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Functional Coupling of β_3 -Adrenoceptors and Large Conductance Calcium-Activated Potassium Channels in Human Uterine Myocytes

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Context: β_3 -Adrenoceptor modulation in human myometrium during pregnancy is linked functionally to myometrial inhibition. Maxi-K⁺ channels (BK_{Ca}) play a significant role in modulating cell membrane potential and excitability.

Objective: This study was designed to investigate the potential involvement of BK_{Ca} channel function in the response of human myometrium to β_3 -adrenoceptor activation.

Design: Single and whole-cell electrophysiological BK_{Ca} channel recordings from freshly dispersed myocytes were obtained in the presence and absence of BRL37344, a specific β_3 -adrenoceptor agonist. The *in vitro* effects of BRL37344 on isolated myometrial contractions, in the presence and absence of the specific BK_{Ca} channel blocker, iberiotoxin (IbTX), were investigated.

Setting: The study was carried out at the Clinical Science Institute.

Patients or Other Participants: Myometrial biopsies were obtained at elective cesarean delivery.

Intervention: No intervention was applied.

Main Outcome Measures: Open state probability of single channel

recordings, whole cell currents, and myometrial contractile activity were measured.

Results: Single-channel recordings identified the BK_{Ca} channel as a target of BRL37344. BRL37344 significantly increased the open state probability of this channel in a concentration-dependent manner (control 0.031 ± 0.004 ; $50 \mu\text{M}$ BRL37344 0.073 ± 0.005 ($P < 0.001$); and $100 \mu\text{M}$ BRL37344 0.101 ± 0.005 ($P < 0.001$). This effect was completely blocked after preincubation of the cells with $1 \mu\text{M}$ bupranolol, a nonspecific β -adrenoceptor blocker, or 100 nM SR59230a, a specific β_3 -adrenoceptor antagonist. In addition, BRL37344 increased whole-cell currents over a range of membrane potentials, and this effect was reversed by 100 nM IbTX. *In vitro* isometric tension studies demonstrated that BRL37344 exerted a significant concentration-dependent relaxant effect on human myometrial tissue ($P < 0.05$), and preincubation of these strips with IbTX attenuated this effect on both spontaneous and oxytocin-induced contractions (44.44 and 57.84% at 10^{-5} M , respectively).

Conclusions: These findings outline that activation of the BK_{Ca} channel may explain the potent uterorelaxant effect of β_3 -adrenoceptor agonists. (*J Clin Endocrinol Metab* 90: 5786–5796, 2005)

PRETERM OR PREMATURE labor is a major cause of disease and mortality in infancy (1, 2) and constitutes an immense cost to healthcare resources (1–3). The aim of successful pharmacological intervention is to stop uterine contractions or maintain uterine quiescence (tocolysis) to prolong gestation. The main tocolytic therapies available to date include the β -adrenergic agents, oxytocin antagonists, calcium channel blockers, magnesium sulfate, and antiprostaglandin agents (1, 2). Current techniques for the diagnosis and treatment of preterm labor are poorly effective (4, 5), largely due to a poor understanding of myometrial smooth muscle physiology and pharmacology.

The β_2 -adrenoceptor agonists are the most commonly used tocolytic agents to date, but their benefits are limited and they are associated with significant adverse cardiovascular effects (6–8). The β_3 -adrenoceptor has many func-

tions in different human tissues and has been intimately linked to smooth muscle relaxation in gastrointestinal tract (9), urinary tract (10), respiratory tract (11), vascular smooth muscle (12), and human myometrium (6, 13, 14). The selective β_3 -adrenoceptor agonist, BRL37344, exerts a potent relaxant effect on human uterine contractions *in vitro* that is of equal potency to the relaxant effect of the β_2 -adrenoceptor agonist ritrodine (6, 13, 14). This relaxant effect appears to be mediated solely through the β_3 -adrenoceptor without obvious effects at the β_1 - or β_2 -adrenoceptors (6). BRL37344 exerts a significantly less potent vasodilatory effect on human umbilical arterial smooth muscle *in vitro* than its counterpart β_2 -adrenoceptor agonist, ritrodine (6). This possibility, that β_3 -adrenoceptor activation may relax uterine contractions without the adverse systemic effects of widespread vasodilatation, is an important principle for clinical development of novel tocolytic compounds.

The exact mechanism by which BRL37344 inhibits contractions in the uterus has yet to be elucidated. β_3 -Adrenoceptors have recently been functionally linked to K⁺ channels in the mammalian heart (15). K⁺ channels are the largest category of ion channels in the cell and hence are the

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Abbreviations: IbTX, iberiotoxin; NP_o, open probability; PSS, physiological saline solution.

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main contributors in the regulation of membrane potential and cell excitability in smooth muscle cells including the myometrium (16–20). The BK_{Ca} channel is a major K⁺ channel type in both nonpregnant and pregnant human myometrium (16, 19, 21) and is essential for many key physiological processes including control of smooth muscle contractions during uterine quiescence and human labor (17, 19, 22). The role of BK_{Ca} channels in the myometrium, and their activation by endogenous agents, is a recognized inhibitory mechanism in the maintenance of uterine quiescence (23–26). Therefore, the aim of our study was to investigate a possible functional coupling between BK_{Ca} channel activity and β_3 -adrenoreceptor-mediated inhibition of uterine contractions.

Materials and Methods

Tissue collection

Biopsies of human myometrium were obtained from women undergoing elective cesarean section in the third trimester of pregnancy, in the Department of Obstetrics and Gynecology, University College Hospital (Galway, Ireland). The biopsies were excised from the midline portion of the upper lip of the incision in the lower uterine segment. Women who had received exogenous prostaglandins, oxytocin, or corticosteroids were excluded from the study. Recruitment was by written informed consent. Ethical Committee approval for the tissue collection was obtained from the Research Ethics Committee, University College Hospital, Galway. For electrophysiological studies tissue samples were immediately placed in sterile Ham's F-12 medium [(Sigma-Aldrich, Dublin, Ireland) supplemented with 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Sigma-Aldrich)]. Tissue samples for isometric recordings were placed in fresh Krebs-Henseleit physiologic saline solution (PSS) of the following composition: 4.7 mM KCl, 118 mM NaCl; 1.2 mM MgSO₄, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose (Sigma-Aldrich). Tissue was stored at 4 C and used within 6 h of collection.

Tissue dispersion / primary human myometrial cells

The preparation of single myometrial cells for electrophysiological recordings was performed using methodology previously described (26). Freshly isolated myometrial cells were obtained by enzymatic digestion of finely minced myometrium with 2 mg ml⁻¹ collagenase (type IA, 300–400 U mg⁻¹) (Sigma-Aldrich) in Hanks' buffered salt solution. The incubation with enzyme was performed at 37 C for 2 h followed by centrifugation (180 \times g) in 50% Percoll (Sigma-Aldrich) for 10 min. The cell layer was removed, washed, and spun in physiological solution to remove excess red blood cells. The cell suspension was then triturated and filtered through an 80- μ m nylon mesh filter. Single cells were placed in a recording chamber (Warner Instrument Corporation, Hamden, CT) and electrophysiological experiments were begun immediately. Morphologically, freshly dissociated uterine myocytes were characterized by a long, slender fusiform shape. All myocytes used for this work were relaxed and adhered to the bottom of the recording chamber with no additional substrate.

