Maxi-K channels localize to caveolae in human myometrium: a role for an actin-channel-caveolin complex in the regulation of myometrial smooth muscle K⁺ current

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Brainard, Adam M., Andrea J. Miller, Jeffrey R. Martens, and Sarah K. England. Maxi-K channels localize to caveolae in human myometrium: a role for an actin-channel-caveolin complex in the regulation of myometrial smooth muscle K⁺ current. Am J Physiol Cell Physiol 289: C49–C57, 2005. First published February 9, 2005; doi:10.1152/ajpcell.00399.2004.—Multiple cell-signaling pathways converge to modulate large-conductance, voltage- and Ca²⁺-sensitive K⁺ channel (maxi-K channel) activity and buffer cell excitability in human myometrial smooth muscle cells (hMSMCs). Recent evidence indicates that maxi-K channel proteins can target to membrane micro-domains; however, their association with other proteins within these macromolecular complexes has not been elucidated. Biochemical isolation of detergent-resistant membrane fractions from human myometrium demonstrates the presence of maxi-K channels in lipid raft microdomains, which cofractionate with caveolins. In both non-pregnant and late-pregnant myometrium, maxi-K channels associate and colocalize with caveolar scaffolding proteins caveolin-1 and caveolin-2, but not caveolin-3. Disruption of cultured hMSMC caveolar complexes by cholesterol depletion with cyclodextrin increases an iberiotoxin-sensitive K⁺ current. Coimmunoprecipitations have indicated that the maxi-K channel also is associated with both α- and γ-actin. Immunocytochemical analysis indicates colocalization of maxi-K channels, actin, and caveolin-1 in primary cultures of hMSMCs. Further experiments using immunoelectron microscopy have shown the proximity of both actin and the maxi-K channel within the same cell surface caveolar structures. Functionally, disruption of the actin cytoskeleton in cultured hMSMCs by cytochalasin D and latrunculin A greatly increased the open-state probability of the channel, while stabilization of actin cytoskeleton with jasplakinolide abolished the effect of latrunculin A. These data indicate that the actin cytoskeleton is involved as part of a caveolar complex in the regulation of myometrial maxi-K channel function.

MYOMETRIAL SMOOTH MUSCLE CELLS (MSMCs) contain an abundance of large-conductance, voltage- and Ca²⁺-sensitive K⁺ channels (maxi-K channels). This channel provides a strong repolarizing current to buffer cell excitation in response to excitatory cell signals (21), and pharmacological block of this channel induces myometrial cell depolarization and enhanced uterine contraction (1). In uterine smooth muscle, the maxi-K channel is able to modulate its current expression via multiple mechanisms, including association with accessory β-subunits (29), alternative splicing of a common transcript (3), and posttranslational modification (47). In addition, studies localizing the maxi-K channel to detergent-resistant membrane fractions in heterologous expression systems (5) and smooth muscle tissue (2) have suggested a role for subcellular localization and trafficking in the regulation and expression of this channel.

Myometrial smooth muscle contains an extensive number of plasma membrane invaginations termed caveolae, which can increase the membrane surface area by 70% (7). These morphologically identifiable invaginations are rich in cholesterol and sphingolipids, and they contain the marker protein caveolin. These structures act as a scaffold to congregate multiple proteins for Ca²⁺ handling in smooth muscle cells, including Ca²⁺ channels, Ca²⁺-binding proteins, and Ca²⁺ pumps (10). In addition, recent investigations have revealed that caveolae can be converging centers to regulate ion channel activity (28). Maxi-K channels associate in lipid rafts in both heterologous expression systems and ureter smooth muscle (2, 5) and may modulate phasic contractions (2). It is interesting to note that signaling molecules known to modulate maxi-K channel activity, including protein kinase C (46), are also associated with raft caveolar domains (31). An assessment of whether myometrial maxi-K channels are directly regulated by cell signaling molecules or indirectly modulated by other proteins assembled in macromolecular complexes containing the channel protein would provide important information regarding the complex regulation of this channel protein.

Studies have demonstrated that the actin cytoskeleton plays a role in anchoring caveolae to the plasma membrane (30, 41). Actin filament disruption by latrunculin A causes redistribution of caveolin-1 and ultimately internalization (30). Depolymerization of the actin cytoskeleton in neuronal cells resulted in maxi-K channel inhibition (20), while channel activity increased in cultured smooth muscle cells (13). The reasons for these contrasting results have not been investigated; however, one potential mechanism is that actin-channel interactions are indirect and complex with other regulatory proteins. Therefore, the actin cytoskeleton may play a role in controlling the proximity of membrane proteins with their signaling molecules by controlling caveolar organization. Channel-cytoskeleton microdomain complexes have been hypothesized for at least two families of K⁺ channels. The inwardly rectifying Kir2.1 channel interacts with filamin, an actin-binding protein that associates with the caveolin-associated protein caveolin-1 (39). Voltage-gated family member Kv4.2 also has been shown to...
interact with filamin. For both channels, filamin regulated surface expression and distribution in either vascular smooth muscle (Kir2.1) (37) or neurons (Kv4.2) (35), and it altered current expression in their respective cell types. To understand the complex regulation of maxi-K channels in MSMCs, we tested whether this channel is compartmentalized in caveolar microdomains. Sequence analysis identified a consensus caveolin-binding motif on the COOH-terminal portion of the maxi-K channel, and coimmunoprecipitation experiments demonstrated that this channel associates with caveolin in human MSMCs (hMSMCs). On the basis of the evidence that actin plays a role in anchoring caveolae in the plasma membrane, we further hypothesized that the maxi-K channel, caveolin, and actin form a macromolecular complex within caveolae. Both immunofluorescence and immunoelectron microscopy demonstrate the proximity of actin and the maxi-K channel within cell surface caveolae. In addition, disruption of the actin cytoskeleton and perturbation of caveolar organization dramatically increase maxi-K channel function. In summary, our data demonstrate that the MSMC maxi-K channels are localized to caveolae on the cell surface as part of a macromolecular complex of proteins that includes actin and caveolin. We report the first data demonstrating that a protein complex containing the maxi-K channel in myometrial smooth muscle may be one mechanism by which to regulate current expression in uterine smooth muscle.

