%; \(P<0.01\)) resulted in contractions that closely resembled oxytocin contractions in SK3\(^{+/+}\)Dox strips (77\(\pm\)6% phasic), which were not significantly affected by apamin (82\(\pm\)5% phasic). Albeit to a lesser degree than that observed in SK3\(^{+/+}\) strips, apamin (0.3 \(\mu\)M) also significantly increased the phasic component of oxytocin contractions in WT strips from 66\(\pm\)3% to 77\(\pm\)2% (\(P<0.01\)). These data indicate that SK3 channels specifically blunt the phasic, L-type Ca\(^{2+}\) channel mediated component of agonist contraction without impairing the capacity of agonists to generate and sustain uterine tone. Indeed, in separate experiments, 1 \(\mu\)M nifedipine completely blocked apamin enhancement of phasic oxytocin contractions but had no effect on oxytocin-induced tone (Fig. 7C). Correspondingly, tonic contractions generated by oxytocin in the presence of nifedipine were unaffected by apamin (Fig. 7C).
This study demonstrates for the first time that the capacity for coordinated uterine contraction is highly dependent on the level of SK3 channel expression in the mouse myometrium; higher levels promoting quiescence and lower levels promoting large periodic phasic contractions. We show that SK3 channels specifically disrupt the development of L-type Ca\(^{2+}\) channel-dependent phasic events. Ca\(^{2+}\) handling and contraction in the mouse myometrium are very similar to that observed in rat and human (16), indicating that the mouse is a valuable animal model for uterine studies. The SK3T/T mouse model employed in our investigations allowed for selective manipulation of SK3 expression (both upregulation and acute downregulation) to determine the specific functional impact of these channels in the intact uterus.

A key feature of SK3 channels is their robust sensitivity to Ca\(^{2+}\). Even very modest increases in subplasmalemmal Ca\(^{2+}\) (\(>0.1\) μM) open these channels (30), promoting K\(^{+}\) efflux and cell hyperpolarization. It should be noted that BK channels, which are also expressed in the uterus, exhibit relatively low Ca\(^{2+}\) affinity at physiological membrane potentials (22), requiring much higher Ca\(^{2+}\) concentrations (\(>3.0\) μM) for activation than do SK channels. Thus, in uterine myocytes, where episodic Ca\(^{2+}\) entry drives action potentials and contraction, SK3 channels likely provide the most immediate negative feedback control. We predicted that by attenuating the rate and magnitude of Ca\(^{2+}\) entry, these channels essentially mute individual contractions as they develop. Indeed, in the current study, we found that half of the uterine strips from mice overexpressing SK3 channels (SK3T/T) failed to generate measurable spontaneous contractions at all, and in those that did exhibit spontaneous activity, contractions were erratic, slow to develop, small in magnitude, and completely inhibited by blockade of L-type Ca\(^{2+}\) channels with nifedipine. Conversely, removal of the SK3 channel influence through downregulation of channel expression (SK3T/T+Dox) or through acute pharmacological blockade (apamin) resulted in a dramatic increase in contractile activity, marked by the development of large, organized events with regular periodicity.

**Fig. 4.** Role of L-type Ca\(^{2+}\) channels. Representative force recordings from SK3T/T uterine strips showing complete abrogation of spontaneous (Spont; A) and Apa (0.3 μM)-induced (B) contractile events by 1 μM nifedipine (Nif). C: summary data for 5 experiments from each protocol.

**DISCUSSION**

This study demonstrates for the first time that the capacity for coordinated uterine contraction is highly dependent on the level of SK3 channel expression in the mouse myometrium; higher levels promoting quiescence and lower levels promoting large periodic phasic contractions. We show that SK3 channels specifically disrupt the development of L-type Ca\(^{2+}\) channel-dependent phasic events. Ca\(^{2+}\) handling and contraction in the mouse myometrium are very similar to that observed in rat and human (16), indicating that the mouse is a valuable animal model for uterine studies. The SK3T/T mouse model employed in our investigations allowed for selective manipulation of SK3 expression (both upregulation and acute downregulation) to determine the specific functional impact of these channels in the intact uterus.

**Fig. 5.** Role of internal Ca\(^{2+}\) stores. Representative force recording (A) and summary data (B) from SK3T/T uterine strips (n = 4) showing the lack of effect of 10 μM cyclopiazonic acid (CPA) on apamin (0.3 μM)-induced contractions. *P < 0.05 vs. +CPA.

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The fact that nifedipine prevented all pro-contractile effects of SK3 inhibition or SK3 suppression indicates that SK3 channels both detect and control Ca\(^{2+}\) entry at a single site, the L-type Ca\(^{2+}\) channel, forming a tight negative feedback loop. We considered that the sarcoplasmic reticulum might contribute to this effect by releasing Ca\(^{2+}\) toward the plasma membrane and promoting SK3 channel activity. However, despite causing a slight enhancement in uterine contractility, CPA, at a concentration previously employed to remove the influence of Ca\(^{2+}\) stores in the myometrium (16, 29), had no notable impact on SK3 dependent modulation of force. Although we cannot be sure that total internal Ca\(^{2+}\) store depletion was achieved, the effects of CPA were essentially indistinguishable from those of a separate endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor thapsigargin (data not shown). Overall, our findings are consistent with previous reports indicating that the sarcoplasmic reticulum normally exerts a modulatory influence on uterine contractility (29) but suggest that this effect is independent of SK3 channel regulation. We surmise that feedback regulation in the uterus is similar to that reported in the urinary bladder, where SK channels attenuate phasic contractions through direct detection and modulation of Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel (12, 13). Interestingly, Herrera et al. (14) have reported that elevated SK3 expression in the urinary bladder increased storage capacity and resulted in residual nonvoided volume with micturition. Our findings suggest that increased SK3-dependent feedback regulation of smooth muscle contractility may similarly contribute to distension and limited expulsive force in the intact uterus.