Electrophysiological recordings of the BK_{Ca} channel

Single-channel recordings using the cell-attached configuration and whole-cell recordings using the perforated patch configuration of the patch clamp technique were performed. Several drops of cell suspension were placed in the recording chamber containing a solution of the following composition: 140 mM KCl, 10 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES, and 30 mM glucose (pH 7.4, 22–25 C). Single K⁺ channel currents were measured in cell-attached patches by filling the patch pipette (2–5 M Ω) with Ringer solution composed of: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 10 mM HEPES, and making a gigaohm seal on a single myocyte. Voltage across the patch was controlled by setting the cellular membrane potential to 0 mV using a high-[K⁺] extracellular solution. Average channel activity in patches was measured as mean open probability (NP_o). The effects of BRL37344 (50 and 100 μ M) on

channel activity were measured by recording approximately 10 sec of continuous recording at +40 mV before and 15 min after drug treatment. In a separate set of experiments, cells were preincubated with 1 μ M bupranolol, a β -adrenoreceptor blocker, for 15 min before cell-attached recordings were taken, and the effect of 50 μ M BRL37344, in the presence of bupranolol, was determined. Subsequently, pretreatment of the cells with the potent selective β_3 -adrenoreceptor antagonist for 15 min, before cell-attached recordings were taken, followed by the effect of 50 μ M BRL37344, was also investigated. Currents were filtered at 1 kHz and digitized at 10 kHz. In all experiments voltage-clamp and voltage-pulse generation were controlled with an Axopatch 200-A patch-clamp amplifier (Axon Instruments, Union City, CA), and data were acquired and analyzed with pClamp 8. For statistical purposes, and to ensure accuracy, NP_o was calculated for single BK_{Ca} channels at a depolarized potential (+40 mV) where channel openings can be clearly differentiated from other channel species. BK_{Ca} channels are easily visualized at +40 mV because there is virtually no "contamination" with other channel species (27). In addition, at such depolarized voltages, there is no other ion channel expressed in these cells with an amplitude of 6–10 pA, making statistical analysis of BK_{Ca} channel activity more accurate (27). Moreover, recording single-channel activity at more depolarized potentials helps compensate the artificial depression of activity otherwise encountered from recording channel activity at room temperature (22–25 C) (27).

For perforated patch experiments, the recording chamber contained a bath solution of the following composition: 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, and 30 mM glucose (pH 7.4; 22–25 C). To measure potassium currents, the tip of the patch pipette (1–5 M Ω) was filled with a solution containing: 60 mM K₂SO₄, 30 mM KCl, 5 mM MgCl₂, 5 mM CaCl₂, 5 mM HEPES, and 40 mM MgSO₄ (pH 7.4). Because divalent ions do not pass through pores in the perforated membrane, the high Ca²⁺ in the pipette solution does not enter the cell, thus obviating the need for artificial Ca²⁺ buffers. In contrast, the K⁺ channels were always exposed to physiological levels of Ca²⁺ (bath solution) in these whole-cell recording experiments and not the 5 mmol of the pipette solution. The remainder of the pipette was back-filled with the same solution to which 6 mg/ml amphotericin B (diluted by sonication from a 50 mg/ml stock in dimethylsulfoxide) was added. Generation of voltage clamp protocols and acquisition of data were carried out using pClamp software (version 8). Voltage-activated currents were filtered at 1 kHz and digitized at 10 KHz. Leakage currents were algorithmically subtracted using short duration, small amplitude negative prepulses.

Isometric tension recordings

Longitudinal myometrial strips (measuring $\sim 2 \times 2 \times 10$ mm) were dissected free from uterine decidua and serosa and mounted isometrically in an organ tissue bath under 2 g tension for recordings, as previously described (6, 26). The tissue baths contained 20 ml Krebs-Henseleit PSS maintained at 37 C, pH 7.4, and were gassed continuously with a mixture of 95% oxygen/5% carbon dioxide. During a period of equilibration of 1 h, the Krebs-Henseleit PSS in the tissue baths was changed every 15 min. The experiments evaluating the effects of BRL37344 and BK_{Ca} channel blockade on spontaneous myometrial contractility (group S), were designed into four subgroups as follows: group S1, spontaneous alone; group S2, spontaneous + BRL37344; group S3, spontaneous + ibuprofen (IbTX); and finally, group S4, spontaneous + IbTX + BRL37344. To evaluate the β_3 -adrenoreceptor agonists effect on oxytocin-induced myometrial contractions (group O) the following subgroups were investigated: group O1, oxytocin alone; group O2, oxytocin + BRL37344; group O3, oxytocin + IbTX; and finally, group O4, oxytocin + IbTX + BRL37344. After equilibration, all group O myometrial strips were incubated with 0.5 nM oxytocin to elicit regular rhythmic contractions. After 30 min, strips from groups S3, O3, S4, and O4 were exposed to IbTX at a bath concentration of 100 nM for a further 30 min. During this 30-min incubation with IbTX, strips from groups S1 and S2 were exposed to Krebs-Henseleit PSS only, whereas strips from groups O1 and O2 were exposed to Krebs-Henseleit PSS containing 0.5 nM oxytocin, previously added. Contractile activity was measured for a 20-min period at which time bath addition of BRL37344 to group S2, O2, S4, and O4 strips took place. BRL37344 was added to the tissue bath in a cumulative manner at bath concentrations 1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M (*i.e.*

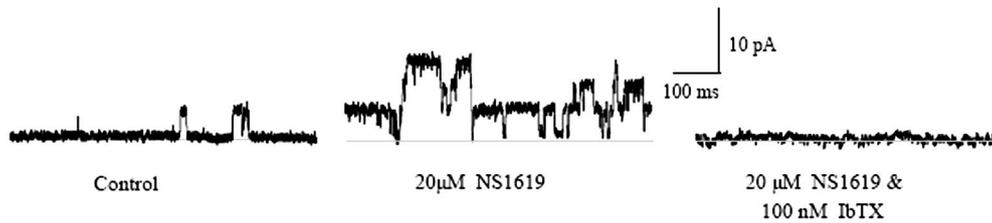


FIG. 1. BK_{Ca} channels are expressed in uterine smooth muscle cells. Representative recordings from the same membrane patch (+40 mV) in the cell-attached configuration before and after the addition of 20 μ M NS1619 and subsequent addition of 100 nM IbTX. IbTX reversed NS1619-stimulated channel activity.

10^{-9} to 10^{-5} M) at 20-min intervals, and the resultant contractile activity was measured for each period and expressed as a percentage of the integral obtained in the 20-min period before any BRL37344 addition (*i.e.* percentage of contractility adjusted for time for each experimental period). The inhibitory effect of BRL37344 was corrected for the reduction in the contractile activity observed in the controls, and the effects of BRL37344 were interpreted as the final additional relaxant effect. This was achieved by subtracting the reduction observed in the control strips from that observed with BRL37344. Measurement of contractile activity was performed by calculation of the integral of the selected area with the PowerLab (AD Instruments, Hastings, UK) hardware unit and Chart version 3.6 software (AD Instruments).