METHODS

Tissue collection. Human myometrial tissue from the lower uterine segment was obtained from the Cooperative Human Tissue Network (Midwestern Division, Columbus, OH), which collected the tissue from nonpregnant (NP) patients undergoing hysterectomies, and from patients who, in the absence of spontaneous or induced labor contractions, underwent elective cesarean section while under spinal anesthesia. Tissue was placed in Hanks’ balanced salt solution or in phosphate-buffered solution (PBS) on ice, and it was either used for culturing or electron microscopy within 1–3 h after collection or placed in liquid nitrogen for future use.

Cell isolation and culturing. Freshly isolated human myometrial tissue was disaggregated with 1 mg/ml collagenase (type 1; Worthington Biochemical, Lakewood, NJ) in Hanks’ balanced salt solution at 37°C with mild agitation for 1 h. Partially digested explants were plated in DMEM-Ham’s F-12 medium supplemented with 5% FBS, 4 ng/ml basic fibroblast growth factor (Sigma, St. Louis, MO), 1 ng/ml epithelial growth factor (Sigma), 50 μg/ml gentamicin (GIBCO-BRL, Grand Island, NY), and 5 μg/ml Fungizone (GIBCO-BRL). This smooth muscle cell-defined medium enhances smooth muscle cell growth (14) and inhibits the growth of other cell types present in myometrial tissue samples (e.g., fibroblasts, endothelial cells). Cells were grown in a humidified incubator with 5% CO2 at 37°C for 2 days until smooth muscle cell outgrowth was observed. At that point, the explants were removed, and adherent cells were trypsinized, plated on fresh dishes, and grown for an additional 2 days in the medium described above (with Fungizone removed) until they reached 70–80% confluence. Subsequently, cells were differentiated in DMEM-Ham’s F-12 medium supplemented with low (0.5%)-concentration FBS before experiments to induce the contractile phenotype similar to that observed in vivo. Cells were stimulated with 30 mM KCl to ensure that cells maintained a contractile phenotype after each passage. Cells were used at low passage number (up to passage 5) because cell phenotype changed irreversibly to a proliferative one with further passages.

Isolation of detergent-resistant membrane rafts. Human uterine tissue (0.75–1 g) was homogenized in 6 ml of MES-buffered saline (24 mM MES, pH 6.5, and 0.15 NaCl) plus 1% Triton X-100, spun down at 3,000 g for 5 min at 4°C, and 4 ml of the supernatant were made to compose 40% sucrose. This solution was placed in a 12.5-ml Beckman centrifuge tube (Beckman Coulter, Fullerton, CA) with a 5–30% sucrose gradient layered on top and then spun at 39,000 rpm for 24 h at 4°C in a Beckman SW-41 rotor. After being spun, 600–μl fractions of the solution were collected. For all lipid raft isolations, detergent solubilization was assessed using fractionation of the transferrin receptor. Insufficient detergent solubilization led to the transferrin receptor floating to a low buoyant density. Increasing the detergent concentration led to full solubilization of the transferrin receptor, while true raft-associated proteins (i.e., caveolin) floated to raft fractions. Extraction in 1% Triton X-100 led to nearly complete solubilization of the transferrin receptor as determined by pelleting insoluble proteins and/or complexes with a high-speed spin in extraction buffer. Therefore, we successfully isolated lipid rafts under these conditions, according to the operational definition of lipid rafts (4, 19, 38).

Preparation of uterine cytosol. NP and LP uterine tissue (0.75–1 g) were homogenized in 1.5 ml of membrane prep solution (0.25 M sucrose, 0.05 M MOPS, 2 mM EDTA, and 2 mM EGTA, pH 7.4) plus a Complete protease inhibitor tablet (Boehringer Mannheim, Indianapolis, IN). This mixture was spun at 10,000 × g for 5 min at 4°C, and the supernatant was spun at 14,000 × g for 15 min and 100,000 × g for 1 h at 4°C. The membrane pellet was solubilized with membrane prep solution with 1% Triton X-100 and was cleared at 14,000 × g for 15 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay (Sigma).

