Contribution of coupling between human myometrial \( \beta_2 \)-adrenoreceptor and the \( \text{BK}_{\text{Ca}} \) channel to uterine quiescence

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Drugs active at the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)-AR) and the large-conductance \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) (\( \text{BK}_{\text{Ca}} \)) channel have been shown, separately, to be involved in mediating uterine relaxation. Our recent studies reveal that the levels of both \( \beta_2 \)-AR and \( \text{BK}_{\text{Ca}} \) channel proteins in pregnant human myometrium decrease by \( \approx 50\% \) after the onset of labor. We present direct evidence in support of a structural and functional association between the \( \beta_2 \)-AR and the \( \text{BK}_{\text{Ca}} \) channel in pregnant human myometrium. Localization of both proteins is predominantly plasmalemmal, with \( 60\% \) of \( \beta_2 \)-AR colocalizing with the \( \text{BK}_{\text{Ca}} \) channel. Coimmunoprecipitation studies indicate that \( \text{BK}_{\text{Ca}} \) and \( \beta_2 \)-AR are structurally linked by direct protein-protein interactions. Functional correlation was confirmed by experiments of human myometrial contractility in which the \( \text{BK}_{\text{Ca}} \) channel blocker, paxilline, significantly antagonized the relaxant effect of the \( \beta_2 \)-AR agonist ritodrine. These novel findings provide an insight into the coupling between the \( \beta_2 \)-AR and \( \text{BK}_{\text{Ca}} \) channel and may have utility in the application of this signaling cascade for therapeutic potential in the management of preterm labor.

\( \beta_2 \)-adrenergic receptor; myometrium; potassium channel; preterm labor; uterine contraction

PRETERM DELIVERY, defined as delivery before 37 completed weeks of gestation, is the single leading cause of perinatal morbidity and mortality, particularly in developing countries (1). Despite advances in perinatal care, the incidence of preterm delivery has increased progressively over the past two decades (23). The impact of this high rate extends to health care, the economy, education, society, and family. To date, a lack of detailed information pertaining to the cellular mechanisms that determine uterine excitability has hampered the development of new, effective treatments for preterm labor. Drugs active at the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)-AR) have long been used as tocolytic agents, but their widespread tissue distribution has led to adverse maternal cardiovascular and metabolic effects (6, 14). Furthermore, the tachyphylaxis that occurs in vivo after exposure to \( \beta_2 \)-AR agonists also compromises their effectiveness (13, 24, 26).

One prominent feature of \( \beta_2 \)-agonists is their ability to activate \( \text{K}^+ \) channels, leading to cellular hyperpolarization (3, 20). We have shown that the protein levels of both the myometrial \( \beta_2 \)-AR and the \( \alpha \)-subunit of the \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) (\( \text{BK}_{\text{Ca}} \)) channel decrease by \( \approx 50\% \) after the onset of labor (7, 8, 21). Anwer et al. (3) demonstrated that isoproterenol, a \( \beta \)-AR agonist, can stimulate \( \text{BK}_{\text{Ca}} \) channels in pregnant rat myometrium. In addition, ritodrine, a \( \beta_2 \)-AR agonist, has also been shown to activate \( \text{BK}_{\text{Ca}} \) channels via a G protein and cAMP-dependent phosphorylation cascade in cultures prepared from pregnant human myometrium (12). \( \text{BK}_{\text{Ca}} \) channels, which are activated by voltage and increased concentrations of intracellular \( \text{Ca}^{2+} \), are abundant in smooth muscle (10, 16, 17), where they play an important role in limiting depolarization, thereby relaxing uterine smooth muscle (2, 15). Although evidence favors phosphorylation as the likely mechanism by which \( \beta_2 \)-agonist regulation of \( \text{BK}_{\text{Ca}} \) channel occurs, little is known regarding the extent of interaction between these two proteins in relation to the mechanisms that determine uterine quiescence and the timing of labor.

We tested the hypothesis that there is a direct association between the \( \beta_2 \)-AR and \( \text{BK}_{\text{Ca}} \) channel that is of physiological and clinical significance during pregnancy and labor. The aim of this study was to explore the interaction between these two proteins in the pregnant human myometrium by investigation of their cellular localization, protein-protein association, and functional correlation with a view to identifying novel signaling cascades as potential therapeutic opportunities for the treatment of preterm labor.