Drugs and solutions

BRL37344 was obtained from Sigma-Aldrich. A stock solution (10^{-2} M) was prepared using deionized water. Serial dilutions were prepared in deionized water on the day of experimentation and were maintained at room temperature for the duration of the experiment. A stock solution of oxytocin (1 mM; Sigma-Aldrich) was made in saline. Serial dilutions were made in deionized water on the day of experimentation and were maintained on ice for the duration of the experiment. A stock solution of IbTX (Sigma-Aldrich) (1×10^{-5} M) was made in saline. Bupranolol was obtained from SIFA Chemicals (Shannon, Ireland). A stock solution (10^{-2} M) was made in deionized water. SR59230a was obtained from Sigma-Aldrich. A stock solution of 20 mM was dissolved in dimethyl-

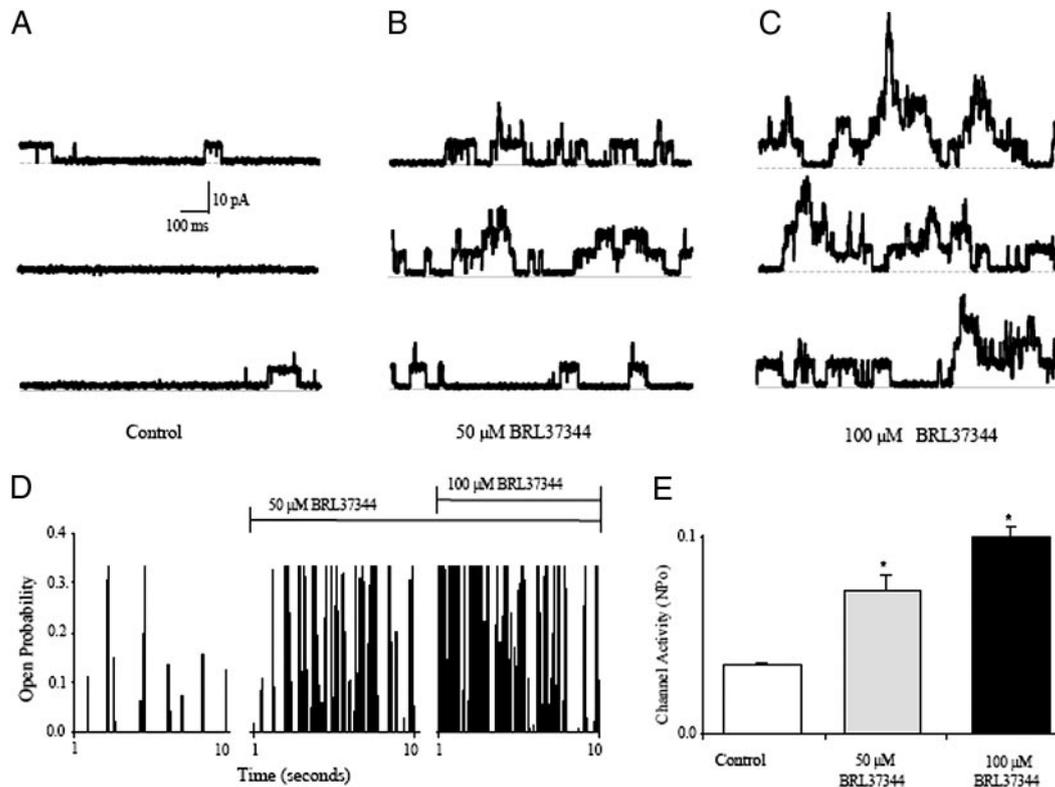


FIG. 2. BRL37344 enhances BK_{Ca} channel activity in human myometrial smooth muscle cells. A, Representative continuous recordings were recorded from the same cell-attached patch (+40 mV) before (control) and 15 min after exposure to 50 μ M (B) and 100 μ M (C) BRL37344. A continuous record in each case was cut into the three presented cases. Channel openings are upward deflections from the baseline (closed state, *dashed line*). D, BRL37344 opens BK_{Ca} channel activity in human myometrial smooth muscle cells in a concentration-dependent manner. Activity plot of BK_{Ca} channel open probability in a single cell-attached patch before and 15 min after exposure to cumulative doses of 50 and 100 μ M BRL37344. *Vertical bars* are channel activity during a 100-msec test pulse to +40 mV. Recording time under each condition was 10 sec, as indicated on the time axis. Breaks in the time axis represent drug incubation periods when channel activity was not recorded. Period of drug exposure is indicated by *horizontal lines above the histogram*. E, Each *bar* represents the average channel open probability obtained from cell-attached patches (+40 mV) before and 15 min after additions of 50 and 100 μ M BRL37344, respectively. *, Significant increase in channel activity compared with control ($n = 6$; $P < 0.001$).

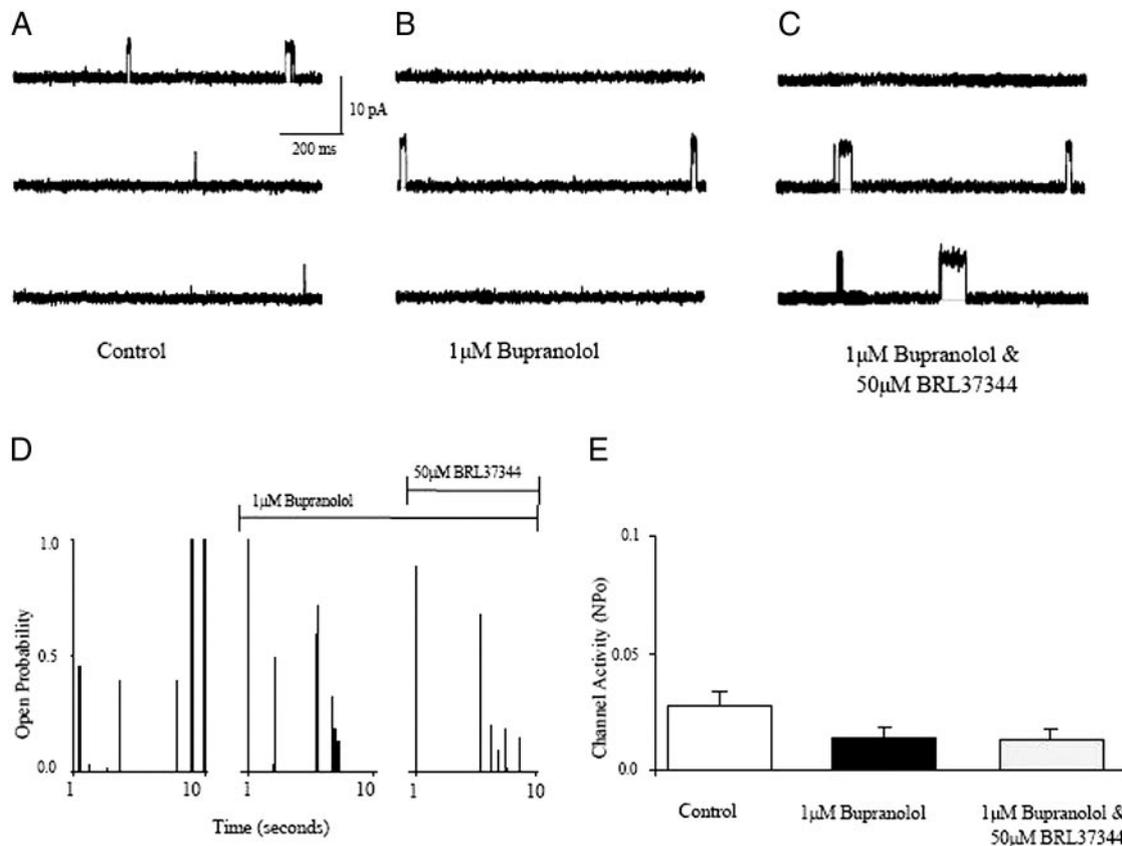


FIG. 3. Stimulatory effect of BRL37344 on BK_{Ca} channel activity involves β -adrenoreceptors. Preincubation of uterine smooth muscle cells with bupranolol, a β -adrenergic blocker, inhibits the stimulatory effect of BRL37344 on BK_{Ca} channel activity. A, Representative continuous recordings were recorded from the same cell-attached patch (+40 mV) before (control) and 15 min after exposure to 1 μ M bupranolol (B) and finally to 1 μ M bupranolol and 50 μ M BRL37344 (C). A continuous record in each case was cut into the three presented cases. D, Channel activity recorded before (control) and 15 min after application of 1 μ M bupranolol (+40 mV), and subsequent addition of 50 μ M BRL37344 did not enhance BK_{Ca} channel activity. E, Each bar represents the average channel open probability obtained from cell-attached patches (+40 mV) before and after additions of 1 μ M bupranolol and 1 μ M bupranolol and 50 μ M BRL37344, respectively.

sulfoxide and further diluted in distilled water. Fresh Krebs-Henseleit PSS was prepared daily. All other chemicals were obtained from Sigma-Aldrich.