Immunoprecipitation. Two milligrams of antibody (maxi-K or pan-caveolin; Transduction Laboratories, Lexington, KY) were bound to 60 μl of protein G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature (RT) with rocking. Ten volumes of 0.2 M sodium borate was added, mixed, and centrifuged at 3,500 × g for 5 min at 4°C. Beads were resuspended in 10 volumes of 0.2 M sodium borate, and then dimethyl pimelimidate-2HCl was added to a concentration of 20 mM and mixed for 30 min at RT. Beads were spun as described above, washed once with 1 ml of 0.1 M ethanolamine, resuspended in 400 μl of 0.1 M ethanolamine, and incubated for 2 h at RT with rocking. Beads were spun as described above and resuspended in 450 μl of PBS, to which 25 μl of each lipid raft fraction containing the opaque band were added and incubated overnight at 4°C with rocking. Beads were washed twice with 1 ml of buffer A (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.5% Triton X-100) and resuspended in 20 μl of 2× SDS. Control immunoprecipitation was performed in the absence of primary antibody.

Actin spin-down assay. Assays were performed as described previously (15). NP and LP human uterine cytosol solutions (1.5 mg) were incubated with 20 nM phallloidin, 2 mM ATP, and 100 μM nocardazole for 30 min at RT. This mixture was spun through a 2 M sucrose cushion at 48,000 rpm for 1 h at 4°C. The pellet was saved and examined using Western blot analysis.

Immunoblotting. Immunoprecipitates, lipid raft fractions, and actin spin-down products were fractionated using SDS-PAGE and stained with Coomassie blue for 1 h or immunoblotted as described previously (3). Primary antibodies used were against the maxi-K channel (1:250 dilution; Transduction Laboratories); caveolin-1, caveolin-2, caveolin-3, and pan-caveolin (1:2,500 dilution; Transduction Laboratories); transferrin receptor (1:1,000 dilution; Zymed Laboratories, South San Francisco, CA); β-tubulin (clone E7; University of Iowa Hybridoma Facility, Iowa City, IA); and α-actin (Sigma) and γ-actin (1:1,000 dilution; Chemicon International, Temecula, CA). Secondary antibodies used included goat anti-mouse (1:3,000 dilution), goat anti-rabbit (1:3,000 dilution), and rabbit anti-sheep (1:3,000 dilution) Jackson ImmunoResearch, West Grove, PA). All antibodies were...
diluted in Tris-buffered saline containing Tween 20 and 3% nonfat dry milk. Blots were detected using enhanced chemiluminescence Western blot detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoelectron microscopy. Thin slices of NP or LP human uterine tissue sample were fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C, and inactive aldehyde groups were quenched with 0.05 M glycine in PBS for 15 min. Tissue was then permeabilized with 0.01% Triton X-100 in PBS for 15 min at RT, blocked in PBS containing 5% BSA, 0.1% coldwater fish skin gelatin, and 5% PBS. Tissue was washed in PBS and incubated overnight at 4°C with gentle agitation with a mouse monoclonal anti-actin antibody (Sigma) diluted 1:500 in PBS. After being washed with PBS, the tissue was incubated with a goat anti-mouse 1.4-nm gold-conjugated secondary antibody (1:2,500 dilution; Nanoprobes, Yaphank, NY) and incubated for 2 h at RT. After being washed with PBS, the tissue was blocked as described above and incubated with rabbit anti-maxi-K polyclonal antibody (1:250 dilution; Chemicon International) in PBS overnight at 4°C. After being washed in PBS, the tissue was incubated with a goat anti-rabbit 10-nm gold-conjugated secondary antibody (Nanoprobes) diluted 1:2,500 in PBS for 2 h at RT. After being washed with PBS, the tissue was postfixed with 2% paraformaldehyde and 0.25% glutaraldehyde in PBS for 5 min and washed with PBS and distilled water. The tissue slices were then enhanced with gold according to the protocol provided by Nanoprobes.

The tissue slices were prepared for transmission electron microscopy by performing osmication for 90 min in osmium fixation solution containing 0.2 M sodium cacodylate, 1% osmium, and 6% potassium ferrocyanide at a ratio of 2:1:1, and they were washed with 0.1 M sodium cacodylate for 20 min followed by a 20-min wash with distilled water. The tissue was then dehydrated using incubation in 50% ethanol for 15 min at RT, 75% ethanol for 15 min at 4°C, 95% ethanol for 30 min at −20°C, and 100% ethanol for 1 h at −20°C. The tissue was subsequently incubated in two parts ethanol and one part Epon-812 (Electron Microscopy Sciences) for 30 min at RT, one part ethanol and two parts Epon-812 for 2 h at RT, and Epon-812 overnight at RT. The tissue was placed in a Beem capsule in fresh Epon-812 and cured overnight at 60°C. After the blocks had cooled, they were sectioned (40-nm sections) and mounted on no. 300 nickel grids backed with Formvar and carbon (Electron Microscopy Sciences). The grids were subsequently stained using a standard electron microscopy grid-staining technique with 5% uranyl acetate and lead citrate. The samples were viewed using a Hitachi H-7000 electron microscope (Hitachi North America, Pleasanton, CA), and images were captured using Kodak 4489 film.