MATERIALS AND METHODS

Subjects and tissue collection. This study was approved by Southern Derbyshire Ethics Committee. The following two groups of women were recruited: 1) singleton term pregnant women (gestational age \( \geq 37 \) wk) undergoing elective cesarean section before the onset of labor and 2) singleton term pregnant women undergoing emergency cesarean section after spontaneous labor (cervical dilatation \( \geq 3 \) cm). Written informed consent was obtained from each participant.

The myometrial biopsy was taken from the mid-upper margin of the lower uterine incision in women undergoing cesarean section. Women suffering from preeclampsia or other medical conditions, such as diabetes or connective tissue diseases, were excluded from this study. All women were undergoing cesarean section because of previous cesarean section or breech presentation. Samples were collected in physiological salt solution (PSS) for immunofluorescence and isometric tension recording studies and snap-frozen in liquid nitrogen followed by storage at \(-80^\circ\text{C} \) for Western blotting and immunoprecipitation studies. Each myometrial sample was used once, and only one sample was obtained per patient.

Double-staining immunofluorescence and confocal microscopy. Fresh myometrial tissues were washed twice with \( \text{Ca}^{2+} \)- and \( \text{Mg}^{2+} \)-free Hanks' balanced salt solution (HBSS), minced finely in
collagenase A in HBSS (2 mg/ml), and incubated in this enzymatic solution for 1 h at 37°C with intermittent, gentle trituration. The cell suspension was carefully layered on a 60% (vol/vol) Percoll gradient and centrifuged at 22°C, 800 g, for 5 min and then washed two times in HBSS (22°C, 800 g for 5 min each) to remove cell debris. The supernatant was discarded, and cytospin slides were prepared immediately by mixing 100 μl of the pellet (containing cells) with an equal volume of PBS followed by centrifugation at 100 g for 10 min.

Cytocentrifuged myometrial cells were fixed for 20 min in 2% (wt/vol) paraformaldehyde and then washed two times with 0.1 M PBS. Cells were subsequently permeabilized with 0.5% Igepal in 0.1 M PBS in a humidity chamber at 4°C for 5 min and then washed with PBS (2 times for 5 min) followed by block with 3% (wt/vol) BSA-1% glycine (wt/vol) in PBS for 15 min at room temperature. Cells were then incubated with both primary antibodies; a mouse monoclonal antibody specific to the α95-1113-subunit of the BKCa channel (anti-BKCa α-subunit antibody; Transduction Laboratories) and a polyclonal anti-β2-AR mapping to residues 338–413 of the carboxy terminus of the receptor (Santa Cruz Biotechnology) diluted in PBS containing 10% (vol/vol) normal horse serum overnight at 4°C. Cells were subsequently washed, incubated for 30 min in the dark with biotinylated anti-mouse IgG (10 μg/ml; Vector Laboratories, Peterborough, UK), washed, and then incubated with Texas Red-avidin DCS (10 μg/ml; Vectorstain Elite; Vector Laboratories) for 30 min followed by further washing. The slides were then incubated for another 30 min with biotinylated anti-rabbit IgG (10 μg/ml; Vector Laboratories) followed by fluorescein-avidin DCS (10 μg/ml Vectorstain Elite; Vector Laboratories) and then washed. Slides were mounted in Vectashield mounting media (Vector Laboratories). Cells were viewed on a Zeiss Axiovert 100 microscope with an LSM 510 confocal scan head (Carl Zeiss, Jena, Germany) and a plan-Apochromat ×63 oil immersion objective lens. Images were captured using multitracking with 488-nm argon and 543 HeNe lasers and analyzed using LSM software version 3.2 (Carl Zeiss). Percentage colocalization is based on the fluorescence intensity of the β2-AR signal relative to that of the BKCa α-subunit, after deducting the background intensity.

Immunoprecipitation and Western blotting. Human myometrium was processed, and immunoprecipitation experiments were performed essentially as described by Matharoo-Ball et al. (22), with the following changes: 1) immunoprecipitation buffer for this study had the following composition: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.1% SDS, 0.3% sodium deoxycholate, 1:500 protease inhibitor, and 1:100 phosphatase inhibitor, and 2) samples were incubated with antibody-specific IgG agarose beads (according to the species of the immunoprecipitating antibody) at 4°C for 2 h instead of 12 h. The immunoprecipitating antibodies were either anti-BKCa α-subunit or anti-β2-AR followed by Western blotting separately (7, 8) with both anti-β2-AR (1:250 dilution) and anti-BKCa α-subunit (1:750) antibodies. Controls were incubated with either