Statistical analysis

Average channel activity (expressed as number of channels \times single-channel open probability, NP_o) in patches with multiple BK_{Ca} channels was determined by the following equation: NP_o = $\sum_{j=1}^n t_j J / T$, where P_o is the single-channel open-state probability, T is the duration of the recording, t_j is the duration of j = 1, 2, ... n channel openings, J is the number of channels open for duration t_j, and N is the maximal number of simultaneous channel openings observed when P_o was high. NP_o calculations were based on approximately 10 sec of continuous recording during periods of stable channel activity. For single-channel activity data, NP_o values are expressed as mean \pm SEM. Comparisons between groups were made by one-way ANOVA, with a *post hoc* Tukey honestly significant difference test to determine significant differences among data groups.

For isometric recordings, multiple comparisons of measured integrals of contractility were performed using two-way ANOVA, followed by *post hoc* Tukey test. The statistical packages SPSS Version 11.0 (SPSS, Inc., Chicago, IL) and Jandel SigmaStat 2.0 (SPSS, Inc.) were used for statistical calculations. *P* < 0.05 was accepted as statistically significant.

Results

Myometrial tissue samples

Myometrial biopsies were obtained from 37 women. The reasons for cesarean section included breech presentation (n = 12), previous cesarean section (n = 16), and maternal medical condition (n = 9). The maternal medical conditions included pelvic disproportion, lower back disease, placenta previa, and large-for-dates fetus. All cesarean sections were performed before the onset of labor under regional anesthesia. The maternal demographic details were as follows: mean age, 34 yr (range, 21–46 yr); median period of gestation, 38 wk (range, 36–41 wk); and median parity, 1 (range, 0–4). The n numbers provided below refer to the patient number included for each experimental group. All of the 37 biopsies contributed to different components of the data presented.

BK_{Ca} channel identification

Single-channel recordings from freshly dispersed myocytes, using the cell-attached configuration of the patch clamp technique, revealed membrane electrical activity to be dominated by a prominent, large conductance [152 ± 19.30

pS ($n = 6$); physiological gradients of potassium] channel carrying outward potassium currents. Channel activity recorded, from a cell-attached patch, with an amplitude range of 6–10 pA at +40 mV, under control conditions revealed minimal gating events ($NP_o = 0.034 \pm 0.006$; $n = 3$). In contrast, exposure to NS1619, a specific BK_{Ca} channel opener, elicited potent channel activation at applied concentration of 20 μ M (0.103 ± 0.0196 ; $n = 3$). Furthermore, application of 100 nM IbTX, which exhibits selectivity for BK_{Ca} channels, reversed the NS1619 activation of this channel (Fig. 1). These properties, the conductance value, and IbTX- and NS1619-sensitivity are in accordance with the characteristics of the BK_{Ca} channels in smooth muscle myocytes described in the literature (28, 29). Therefore, we identified this protein as the high-conductance, NS1619-sensitive, IbTX-sensitive BK_{Ca} channel, which is reported to be the predominant K⁺ channel species in human myometrial smooth muscle (16–19, 21).

BK_{Ca} channel activity

A representative recording, demonstrating minimum BK_{Ca} channel activity under control conditions ($NP_o = 0.03$), is shown in Fig. 2A. Cumulative additions of BRL37344 to the extracellular bathing solution resulted in a potent activation of BK_{Ca} channel activity in a concentration-dependent fashion, as illustrated in Fig. 2, B and C. BK_{Ca} channel activity was increased after the addition of 50 ($NP_o = 0.08$) (Fig. 2, B and D) and 100 μ M BRL37344 ($NP_o = 0.10$) (Fig. 2, C and D). The average NP_o (Fig. 2E) before and after addition of BRL37344 was as follows: control, 0.03 ± 0.004 ($n = 6$); 50 μ M BRL37344,

0.07 ± 0.005 ($n = 6$; $P < 0.001$); and 100 μ M BRL37344, 0.10 ± 0.006 ($n = 6$; $P < 0.001$). BRL37344 activated the BK_{Ca} channel with a maximal activation effect observed at the highest concentration of 100 μ M BRL37344, with a statistically significant effect also achieved at the lower concentration investigated, 50 μ M BRL37344. Open state channel activity was stimulated on average 2- and 3-fold by 50 and 100 μ M BRL37344, respectively. In general, there was a 5- to 10-min latency period before observation of BRL37344-stimulated channel activity, and this effect appeared to be maximal within 15–20 min. In all patches, this activity persisted until either seal integrity was lost, or the experiment was terminated.

A further set of experiments on cell-attached patches indicated that the stimulatory effect observed with BRL37344 was mediated via the β_3 -adrenoceptor. Pretreatment of uterine myocytes with bupranolol, a nonselective β_1 -, β_2 -, and β_3 -adrenoceptor antagonist, and the more potent specific β_3 -adrenoceptor antagonist, SR59230a, respectively, completely blocked the stimulatory effect of BRL37344 on BK_{Ca} channel activity. The typical response to BRL37344 in the presence of either bupranolol or SR59230a is illustrated by the traces in Fig. 3, A–D, and Fig. 4, A–D, respectively. The average effects are summarized in Figs. 3E and 4E, respectively. BK_{Ca} channel activity remained unchanged after 15 min exposure to bupranolol or SR59230a, when compared with control channel activity. However, both antagonists completely prevented the stimulatory action of the BRL37344-BK_{Ca} channel response to subsequent addition of