Immunocytochemistry. Cultured hMSMCs were fixed in 2% paraformaldehyde and 0.01% Triton X-100 for 30 min at RT, blocked with 10% heat-inactivated FBS and 1% heat-inactivated donkey serum (blocking buffer; 30 min at 37°C). For colocalization studies, hMSMCs were incubated with mouse maxi-K channel antibody (1:250 dilution; Transduction Laboratories), biotin-conjugated donkey anti-mouse secondary antibody (1:1,000 dilution; Jackson ImmunoResearch). Signal was enhanced using mouse anti-biotin antibody (1:500 dilution; Jackson ImmunoResearch), a biotin-conjugated donkey anti-mouse secondary antibody, and a Cy3-conjugated streptavidin (1:1,000 dilution; Jackson ImmunoResearch). Cells were incubated in blocking buffer and then with a pan-caveolin antibody (1:500 dilution) or a caveolin-1 antibody (1:500 dilution) and Cy5-conjugated donkey anti-rabbit secondary antibody (1:1,000 dilution; Jackson ImmunoResearch). Cells were blocked and incubated with either Cy3-conjugated anti-α-actin (1:500 dilution; Sigma) or γ-actin antibodies (1:500 dilution; Chemicon International). Cells treated with γ-actin antibody were incubated with a biotin-conjugated donkey anti-sheep secondary antibody and a Cy3-conjugated streptavidin (both 1:1,000 dilution; Jackson ImmunoResearch). Signals were visualized using a confocal scanning microscope (model 510; Zeiss, Oberkochen, Germany), and images were obtained using LSM 5 image browser software (Zeiss, Jena, Germany). For controls, cultured hMSMCs were incubated either with primary antibodies alone for 30 min at 37°C or with secondary antibodies alone for 15 min at 37°C. Leakage of fluorescent signals into the neighboring channels was controlled by turning off all but one laser at a time and recording images in all three channels. Laser power, pinhole size, and detector gain were then adjusted to ensure that images appeared in their respective channels only.

Electrophysiology. All patch-clamp experiments were performed at RT (−22°C). Cultured hMSMCs were placed in a pH 7.4 solution containing (in mM) 145 KCl, 2 CaCl2, 1 MgCl2, and 5 HEPES. Borosilicate glass pipettes of 6−15 MΩ were filled with a pH 7.2 solution containing (in mM) 145 KCl, 2 CaCl2, 1 MgCl2, and 5 HEPES. High (3−30 GΩ)-resistance patch seals were used for cell-attached measurements. A membrane potential of +40 mV was applied to membrane patches for up to 2 min using an Axopatch 200B voltage-current amplifier (Axon Instruments, Union City, CA). The elicited currents were recorded using pCLAMP 6.0 software (Axon Instruments). The number of open events was measured using the Fchegan function of pCLAMP 6.0 software. The open-state probability was calculated using the pStat program of pCLAMP 6.0. Statistical significance was calculated using Student’s t-test for paired observations (SigmaPlot software; SPSS, Chicago, IL). Differences were considered significant at P < 0.05. Whole cell recording was performed and analyzed as described previously (3). Briefly, current was measured using an Axopatch 200-B amplifier (Axon Instruments). Signals were filtered with a cutoff frequency of 5 kHz. Data acquisition was controlled using commercially available pCLAMP 6.0.3 software (Axon Instruments), and data were digitized using a Digidata 1200 interface (Axon Instruments). Ibrevotoxin (100−200 nM; Sigma) was used to confirm the presence of maxi-K channel current. Membrane area was estimated on the basis of integrating capacitive currents generated by a 5-nV pulse after cancelation of the patch-pipette capacitance. Currents were measured using a holding potential of −80 mV and prepulsing to −100 mV, and they were elicited at step potentials from −80 to +120 in 20-mV intervals. The bath solution contained (in mM) 135 NaCl, 4.7 KCl, 1 MgCl2, 10 glucose, 2 CaCl2, and 5 HEPES (pH 7.4). The pipette solution contained (in mM) 140 KCl, 0.5 MgCl2, 1 EGTA, 5 ATP, and 5 HEPES (pH 7.2). Mean sustained K+ current amplitudes were calculated using the Clampfit 6.0.4 software program (Axon Instruments) and plotted as pA/pF to normalize for differences in cell size. Results are plotted as means ± SE.

RESULTS

Maxi-K channels and caveolin-1 and caveolin-2, but not caveolin-3, associate with lipid rafts in human LP myometrium. One defining characteristic of lipid rafts is their resistance to solubilization at low temperatures in nonionic detergents and their relative buoyancy due to an enriched lipid content (6). We isolated low-density, Triton X-100-insoluble complexes from NP and LP human myometrium at 4°C. Western blot analysis of sucrose gradient fractions probed with channel antibodies demonstrated that maxi-K channels were present in low-density, 1% Triton-insoluble fractions (Fig. 1A, boxes). Detergent solubilization at RT disrupted maxi-K channel-raft association as shown by the detection of channels in the high-density fractions at RT (Fig. 1A). This study is the first to demonstrate that myometrial maxi-K channels are located in lipid raft compartments, and this finding is consistent with recent reports of channel compartmentation in smooth muscle (2).
A maxi-K channel (maxi-K channel) is localized to lipid rafts in both nonpregnant (NP) and late-pregnancy (LP) human myometrium. Sucrose density gradient centrifugation of 1% Triton X-100-solubilized extracts from NP ($n=10$) and LP ($n=11$) human myometrial samples were examined using Western blot analysis. Representative immunoblots show low-density, raft-associated distribution of maxi-K channels ($A; n=7$) and caveolin-1 and caveolin-2 ($B$ and $C, n=4–7$) in contrast to the high-density, nonfloating distribution of caveolin-3 ($D; n=3$) and the transferrin receptor ($E; n=5–6$). Positive controls (+) were human endothelial lysate for caveolin-1, RSV-3T3 lysate for caveolin-2, rat skeletal muscle lysate for caveolin-3, and rat cerebrum lysate for the maxi-K channel. Controls include lipid raft preparations at room temperature and the human transferrin receptor, which does not associate in lipid rafts. Boxes in $A$ refer to fractions used for the immunoprecipitation experiments shown in Fig. 2.