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**Fig. 1.** Double-fluorescence labeling on the plasma membrane of myometrial cells from nonlaboring (A–C) and laboring (D–F) pregnant women. Cells were incubated with selective antibody to α-subunit Ca2+-activated K+ (BKCa) channels (Texas red; A and D) and β2-adrenergic (β2-AR) receptors (fluorescein; C and F). After superimposition (B and E), the yellow signal demonstrates the colocalization of β2-AR and BKCa channels. Magnification is 630-fold in A–F. The photomicrographs of the 2 cells shown are representative of at least 5 cells.
mouse or rabbit IgG (DAKO, Glostrup, Denmark) instead of primary antibody. Western blotting was carried out as described previously (7, 8). Immunoblots were then processed (Immun Star; Bio-Rad Laboratories, Hertfordshire, UK) and viewed using an imaging densitometer (ChemiDoc; Bio-Rad). Each experiment was repeated in triplicate.

**Isometric tension recording.** Longitudinal myometrial strips (∼2 × 2 × 10 mm) were mounted under 2 g tension in an organ bath (Lettica 01; AD Instruments, Oxfordshire, UK) for isometric tension recording in 10 ml of aerated (95% O₂ + 5% CO₂) PSS at 37°C. Myometrial contractions were stimulated by 10⁻⁹ M oxytocin (Sigma-Aldrich, Poole, UK). Mechanical responses of myometrial strips were measured by Quadbridge (PowerLab; AD Instruments) and recorded using Chart version 4.2 (PowerLab; AD Instruments). After 1 h of equilibration, cumulative increases of ritodrine (10⁻⁹ to 10⁻³ M; Sigma-Aldrich) were applied at 20-min intervals, and the contractile activity was measured during each period.

The effects of the BKCa channel blocker paxilline (Sigma-Aldrich) and β²-AR antagonist ICI-118551 (Tocris Cookson, Bristol, UK) on ritodrine-mediated responses were tested by preincubating myometrial strips for 30 min with either 10⁻⁶ M paxilline or 10⁻⁷ M ICI-118551, followed by cumulative additions of ritodrine. Data are presented as the activity integral calculated during the 20-min period after addition of each ritodrine concentration as a percentage of the control integral obtained for 20 min in oxytocin alone. Concentration-response curves of the activity integral were analyzed by fitting the data to the following equation:

\[
y = (y_{\text{max}}) + \left(y_{\text{max}} - y_{\text{min}}\right) \left(1 + 10^{\log EC_{50}\cdot X}\right)^{-1}
\]

where \(y\) is the response, \(y_{\text{max}}\) is the maximum relaxation achieved, \(y_{\text{min}}\) is the minimum relaxation achieved, and \(X\) is the drug concentration. Significance was determined by F-test and Student’s t-test as appropriate. A \(P\) value <0.05 was considered statistically significant.

**RESULTS**

With the exception of the functional studies, results were compared using myometrium from nonlaboring and laboring women on the basis that parturition may be preceded by a physical dissociation between the β²-AR and the BKCa channel that would be detectable by immunoprecipitation or colocalization studies.

Dispersed myometrial cells in cytopsins appeared relaxed and elongated, demonstrating their smooth muscle phenotype. However, because of the hypertrophy myometrial cells undergo during pregnancy, myocytes are often ∼600 μm long. This has the effect that, even after fixation, the extremities of the cells are often raised above the field of view, imparting a distinct appearance to certain cells (Fig. 1, D–F, and Fig. 2, D–F). The smooth muscle nature of these cells has been confirmed by anti-α-actin staining and electrophysiological

![Fig. 2. Sagittal section of myometrial cells demonstrated double-fluorescence labeling of myometrial cells from nonlaboring (A–C) and laboring (D–F) pregnant women. Cells were incubated with selective antibody to α-subunit BKCa channels (Texas red; A and D) and β²-AR (fluorescin; C and F). After superimposition (B and E), the yellow signal demonstrates the colocalization of β²-AR and BKCa channels. Magnification is 630-fold in A–F. The photomicrographs of the 2 cells shown are representative of at least 5 cells.](http://ajpcell.physiology.org/)

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Characteristics showed strong labeling of myometrial β2-ARs. This signal localized to a structure coincident with the BKCa channel in myometrial cells of pregnant labor and nonlabor tissues (Fig. 1, A, C, D, and F). Quantitative double-immunofluorescence studies revealed that 63.7 ± 16.8% of the nonlabor (n = 5) β2-AR signal (Fig. 1B) and 61.7 ± 4% of the labor β2-AR signal (n = 5; Fig. 1E) colocalized with myometrial BKCa channels (P = 0.86). Laser scanning by slicing through the cytosol every 20 μm of both nonlabor (n = 5) and labor (n = 5) provided very little evidence of immunofluorescence intracellularly (Fig. 2).