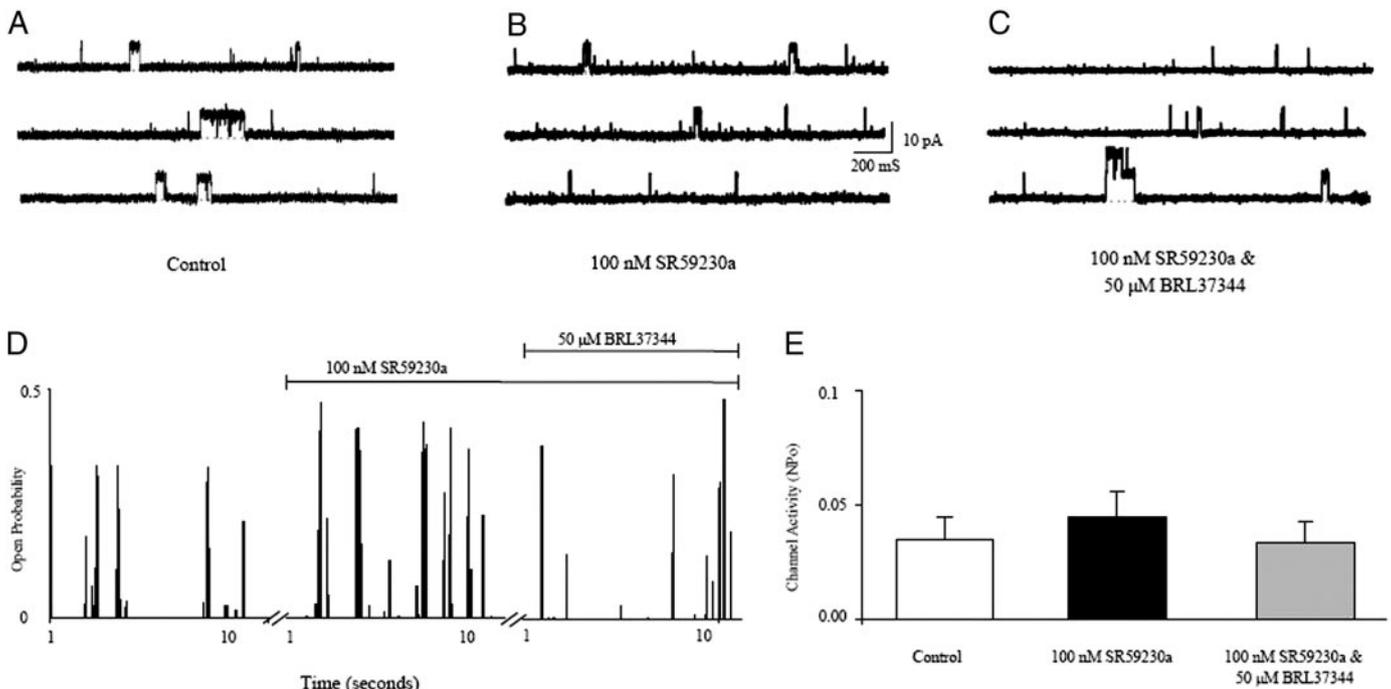


FIG. 4. Stimulatory effect of BRL37344 on BK_{Ca} channel activity involves β_3 -adrenoreceptors. Preincubation of uterine smooth muscle cells with SR59230a, a specific β_3 -adrenergic antagonist, inhibits the stimulatory effect of BRL37344 on BK_{Ca} channel activity. A, Representative continuous recordings were recorded from the same cell-attached patch (+40 mV) before (control) and 15 min after exposure to 100 nM SR59230a (B) and finally to 100 nM SR59230a and 50 μ M BRL37344 (C). A continuous record in each case was cut into the three presented cases. D, Channel activity recorded before (control) and 15 min after application of 100 nM SR59230a (+40 mV), and subsequent addition of 50 μ M BRL37344 did not enhance BK_{Ca} channel activity. E, Each bar represents the average channel open probability obtained from cell-attached patches (+40 mV) before and after additions of 100 nM SR59230a and 100 nM SR59230a and 50 μ M BRL37344, respectively.

50 μM BRL37344. The average NP_o before and after addition of bupranolol and further application of BRL37344 was as follows: control, 0.028 ± 0.006 ($n = 3$); 1 μM bupranolol, 0.014 ± 0.006 ($n = 3$; $P = 0.184$); and 50 μM BRL37344, 0.013 ± 0.007 ($n = 3$; $P = 0.156$; Fig. 3, D and E). The average NP_o before and after addition of SR59230a and further application of BRL37344 was as follows: control, 0.035 ± 0.01 ($n = 5$); 100 nM SR59230a, 0.045 ± 0.011 ($n = 5$; $P = 0.776$); and 50 μM BRL37344, 0.034 ± 0.009 ($n = 5$; $P = 0.996$; Fig. 4, D and E).

Whole-cell studies from single human uterine myocytes were performed to further characterize the effects of BRL37344 on ionic currents. As illustrated in Fig. 1, uterine myocytes exhibited prominent outward currents that increased with membrane depolarization. Application of 50 μM BRL37344 increased these steady-state outward currents at all positive voltages (Fig. 5A). A complete current-voltage relationship illustrating the stimulatory effect of BRL37344 is presented in Fig. 5B ($n = 6$). This effect of BRL37344 was observed at all voltages where outward current was elicited.

The effect of BRL37344 was reversed by 100 nM IbTX ($n = 5$). A summary of the stimulatory effect of 50 μM BRL37344 followed by application of IbTX is presented in Fig. 5C. These whole-cell studies clearly demonstrate that BRL37344 stimulates outward current in human uterine smooth muscle cells, and blockage of these outward currents with IbTX strongly suggests the involvement of the BK_{Ca} channels.

In vitro contractility

To further characterize the potential involvement of BK_{Ca} channel function in the response of human myometrium to BRL37344, tension studies were performed. BRL37344 exerted a cumulative inhibitory effect on both spontaneous ($n = 5$) and oxytocin ($n = 5$)-induced contractility in isolated pregnant human myometrium. Fig. 6, A and B, demonstrate, respectively, a representative recording of spontaneous myometrial contractility and the relaxant effects of cumulative additions of BRL37344 (1 nM, 10 nM, 100 nM, 1 μM , and 10