Previous studies have shown that human myometrium contains an abundance of caveolae, which can increase the MSMC surface by $\sim 70\%$ (16). Caveolae contain the marker protein caveolin, of which there are three known isoforms (44). Previous reports suggest the presence of caveolin-1 and -2 in rat uterine smooth muscle cells (40). We tested for the presence of each caveolin isoform in detergent-resistant membrane fractions from human myometrium. Our results indicate that the caveolin-1 and caveolin-2 isoforms are present in low-density Triton X-100-insoluble fractions from NP and LP human myometrium (Fig. 1, $B$ and $C$). Surprisingly, caveolin-3, which has been described in muscle tissue, was not detected in low-density raft fractions in either NP or LP myometrium (Fig. 1$D$). While this result was surprising, it has been reported that levels of caveolin protein can change during pregnancy (40). Our results suggest that temporal changes in the expression of different caveolin isoforms may exist. These changes could be due to changes in promoter activity between the caveolin isoforms in response to hormonal changes during pregnancy. These results demonstrate the presence of caveolins in human myometrium and indicate that caveolar microdomains exist in this tissue throughout pregnancy. Finally, to ensure complete solubilization of non-raft proteins, the transferrin receptor, which is not associated with lipid rafts (4, 19, 38), was used as a marker for Triton X-100-soluble membranes in each experiment. Gradient fractions from NP and LP samples were probed for the human transferrin receptor. As predicted, the transferrin receptor signal was detected in the high-density, detergent-soluble fractions (Fig. 1$E$) even after prolonged exposure of the blot.

Maxi-K channels are part of a macromolecular complex containing both caveolin and actin on the cell surface in myometrial cells. Recent evidence has demonstrated that different K$^+$ channel isoforms can localize to distinct raft populations (28). While caveolae share many of the biochemical properties of noncaveolar raft domains, isolation of detergent-resistant membrane fractions does not discriminate between distinct cell surface microdomains. To determine whether the maxi-K channel and caveolin isoforms are localized to the same membrane area, immunocytochemistry was performed on cultured LP human myometrial cells using maxi-K channel (Fig. 2$A$, green) and caveolin-1 antibodies (Fig. 2$A$, red). Maxi-K channels are localized primarily on the plasma mem-

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![Fig. 1](image1.png)

Fig. 1. The large-conductance, voltage- and Ca$^{2+}$-sensitive K$^+$ channel (maxi-K channel) is localized to lipid rafts in both nonpregnant (NP) and late-pregnancy (LP) human myometrium. Sucrose density gradient centrifugation of 1% Triton X-100-solubilized extracts from NP ($n=10$) and LP ($n=11$) human myometrial samples were examined using Western blot analysis. Representative immunoblots show low-density, raft-associated distribution of maxi-K channels ($A; n=7$) and caveolin-1 and caveolin-2 ($B$ and $C, n=4–7$) in contrast to the high-density, nonfloating distribution of caveolin-3 ($D; n=3$) and the transferrin receptor ($E; n=5–6$). Positive controls (+) were human endothelial lysate for caveolin-1, RSV-3T3 lysate for caveolin-2, rat skeletal muscle lysate for caveolin-3, and rat cerebrum lysate for the maxi-K channel. Controls include lipid raft preparations at room temperature and the human transferrin receptor, which does not associate in lipid rafts. Boxes in $A$ refer to fractions used for the immunoprecipitation experiments shown in Fig. 2.

![Fig. 2](image2.png)

Fig. 2. Immunocytochemistry and immunoprecipitation studies showing that the maxi-K channel is associated with caveolin-1 and caveolin-2, but not with caveolin-3. Images are pseudocolored to facilitate comparison. $A$: representative immunocytochemical localization of the maxi-K channel (Channel; green), caveolin-1 (Cav-1; red), the merged image with yellow indicating signal overlap, and differential interference contrast image (DIC; $n=6$). $B$: detergent-insoluble fractions from either NP or LP samples were pooled, and the maxi-K channel and caveolin antibodies were used to immunoprecipitate associated proteins. Samples were studied using Western blot analysis with antibodies against caveolin-1, caveolin-2, caveolin-3, and the maxi-K channel ($n=3–5$). Human endothelial cell lysate was used for a positive control for caveolin-1, RSV-3T3 lysate for caveolin-2, and rat skeletal muscle lysate for caveolin-3. $C$: same fractions immunoprecipitated in the absence of an antibody were examined using Western blot analysis with antibodies against caveolin-1, caveolin-2, caveolin-3, and the maxi-K channel ($n=2$).

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brane, whereas caveolin is localized to intracellular and plasma membrane compartments. The proteins colocalize primarily on the MSMC membrane as indicated by the signal overlap in the merged figure (Fig. 2A, yellow) and the corresponding area in the differential interference contrast image (DIC). Control studies using primary or secondary antibodies alone did not produce fluorescent signals.