In light of the double-immunofluorescence studies, we examined whether β2-AR and BKCa channels were structurally associated. Coimmunoprecipitation experiments were performed with the same antibodies as for the double-staining immunofluorescence studies. Thus myometrial membrane proteins were immunoprecipitated with either anti-β2-AR or anti-α-BKCa antibody. Both β2-AR (52 kDa) and BKCa α-subunit (125 kDa) protein bands were demonstrable after immunoblots were probed with either anti-β2 AR or anti-BKCa α-subunit antibody (Fig. 3). Positive results were demonstrated in proteins from both pregnant women with (n = 5) and without (n = 5) labor. No specific bands were detected when the precipitating antibodies were replaced by rabbit or mouse IgG.

On the basis of the observed colocalization and coimmunoprecipitation findings, we performed additional isometric tension recordings of nonlabor myometrium to investigate the effect of the specific BKCa channel blocker paxilline on the relaxant effect of the β2-AR agonist ritodrine. The relaxation observed with cumulative additions of ritodrine (n = 15) was antagonized by 1 × 10−6 mol/l paxilline (●) and 10−5 mol/l ICI-118551 (▲). Paxilline and ICI-118551 produce rightward displacements of the curve, with a significant shift in EC50 (●, P < 0.01) for both. Data of activity integral were presented as the mean ± SE percentage of the results obtained before any drug application for each individual strip. The concentration-response curves were analyzed by fitting to the equation: $y = (y_{\text{min}}) + (y_{\text{max}} - y_{\text{min}})/[1 + 10^{10 \log EC_{50}(y)}]$, where $y$ is the response, $y_{\text{max}}$ is the maximum relaxation achieved, $y_{\text{min}}$ is the minimum relaxation achieved, and $X$ is the drug concentration.

**Fig. 4.** Concentration-response curves for the effect of cumulative additions of ritodrine on myometrial contractility (percentage contraction achieved). Response is shown in the absence (control, ●) and presence of 10−6 mol/l paxilline (●) and 10−5 mol/l ICI-118551 (▲). Paxilline and ICI-118551 produce rightward shifts observed with paxilline mirrored that obtained with the specific β2-AR antagonist ICI-118551 [EC50 = 0.87 × 10−4 (SE 0.9) mol/l; P < 0.01].

**DISCUSSION**

We present herein immunochemical evidence for an apparently direct protein-protein interaction between the human myometrial β2-AR and the BKCa channel. Furthermore, our pharmacological data lend support to the hypothesis that a functional signaling complex formed by these two membrane proteins mediates relaxation in pregnant human myometrium and may constitute a cellular mechanism whereby uterine quiescence is maintained throughout pregnancy. We and others have shown that the BKCa channel, activated by raised intracellular Ca2+ levels, is of significance in maintaining the resting membrane potential of isolated myometrial cells and, by opposing depolarization, would, at a tissue level, prevent uterine contractions (2, 16). The BKCa channel, by integrating multiple inputs from diverse stimuli, influences and affects numerous cellular pathways. However, the mechanisms by which signaling is spatially and specifically routed are not fully understood.

In many smooth muscle tissues, including those of the respiratory tract and vasculature (5, 19), BKCa channels demonstrate relaxant effects. Experimental evidence suggests that β2-AR agonists cause relaxation via G protein-dependent pathways that activate adenylate cyclase and increase cytosolic levels of cAMP, leading to phosphorylation of the channel (11). Recent findings in tracheal smooth muscle have demonstrated that β-adrenergic stimulation can also activate BKCa channels independently of channel phosphorylation via the α-subunit of Gs (18). Our functional studies showing that ritodrine-mediated relaxation is blocked by both paxilline and
ICI-118551 to the same extent (maximal relaxation of ~50%) suggests that the BKCa channel and β2-AR probably form a regulatory pathway that is prominent in the control of myometrial excitability. It is not possible to conclude from our results whether phosphorylation of the BKCa channel is necessary for the β2-mimetic ritodrine to exert its effects. However, in human cultured myometrial cells, the effect of ritodrine appears to involve both cAMP and protein kinase A (PKA)-mediated phosphorylation in addition to a direct GTP activation of BKCa channel activity in inside-out patches (12).