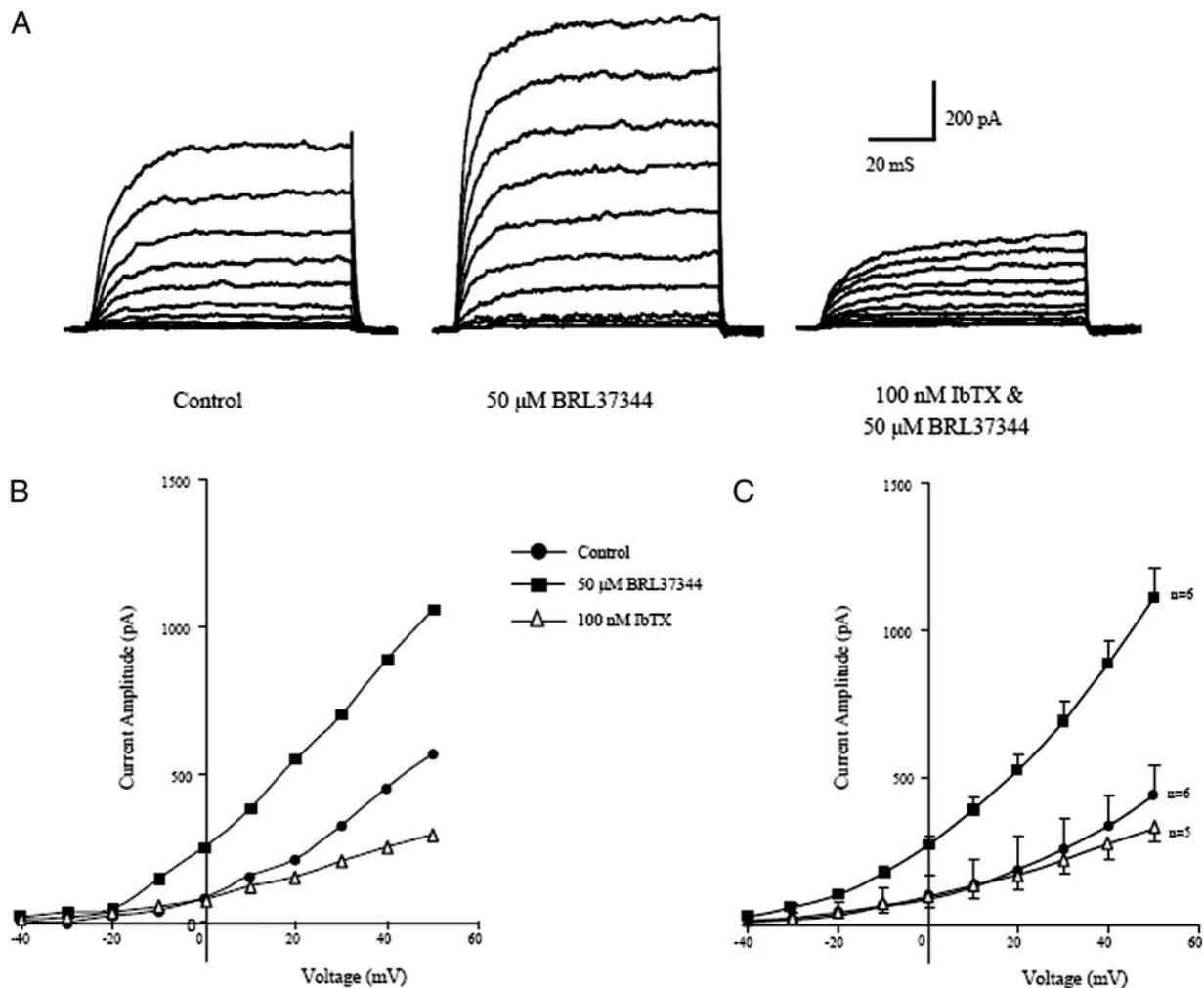


FIG. 5. BRL37344 increases whole-cell currents in human uterine smooth muscle myocytes. A, Perforated patch recordings from the same myocyte before and 15 min after exposure to 50 μM BRL37344, and then 5–10 min after cumulative addition of 100 nM IbTX. Tracings were taken from a range of potentials (–40 to +50 mV), with a holding potential of –60 mV. B, The complete current (pA)-voltage (mV) relationship for steady-state outward current from the same cell as in A. Treatment conditions are the same as described for control, 50 μM BRL37344, and BRL37344 and 100 nM IbTX. C, Average current-voltage relationship for uterine myocytes before and after 50 μM BRL37344, and then cumulative addition of 100 nM IbTX. Each point represents the mean number of cells \pm SEM.

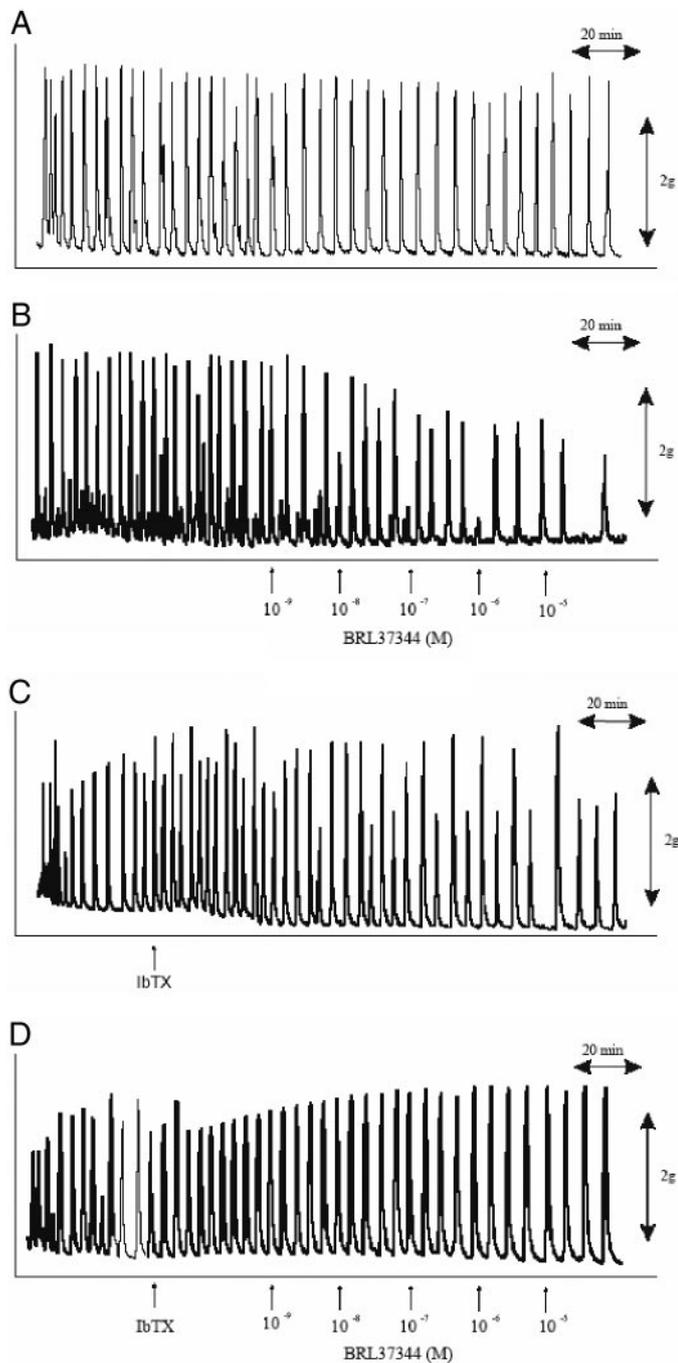


FIG. 6. Effects of BRL37344 on spontaneous myometrial contractions. A, Representative recordings of spontaneous contractions of pregnant human myometrium under control conditions. B, The effects of cumulative additions of BRL37344 (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) at 20-min intervals. C and D, The uterorelaxant effect was significantly attenuated by preincubation with the BK_{Ca} channel antagonist IbTX.

μ M). ANOVA revealed a significant effect on contractility of both addition of BRL37344, and also of increasing BRL37344 concentrations (group S1 vs. group S2; $n = 5$; $P < 0.05$). *Post hoc* analysis showed a statistically significant relaxant effect of BRL37344 at bath concentrations of 10^{-6} M ($n = 5$; $P = 0.016$) and 10^{-5} M ($n = 5$; $P = 0.007$) when compared with the

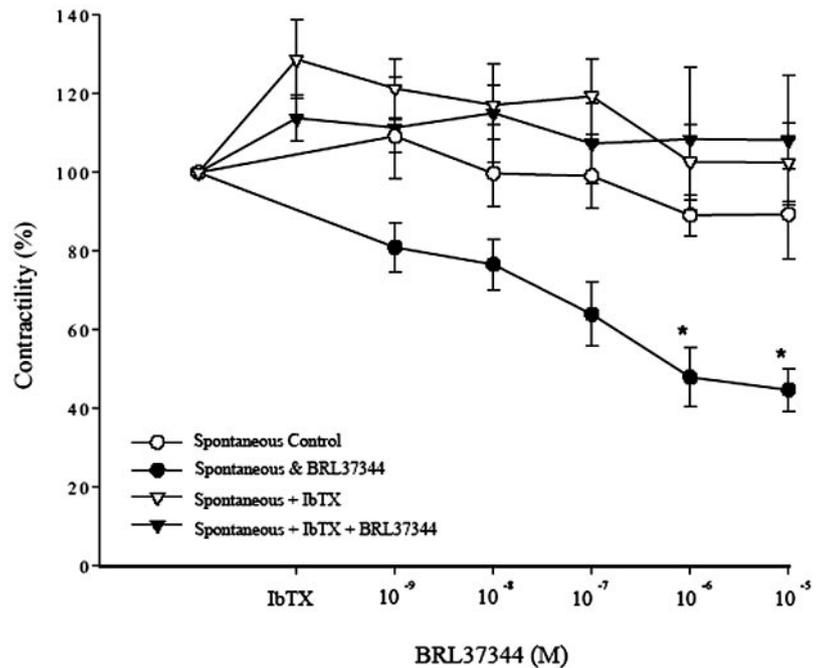
respective control values measured from spontaneously contracting strips.