To determine whether maxi-K channels and caveolins are associated in human myometrium, coimmunoprecipitations were performed using detergent-insoluble fractions containing the channel (boxed fractions in Fig. 1A). Immunoprecipitations using a maxi-K channel antibody were immunoblotted with caveolin-1, caveolin-2, and caveolin-3 isoform-specific antibodies. As shown in Fig. 2, B and C, both caveolin-1 and caveolin-2 isoforms coimmunoprecipitated with the channel in human NP and LP myometrium. As predicted on the basis of the results shown in Fig. 1D, caveolin-3 was barely detected in NP and LP myometrium (Fig. 2B). The association between the maxi-K channel and caveolin isoforms was further verified by coimmunoprecipitating the maxi-K channel with a pan-caveolin antibody (Fig. 2B). Control immunoprecipitation experiments performed in the absence of primary antibody did not detect either protein (Fig. 2C). These coimmunoprecipitation results suggest that there is a direct association between maxi-K channels and caveolin isoforms in human myometrium and that this interaction is present at NP and LP stages. In support of this hypothesis, the maxi-K channel contains a putative caveolin-binding motif in the COOH-terminal end of the channel that has been implicated in proteins directly associating with the scaffolding protein caveolin (9).

To identify potential proteins associated with the maxi-K channel, immunoprecipitation experiments were performed with LP human myometrial protein using an antibody against the maxi-K channel. This was separated using SDS-PAGE and stained with Coomassie blue (Fig. 3A). A prominent ~43-kDa band was immunoprecipitated with the maxi-K channel antibody. This band was identified using matrix-assisted laser desorption time-of-flight mass spectrometry in the LP sample as a mixture of α- and γ-actin. To elucidate the nature of the interaction between actin and the maxi-K channel, actin spin-down assays were performed using previously described methods (15). NP and LP human uterine cytosol (1.5 mg) were incubated with 20 nM phalloidin, 2 mM ATP, and 100 μM nocodazole for 30 min at RT and spun through a sucrose cushion. Pellets were then immunoblotted for the maxi-K channel as well as for α- and γ-actin. The interaction between the maxi-K channel and actin was more prevalent in NP than in LP myometrium. In addition, although similar amounts of α-actin were spun down at these two phases of pregnancy, γ-actin was more predominant at LP stages, similar to what has been reported previously in the literature (7). Positive control experiments using the actin-binding protein filamin indicate that actin is able to pellet associated proteins. Negative controls using β-tubulin (which would be disrupted by nocodazole) were not pelleted after actin polymerization. These experiments demonstrate that the maxi-K channel is associated with actin in both NP and LP human myometrium. Even though the interactions between actin and the maxi-K channel are stronger in NP than in LP myometrium, the availability of fresh human uterine tissue from LP samples was greater; thus characterization of these macromolecular interactions was performed in LP hMSMCs.

Given the interactions between these proteins, immunocytochemistry was performed on cells cultured from LP human myometrium to assess whether caveolin, maxi-K channels, and actin are located within a macromolecular complex. Cells were incubated with antibodies specific for the maxi-K channel (Fig. 4A, green), caveolin-1 (Fig. 4B, red), and γ-actin (Fig. 4C, blue). γ-Actin was used because this is the primary actin isoform after 20 wk of gestation in human myometrium (7). Similar to what is shown in Fig. 2, A and B, the maxi-K channel, caveolin-1, and γ-actin colocalize on the cell surface of LP hMSMCs. These representative images are pseudocolored to facilitate comparison. A: immunocytochemical localization of the maxi-K channel in cultured hMSMCs (green). B: caveolin-1 (red). C: γ-actin (blue). D: merged image (white) shows overlap of signals on the cell membrane (n = 6).

Fig. 3. Maxi-K channels associate with actin in LP hMSMCs. A: LP human myometrial protein was immunoprecipitated with an antibody against the maxi-K channel, separated using SDS-PAGE, and stained with Coomassie blue. The boxed band from the LP extract was excised from the gel and identified using matrix-assisted laser desorption time-of-flight mass spectrometry as a mixture of α- and γ-actin. B: NP and LP hMSMC cytosol was incubated in the presence of phalloidin and spun through a sucrose cushion. The proteins colocalize primarily on the MSMC membrane as indicated by the signal overlap in the merged figure (Fig. 2A, yellow) and the corresponding area in the differential interference contrast image (DIC). Control studies using primary or secondary antibodies alone did not produce fluorescent signals.

Fig. 4. Maxi-K channels, caveolin, and γ-actin colocalize on the cell surface of LP hMSMCs. These representative images are pseudocolored to facilitate comparison. A: immunocytochemical localization of the maxi-K channel in cultured hMSMCs (green). B: caveolin-1 (red). C: γ-actin (blue). D: merged image (white) shows overlap of signals on the cell membrane (n = 6).
Fig. 5. Both the maxi-K channel and actin are localized to caveolar invaginations in human myometrium. A: electron microscopic image showing NP human uterine tissue sections with an abundance of caveolae (n = 8; area in box). B: immunoelectron microscopic image showing cross sections of LP human uterine tissue treated as described in METHODS and examined using transmission electron microscopy. These images show the localization of both the maxi-K channel (black arrow) and α-actin (white arrow) within caveolae (n = 12).

channel is localized primarily to the cell surface, and caveolin is located on both the membrane surface and intracellularly (Fig. 4, A and B). Immunocytochemical analysis with the γ-actin antibody shows an abundant cytoskeletal network in hMSMCs (Fig. 4C). Merging these images indicates partial colocalization of these three proteins with a high degree of overlap on the myometrial smooth muscle cell membrane (Fig. 4D, white). Control experiments performed in the presence of primary or secondary antibodies alone did not produce detectable fluorescence. To examine this colocalization with greater resolution and to establish the proximity of maxi-K channels to caveolae, ultrastructural analysis of human myometrium was performed. The LP myometrial cell surface was abundant, with deeply invaginated flasklike structures that lacked a cytosolic coat, structurally consistent with caveolae (Fig. 5A, boxed). Using immunoelectron microscopy, we were able to confirm the localization of the maxi-K channel within caveolar invaginations (Fig. 5B, black arrow). Importantly, as shown in Fig. 5B, the maxi-K channel (1.4-nm gold particle, black arrow) and actin (10-nm gold particles, white arrow) are both located within cell surface caveolae in LP human myometrium.