It is important to note in our studies that SK3 expression in SK3\(^{+/+}\), WT, and SK3\(^{+/+}\)Dox uterus corresponded directly with general uterine contractility among the three groups (SK3\(^{+/+}\) < WT < SK3\(^{+/+}\)Dox), suggesting that modulation of uterine function is directly related to the level of SK3 expression. Similar to our previous findings in mesenteric arteries (28), we detected residual uterine SK3 expression (Fig. 1) after 5 days of Dox treatment. This expression was not further reduced by extended Dox treatment (14 days), indicating that smooth muscle SK3 expression is suppressed in this model but not entirely obliterated. For our studies, apamin was used at a concentration previously found to elicit effective but submaximal inhibition of SK channel currents and promote smooth muscle contraction without interfering with other K\(^{+}\) channels (11, 13). An important functional influence of accessory SK channels (e.g., SK1 and/or SK2) seems unlikely in our experiments since apamin had no effect on uterine contractions where SK3 was selectively downregulated (i.e., Dox-treated SK3\(^{+/+}\) mice).

Dynamic expression of myometrial K\(^{+}\) channels could be instrumental in modulating uterine excitability under different physiological conditions, particularly over the course of pregnancy. Although changes in BK channel levels have been suggested in this role (2, 26), there is currently no clear correlation between BK channel expression and uterine function, and an obligatory downregulation of BK channel subunits at term has not been established (6). Although the expression pattern of uterine SK3 channels over the course of pregnancy is currently unknown, recent data suggest these channels are expressed in human myometrium, and that their levels are substantially downregulated at term (17). Recent functional evidence from Modzelewksa et al. (19, 20) revealed that pharmacological blockade of SK channels potently antagonizes nitric oxide-mediated relaxation of human uterus and that this influence is significantly reduced in uterus taken from pregnant women at term. Our findings indicate that SK3 channels are key determinants of the magnitude and nature of uterine contraction and that their expression level is a pivotal factor.
Taken together, it is tempting to speculate that increased expression of myometrial SK3 during gestation might play a critical permissive role in uterine quiescence by disrupting concerted contractile events. Conversely, reduced SK3 levels at term may aid in the formation of the robust phasic contractions associated with labor.

We also show that the characteristics of oxytocin contractions are highly dependent on the status of myometrial SK3.

Fig. 7. Specific effect of SK3 channels on phasic and tonic components of steady-state oxytocin contractions. A: representative recordings showing contractions in response to 0.1 μM oxytocin in uterine strips from WT, SK3<sup>T/T</sup>, and SK3<sup>T/T</sup>+Dox mice before and after addition of 0.3 μM apamin. B: summary of steady-state oxytocin contractions showing mean phasic contraction frequency (±SE) as well as the relative phasic and tonic components of force during the steady state, before and after apamin addition; WT (n = 8), SK3<sup>T/T</sup> (n = 6), and SK3<sup>T/T</sup>+Dox (n = 6). C: representative recordings from SK3<sup>T/T</sup> uterine strips (n = 5, top, and n = 4, bottom), showing apamin enhancement of the phasic nifedipine sensitive, but not the sustained tonic component of oxytocin (0.1 μM) contraction.
expression. Specifically, increased expression of SK3 channels severely impairs oxytocin-dependent increases in event frequency and amplitude while allowing Ca\(^{2+}\) release from internal stores and the development of sustained tone. Thus, at term, a precipitous drop in SK3 expression could play a particularly important role in unmasking L-type Ca\(^{2+}\) channel activity and converting disorganized low-grade tonic uterine contractions (in the presence of endogenous oxytocin) into coordinated forceful and productive phasic contractions sufficient for parturition (21). This idea is consistent with the original observation that parturition is severely compromised in the SK3\(^{−/−}\) mouse (4), a model characterized by SK3 channel overexpression. Our current data suggest that excessive SK3 expression in the maternal myometrium critically limits the contractile capacity of the uterus in these animals at term and that Dox-mediated downregulation of SK3 expression is required to remove the brake on Ca\(^{2+}\) entry and allow for uterine contractions of sufficient intensity and coordination for the progression of labor. Finally, we postulate that dysregulation of SK3 channel expression might also contribute to complications during gestation. For instance, abnormally low levels of myometrial SK3 expression during midpregnancy might promote the premature development of coordinated phasic contractions, contributing to preterm labor. The precise factors contributing to sustained apamin-insensitive oxytocin-induced force in our study are unknown. We found that pretreatment of uterine strips with the combination of caffeine (10 μM) and CPA (10 μM) to simultaneously open and empty internal stores reduced this tonic contraction by >70% (data not shown). It is unknown whether the remaining contraction results from additional Ca\(^{2+}\) release or entry or from sensitization of the contractile apparatus.

In summary, our findings reveal a central modulatory role of SK3 channels in the control of uterine function, involving attenuation of the coordinated gating of L-type Ca\(^{2+}\) channels and depression of concerted phasic contractions. Elucidating the specific influence of SK3 channels on myometrial membrane potential, Ca\(^{2+}\) dynamics, and contractility over the course of pregnancy may provide a new direction for future studies of uterine function and advance the search for novel tocolytic therapies.

REFERENCES