Incubation of the strips with IbTX, resulted in a nonsignificant increase in the integrals of contractile activity measured as seen in Fig. 6C, (group S1 vs. group S3; $n = 5$; $P > 0.05$). However, the presence of IbTX did significantly attenuate the relaxant effect by BRL37344. In Fig. 6D, a representative recording demonstrating the effects of preincubation with the BK_{Ca} channel antagonist IbTX on the uterorelaxant effects of BRL37344 is shown. Comparison of the inhibitory effects of BRL37344 on spontaneous contractions of myometrial strips, in the presence or absence of IbTX, revealed a significant difference across the groups (group S2 vs. group S4; $n = 5$; $P < 0.05$). *Post hoc* analysis revealed that preincubation with IbTX (100 nM) significantly attenuated the uterorelaxant effect of BRL37344, resulting in no significant difference when compared with control values (group S3 ($n = 5$) vs. group S4 ($n = 5$); $P > 0.05$) at all BRL37344 bath concentrations. In Fig. 7, the average effect ($n = 5$) of cumulative additions of BRL37344 on spontaneous contractions of myometrial strips, in the absence and presence of IbTX, are shown in graphical form alongside the integrals observed in control strips.

A similar trend was observed with contractions induced with oxytocin. Fig. 8A represents a typical recording of oxytocin elicited myometrial contractility, and in Fig. 8B, the effects of cumulative additions of BRL37344 (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) to the tissue bath are shown. ANOVA revealed a significant effect on contractility of both addition of BRL37344 and also of increasing BRL37344 concentrations (group O1 vs. group O2; $n = 5$; $P < 0.05$). The inhibitory effect of BRL37344 was corrected for the reduction in the contractile activity observed in the controls. *Post hoc* analysis showed a statistically significant relaxant effect of BRL37344 at bath concentrations of 10^{-5} M ($n = 5$; $P = 0.023$) when compared with respective control values measured from strips exposed to oxytocin alone.

Incubation of strips with IbTX, in addition to oxytocin, did not significantly alter the integrals of contractile activity measured, although an increasing trend in frequency was observed (group O1 vs. group O3; $n = 5$; $P > 0.05$; Fig. 8C). However, addition of IbTX, before bath addition of BRL37344, significantly attenuated the relaxant effect exerted by BRL37344. Fig. 8, C and D, show a representative recording demonstrating the effects of preincubation with the BK_{Ca} channel antagonist IbTX, and on the uterorelaxant effects of BRL37344. Comparison of the inhibitory effects of BRL37344 on oxytocin-induced contractility of myometrial strips, in the presence or absence of IbTX, revealed a significant difference across the groups (group O2 vs. group O4; $n = 5$; $P < 0.05$). *Post hoc* analysis revealed that preincubation with IbTX significantly attenuated the uterorelaxant effect of BRL37344, resulting in no significant difference when compared with control values (group O3 ($n = 5$) vs. group O4 ($n = 5$); $P > 0.05$) at all BRL37344 bath concentrations. In Fig. 9, the average effect ($n = 5$) of cumulative additions of BRL37344 on oxytocin-induced contractions of myometrial strips, in the absence and presence of IbTX, are shown in graphical form alongside the integrals observed in control strips.

FIG. 7. Dose-response curves for BRL37344 and spontaneous myometrial contractions. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of BRL37344 (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) at 20-min intervals on spontaneously contracting pregnant myometrium in the presence (\blacktriangle) and absence (\bullet) of IbTX. Control traces showing uninterrupted spontaneous contractions in the presence (\triangle) and absence (\circ) of IbTX in pregnant myometrium are shown for comparison. The symbols used represent the mean values within each group. Vertical error bars represent the SEM.



Discussion

Myometrial smooth muscle cells are richly endowed with BK_{Ca} channels (16, 30, 31). These channels are sensors of voltage and intracellular Ca²⁺, and are responsible for membrane repolarization that follows depolarization and the accompanying increase in cytosolic free Ca²⁺ during an action potential (32). Activation of K⁺ channels, including BK_{Ca} channels, in smooth muscle cells, results in hyperpolarization and muscle relaxation, whereas their inhibition or blockade induces depolarization and muscle contraction (19, 20, 33). Thus, myometrial excitability is closely linked to the membrane potential, and BK_{Ca} channels are important regulators of smooth muscle contractility (16). They are important targets for differential regulation by cAMP- and cGMP-dependent protein kinases (34–36) in pregnant and nonpregnant myometrial cells, respectively. β -Adrenergic agonists appear to act via a cascade of events that involves stimulatory G proteins, adenylate cyclase, cAMP, and finally, PKA activation, hence this study aimed to investigate a functional link between activation of BK_{Ca} channels and the uterorelaxant effect exerted by the β_3 -adrenoreceptor agonist, BRL37344.

The findings reported here clearly demonstrate functional coupling of β_3 -adrenoreceptors to BK_{Ca} channel activity from freshly isolated human myocytes in cell-attached single-channel recordings. BRL37344 significantly increased whole-cell (perforated patch) K⁺ currents and stimulated the activity of single BK_{Ca} channels in cell-attached patches dramatically. BRL37344 significantly increased the open state probability of these channels in a concentration-dependent manner. In general, BRL37344-stimulated channel activity appeared to be maximal within 15–20 min. Pretreatment of the cells with bupranolol, a nonspecific β -adrenoreceptor antagonist, completely blocked the stimulatory effect by BRL37344 on BK_{Ca} channel activity. These

results concur with data previously published that demonstrate that the BRL37344 elicited uterorelaxant effect is antagonized by bupranolol (6). This previous report outlined the relevant concentration range for use in this study.

Because involvement of the β_3 -adrenoreceptor is the central finding of this study, we then investigated the BRL37344 stimulatory effect on the BK_{Ca} channel using the more potent and selective β_3 -adrenoreceptor antagonist, SR59230a. Pretreatment of myometrial cells with this antagonist provided more convincing evidence for direct functional connection between the relaxatory effects of BRL37344 and activation of BK_{Ca} myometrial channels. The stimulatory action of BRL37344 was completely abolished by pretreating the cells with SR59230a.

In the functional studies carried out during our study, the uterorelaxant effect mediated by BRL37344 was evident on both spontaneously occurring and agonist-induced contractions in human myometrium, and was significantly attenuated by BK_{Ca} channel blockade. After preincubation with IbTX, the contractility measured after BRL37344 exposure was not significantly different to that of control strips. Taken together, these findings strongly suggest that BRL37344 activates the BK_{Ca} channels by virtue of its agonist action at β_3 -adrenoreceptors and that this mechanism explains, at least in part, the relaxant property of BRL37344 on human uterine smooth muscle.

During this study, we used a model of spontaneous and agonist-induced contractility. The advantage of investigating oxytocin-induced contractions is that, unlike spontaneous contractions *in vitro*, strips exposed to oxytocin generate regular rhythmic contractions that are reproducible over a period of hours and hence serve as a reliable control (37). Although spontaneous contractility may be less reliable for long duration experiments, this model provides the opportunity to study indigenous uterine contractions, in a model