Disruption of the actin cytoskeleton alters maxi-K channel function. To functionally characterize the interaction between the maxi-K channel and actin in human myometrium, cell-attached single-channel analysis was performed using cultured hMSMCs from LP human myometrium in the presence and absence of actin toxins. Bath application of cytochalasin D, which partially disassembles actin filaments, significantly increased the open-state probability of the channel from 0 to 0.54 (Fig. 6A). Cytochalasin D has been implicated to increase intracellular Ca2+, which would increase channel activity (17). Thus latrunculin A, which depolymerizes actin filaments, also was used. Introduction of latrunculin A to the bath increased the open-state probability of the maxi-K channel from 0.003 to 0.46 (Fig. 6B). Pretreatment of cultured hMSMCs for 5 min with 1 μM jasplakinolide, an actin polymerizer, inhibited the effect of latrunculin A (Fig. 6C), with a small increase in open-state probability from 0.005 to 0.035. Backfilling the pipette with 200 nM iberiotoxin, a specific maxi-K channel inhibitor, blocked the large-conductance K+ channel current that was activated by cytochalasin D (Fig. 6D), indicating that this channel was the maxi-K channel (results were the same with latrunculin A; data not shown). The significant increase in maxi-K channel activity after actin depolymerization (Fig. 6E) indicates that the actin cytoskeleton modulates maxi-K channel current in cultured hMSMCs.
Disruption of lipid rafts by cyclodextrin increases maxi-K channel activity. To establish how caveolin-associated maxi-K channels contribute to the total outward K⁺ myometrial current, cultured LP hMSMCs were electrophysiologically characterized after a 1-h, 37°C incubation in 2% cyclodextrin, a cholesterol-depleting agent that disrupts caveolae (Fig. 7A). After incubation in cyclodextrin, K⁺ current density in cultured hMSMCs increased 2.4-fold from control levels (Fig. 7, B and D). This effect was blocked by 100 nM iberiotoxin, a maxi-K channel blocker, indicating that this increase in current was a result of maxi-K channel activity (Fig. 7, C and D). Iberiotoxin was able to suppress current to a greater extent than the control recordings, suggesting that not all channels are lipid raft associated (Fig. 7D). Overall, our results suggest that myometrial maxi-K channels are associated with caveolar lipid raft domains (Fig. 7E). These channels interact with both caveolin and actin, and disruption of this complex can alter maxi-K channel activity. A schematic of the potential association between the maxi-K channel, caveolins, and actin is shown in Fig. 7E.

DISCUSSION

To comprehend mechanisms underlying quiescent and active contractile states of the uterus during pregnancy, it is necessary to understand the basis for myometrial electrical activity. Sarcolemmal depolarization results in Ca²⁺ influx and contraction of myometrial smooth muscle. The roles of Ca²⁺ as a major charge carrier in mediating depolarizing membrane potential changes as well as providing a contractile signal for electromechanical coupling have been well characterized in the myometrium (22, 27, 33, 45). Recent evidence indicates that key components for Ca²⁺ handling in smooth muscle are localized in caveolae, which may serve as initiation sites for Ca²⁺ sparks in myocytes (10, 26). These studies correlate to recent findings indicating that disruption of caveolae by cholesterol depletion inhibits intracellular Ca²⁺ signals and generation of smooth muscle force (2). Because acute increases in intracellular Ca²⁺ activate maxi-K channels, leading to membrane hyperpolarization and uterine relaxation, understanding the proximity of these channels to Ca²⁺ signaling machinery in caveolae will provide insight into methods that regulate myometrial contractility.

The role of caveolae in converging multiple signaling processes has been described in various tissues (36). To date, little is known about the role of caveolae in the uterus, despite morphological data demonstrating an abundance of caveolae in myometrial smooth muscle (7). Although quantitative studies indicate that the number of caveolae do not differ between NP, term-pregnant (i.e., LP), and laboring human myometrium (8), the levels of caveolin-1 and -2 proteins do change. Each appears to demonstrate low expression during the first part of the pregnancy and then gradually increases in expression until the day of delivery in the pregnant rat (40). Although these results appear contrary, caveolins directly bind signaling molecules, and the dynamic nature of these interactions could elicit changes in myometrial excitability without altering the abundance of caveolae. Both caveolin-1 and caveolin-2 are suppressed by estrogen (40), indicating that hormonal status regulates caveolar proteins and perhaps associated signaling processes during pregnancy.