The discovery of a signaling complex in rat hippocampal neurons comprising the β2-AR, L-type Ca\(^{2+}\) channels, phosphatase 2A, and adenylate cyclase supports the existence of molecular signaling assemblies that allow for specificity of cellular responses (9). Davare et al. (9) have elegantly demonstrated that addition of the β2-agonist albuterol directly to the patch pipette increased the activity of single L-type Ca\(^{2+}\) channels soon after seal formation of cell-attached recordings. This effect was not observed after bath application of albuterol, suggesting that the close proximity facilitates specificity and rapid interaction of the β2-AR, L-type Ca\(^{2+}\) and key signaling molecules. Moreover, a recent study (21) postulated that β2-AR modulation of membrane excitability occurs, with the latter providing a scaffold that couples BKCa with L-type Ca\(^{2+}\) channels in brain, lung, aortic, and bladder tissues. The human myometrial BKCa channel may also function as part of a similarly complex intracellular network comprising enzymes, scaffolding proteins, and second messenger molecules. In support of this, downstream signaling intermediaries (activated by agonist binding to the β2-AR), such as G\(_{\alpha}\), PKA, adenylate cyclase, and the cAMP pathway, act directly to modulate myometrial BKCa channel activity. Electrophysiological evidence demonstrates that there is differential coupling between the myometrial BKCa channel and the β-adrenoreceptor, since isoprenaline enhanced macroscopic outward current in myometrial cells of term, pregnant women while reducing outward current in myocytes of nonpregnant myometrium. Outward K\(^+\) currents in rat myometrium have also been shown to be influenced in opposing directions by PKA and norepinephrine, depending upon the reproductive status of the animal. Our findings, which demonstrate a significant coexistence of β2-AR and BKCa channels, support the notion that membrane ion channels may be direct targets for the regulatory action of β2-AR in target organs, including human myometrium. This close, intimate association would accelerate and concentrate direct signaling between β2-AR and BKCa channel to achieve relaxation.

The control of human myometrial quiescence during pregnancy and its transformation to a highly contractile state are not fully understood. However, the pivotal role of the BKCa channel and its modulation by an array of chemical mediators identifies it as a key sensor through which many cellular processes may be implemented. These include control of vascular tone, cytokine and hormone secretion, redox processes, and neuronal firing. The β2-AR has a similarly ubiquitous cellular distribution. This suggests that, to execute specific cellular functions in response to a physiological stimulus, subcellular compartmentation must be organized such that coupling between receptors and ion channels is optimized. We provide evidence for a protein-protein interaction between the BKCa channel and β2-AR. The fact that an anti-β2-AR antibody could precipitate BKCa channel protein and vice versa is further evidence to indicate that these two proteins exist in close proximity. The β2-AR has a molecular mass between 56 and 85 kDa (4), with 67 kDa reported as being the mature form of the receptor. In our earlier study, the myometrial β2-AR was reported to have a molecular mass of 67 kDa, whereas immunoprecipitation followed by Western blotting, as described herein, identifies a 52-kDa protein. We propose that the reported size differences may be attributed to the heavily glycosylated or phosphorylated nature of this protein. A 56-kDa β2-AR protein was observed in β2-AR-transfected COS-7 cells along with a 43-kDa deglycosylated form of the receptor (25). Although glycosylation does not interfere with β2-AR function, it is considered to be pivotal in determining the correct delivery of the β2-AR to the cell membrane. It appears that the immunoprecipitation protocol we used preferentially detects the truncated form of the β2-AR, reflecting possible chemical modifications to the parent molecule at a site distinct from the antibody recognition site.

Our results provide compelling evidence in favor of a direct interaction between the β2-AR and BKCa channels as a novel component in the mechanism of uterine relaxant and therefore gestational quiescence. Here we show a close association of this receptor-channel coupling at a molecular and functional level. We did not observe any significant difference in either colocalization or dissociation of the two proteins between nonlabor and labor tissues. It is unclear whether myometrial relaxation and the transition to contractions at term involves a direct and immediate interaction between β2-AR and BKCa channels, an action through second-messenger pathways, or indeed activation of alternative signaling pathways that favor contractility. However, it is interesting to speculate from a clinical perspective that bypassing the β2-AR and targeting BKCa channels in human myometrium instead may, in time, deliver a drug superior in terms of its tocolytic efficacy and apparent absence of desensitization, ultimately alleviating the economic and emotional burden of prematurity.

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