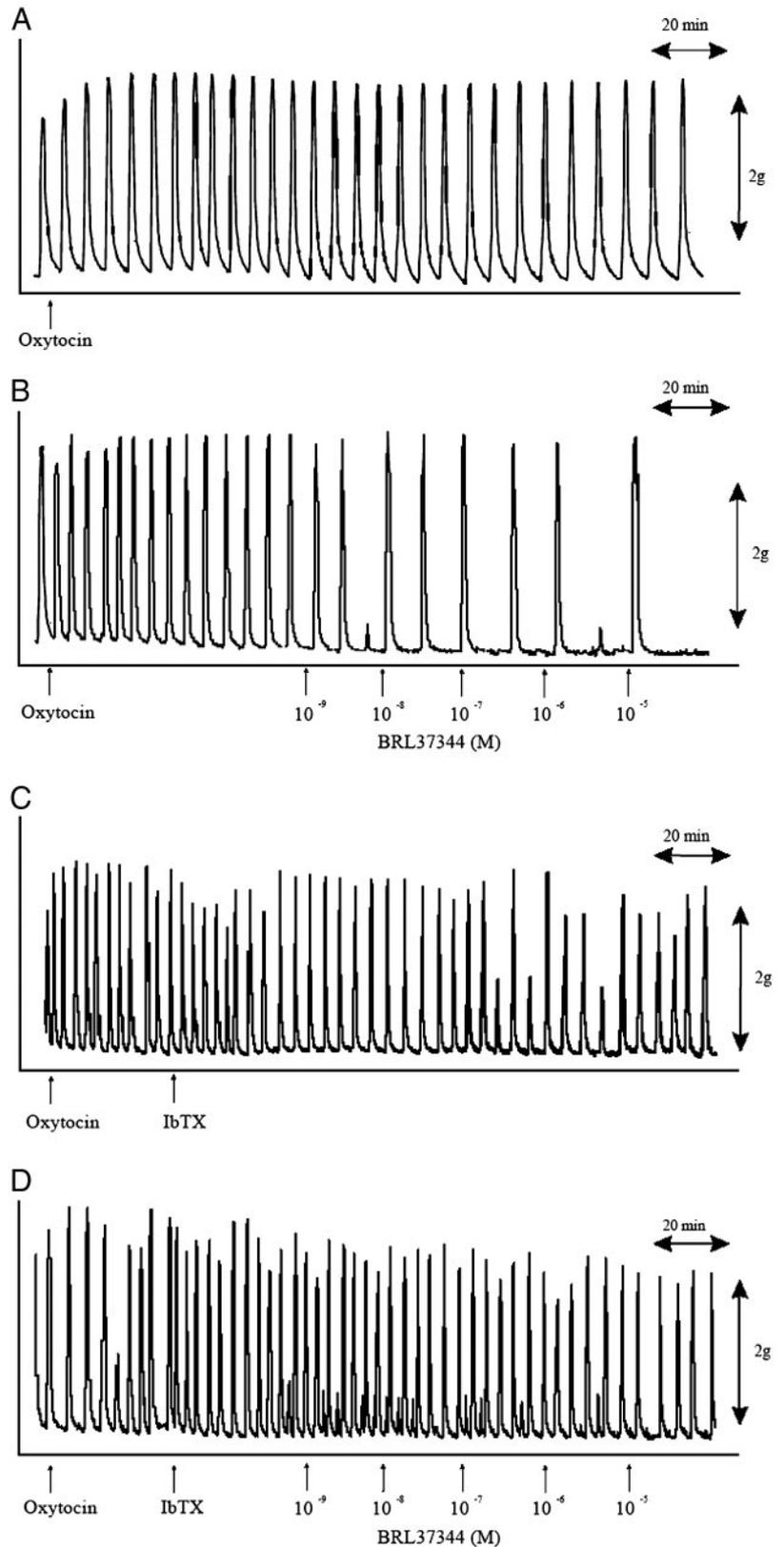
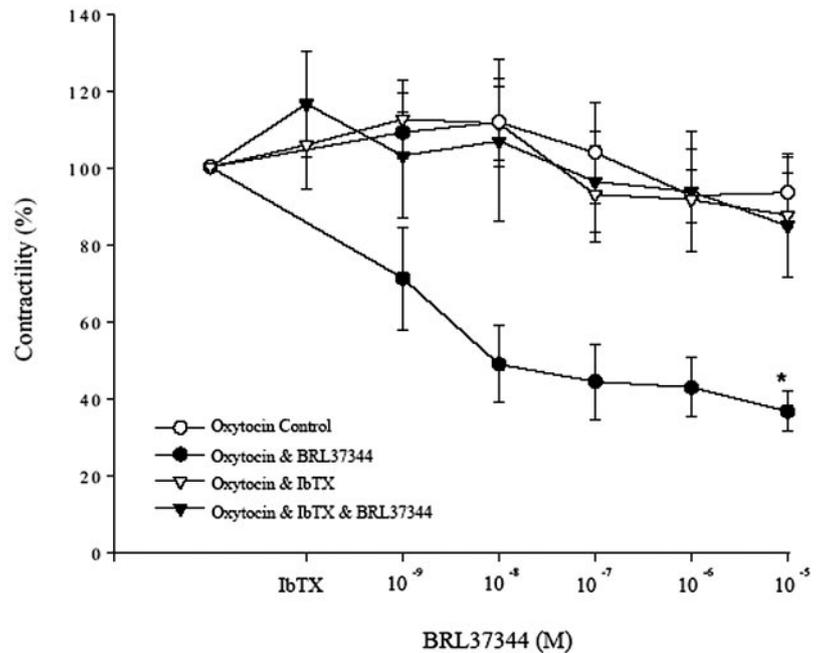


FIG. 8. Effects of BRL37344 on oxytocin-induced myometrial contractions. A, Representative recordings of oxytocin-induced contractions of pregnant human myometrium. B, The effects of cumulative additions of BRL37344 (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) at 20-min intervals. C and D, The uterorelaxant effect was significantly attenuated by preincubation with the BK_{Ca} channel antagonist IbTX.

that may be closest to spontaneous human labor, *i.e.* no agonist used. Finally our methodology of measuring integrals of tension for a set experimental time period incorporates all param-

eters of myometrial contractility, *i.e.* amplitude, frequency, and duration of contractions. This is regarded as the optimum approach by myometrial physiologists (37).

FIG. 9. Dose-response curves for BRL37344 and oxytocin-induced myometrial contractions. Graphical representation of the effects of cumulatively increasing tissue bath concentrations of BRL37344 (1 nM, 10 nM, 100 nM, 1 μ M, and 10 μ M) at 20-min intervals on oxytocin-induced contracting pregnant myometrium in the presence (\blacktriangle) and absence (\bullet) of IbTX. A control trace showing uninterrupted oxytocin-induced contractions in pregnant myometrium in the presence (\triangle) and absence (\circ) are shown for comparison. The symbols used represent the mean values within each group. Vertical error bars represent the SEM.



Our findings indicate that β_3 -adrenoreceptor modulation induces uterorelaxation, at least partially, via activation of BK_{Ca} channel activity. This strongly suggests β_3 -adrenoreceptor activation induced a soluble signal molecule to activate BK_{Ca} channels, possibly a signal transduction molecule. However, the effector mechanisms or signaling pathways involved in this process were not addressed in this study. This remains an exciting study requiring further evaluation. Secondly, the possibility that BRL37344 may have other mechanisms of action, was not evaluated in this study. One further limitation of this study was that the effects of BRL37344 on both BK_{Ca} channel activity and myometrial contractions were studied in myometrium excised from the upper portion of the lower uterine segment. Although there are no data indicating differential regional distribution of β_3 -adrenoreceptors in human uterine tissue, one cannot conclude that similar sensitivity to BRL37344 exists in myometrium from the uterine upper segment or fundus. However, there is reasonable evidence that the functional effects and contractile properties of isolated myometrium from the upper and lower segments of pregnant women are similar (38). In addition, there are obvious ethical constraints in obtaining biopsies from the upper segment of the human uterus at cesarean section.

In conclusion, this study reports, using single-channel and whole-cell electrophysiological recordings and tissue bath functional studies, that the β_3 -adrenoreceptor agonist, BRL37344, exerts its potent relaxant effect in human pregnancy, at least partially, via BK_{Ca} channel activation. These novel data further highlight the physiological role of the β_3 -adrenoreceptors in human myometrium and their possible endogenous role in the maintenance of uterine quiescence during pregnancy. Finally, these findings also support the possibility that BRL37344, or pharmacologic modulation of the β_3 -adrenoreceptor, may confer therapeutic benefit in preterm labor management by a mechanism that appears to be mediated by BK_{Ca} channel activity.

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