Our data, which complement the caveolin-binding motif in the channel, suggest a direct association between the maxi-K channel and caveolin-1 and -2 in human myometrium in both NP and LP states. Although caveolin-3 is found in vascular smooth muscle (12), our studies and others have not detected this isoform in great abundance in uterine tissue (18). In addition, we found only a weak association between the maxi-K channel and caveolin-3. While our data suggest a role for a channel-caveolin-actin complex in the regulation of maxi-K current in the uterus, the complexity and physiological role of channel-caveolin interactions require additional study. It is tempting to hypothesize that this interaction is important for channel transport. Caveolae could be involved in the trafficking of the maxi-K channel to the plasma membrane, with caveolin acting as a tether helping to anchor the channel to a caveolar vesicle. It is interesting that, using immunoelec-

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**Fig. 7.** Disruption of the lipid bilayer and caveolae increases maxi-K channel activity in cultured LP hMSMCs. Whole cell recordings of cultured LP hMSMCs before (A; n = 6) and after (B; n = 5) incubation with 2% cyclodextrin at 37°C and after incubation with 2% cyclodextrin and 200 nM iberiotoxin (C; n = 2). Cyclodextrin increased K⁺ current density 2.4-fold. D: graphic representation of the increase in current density due to cyclodextrin. *P ≤ 0.05, significant differences from control recordings. Current density (pA/pF) was used because of differences in cell size. E: model of proposed interaction of the maxi-K channel with actin and caveolin localized within caveolae.
tron microscopy, we could detect electron-dense particles corresponding to the maxi-K channel in unfused caveolae beneath the cell surface (Fig. 5B).

Maxi-K channels contain a putative caveolin-binding motif in the COOH terminus (9). Recent studies have demonstrated loss of membrane expression in maxi-K channels lacking this region, perhaps implicating this region in transport (42). Caveolae are also thought to be able to open and close when on the membrane (32), and perhaps localization of the maxi-K channel to cell surface caveolae allows cells to dynamically regulate their electrical excitability by the opening and closing of the caveolar neck. As shown in ureter smooth muscle, disruption of lipid rafts, which contain maxi-K channels, alters phasic contraction (2). However, it is unlikely that all maxi-K channels are localized to caveolar microdomains; there may be a basal amount on the cell surface, with additional channels added as needed to buffer cell excitation. Therefore, caveolae could open and expose more channels to the membrane, buffering cell excitability and thereby keeping the cells relaxed, and may thus explain partially the increase in current density observed after caveolar disruption (Fig. 7A). In contrast, caveolae could close off from the membrane, engulfing channels with them to promote conditions required for uterine contraction.

The maxi-K channel plays a role in relaxing the uterus by keeping it in a quiescent state throughout pregnancy; however, its role at the onset of labor is unknown. Studies have shown a switch in channel phenotype at the onset of labor (23, 24) from a typical voltage and Ca$^{2+}$-sensitive maxi-K current to one that is constitutively active, lacking sensitivity to these factors. While this may represent a novel channel type, it may also represent differential regulation of the maxi-K channel. There is precedent for this channel to be regulated differently by PKA during pregnancy (34), which is likely due in part to alternative splicing that results in expression of different maxi-K channel isoforms. Although our studies were performed with LP non-laboring human myometrium and primary cultured myometrial cells from this stage, our results suggest that changes in channel macromolecular complexes, including caveolin, may lead to dynamic regulation of proteins associated with the maxi-K channel. The culturing of myometrial cells is an experimental limitation because phenotypic changes can occur. However, results obtained using these cells are in agreement with data collected using myometrial tissue and suggest that changes in channel-containing macromolecular complexes may regulate channel activity and thereby contribute to changes in the current phenotype observed at the onset of labor.

Our data demonstrate an interaction between the maxi-K channel and actin that may be regulated in part by caveolae (Fig. 7B). Caveolae are thought to be membrane-organizing centers that recruit lipids and proteins for participation in intracellular trafficking and signal transduction (25). As functionally noted, disruption of the actin cytoskeleton could elicit a series of events that lead directly to channel activation and/or release of an inhibitory factor. In either case, disruption of the actin cytoskeleton in cultured LP hMMSMCs produces a phenotype similar to that observed during labor. It is important to note that on the basis of our biochemical fraction data (Fig. 1A), not all maxi-K channels are associated with rafts and/or caveolin. The actin pull-down assays performed in the present study cannot discriminate raft- and non-raft-associated channels. Although our immunolocalization data suggest that the three proteins colocalize on the cell surface, it is possible that non-raft- as opposed to raft-associated maxi-K channels associate with actin.

Remodeling of the actin cytoskeleton in the myometrium has not been examined during pregnancy; however, recent reports have indicated that localized mechanical stress can induce actin remodeling and stiffening in smooth muscle cells (11). Whether this occurs in the myometrium during labor and how this may affect electrical activity are interesting points to consider. Actin has been implicated in the spatial organization of caveolae on the plasma membrane (39). This finding, coupled with our data showing the proximity of the maxi-K channel to actin within the caveolae, may indicate that their compartmentation is one mechanism by which to regulate uterine function. While the link between the maxi-K channel, caveolin, and actin is not known, it may involve additional actin-binding proteins (i.e., filamin) or a lipid intermediate. Nevertheless, understanding this interaction will be an important step in identifying other mechanisms that may regulate K$^+$ channel activity.

The increase in the rate of premature births (43) and the need for more effective tocolytic therapy have prompted new studies to understand the proteins involved in this process. With much to elucidate about the regulation of the maxi-K channel during pregnancy and at the onset of labor, these data are the first to show that maxi-K channels target to caveolar membrane microdomains, associate with caveolin, and interact with the actin cytoskeleton in hMMSMCs.

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