Stimulation of β3-adrenoceptors relieves rat urinary bladder smooth muscle via activation of the large-conductance Ca\(^{2+}\)-activated K\(^+\) channels

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Hristov KL, Cui X, Brown SM, Liu L, Kellett WF, Petkov GV. Stimulation of β3-adrenoceptors relaxes rat urinary bladder smooth muscle via activation of the large-conductance Ca\(^{2+}\)-activated K\(^+\) channels. Am J Physiol Cell Physiol 295: C1344–C1353, 2008. First published September 17, 2008; doi:10.1152/ajpcell.00001.2008.—We investigated the role of large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels in β3-adrenoceptor (β3-AR)-induced relaxation in rat urinary bladder smooth muscle (UBSM). BRL 37344, a specific β3-AR agonist, inhibits spontaneous contractions of isolated UBSM strips. SR59230A, a specific β3-AR antagonist, and H89, a PKA inhibitor, reduced the inhibitory effect of BRL 37344. Iberiotoxin, a specific BK channel inhibitor, shifts the BRL 37344 concentration response curves for contraction amplitude, net muscle force, and tone to the right. Freshly dispersed UBSM cells and the perforated mode of the patch-clamp technique were used to determine further the role of BK channel stimulation by BRL 37344 on BK channel activity. BRL 37344 increased spontaneous, transient, outward BK current (STOC), which can modulate the UBSM resting membrane potential (20, 39, 42). In contrast, the control of so-called “Ca\(^{2+}\) sparks” caused by Ca\(^{2+}\) release from the ryanodine receptors (RyRs) of the sarcoplasmic reticulum (SR), adjacent to the plasma membrane (20, 42). In both animal and human UBSM, the Ca\(^{2+}\) spark’s activation of the BK channel is manifested in the form of spontaneous, transient, outward BK currents (STOCs), which can modulate the UBSM resting membrane potential (20, 39, 42).

Norepinephrine, released from sympathetic nerves, relaxes UBSM via stimulation of β-adrenergic receptors (β-ARs), which is the most plausible major mechanism that sustains bladder relaxation during filling (1, 15, 48). In guinea pig UBSM, isoproterenol, a non-selective β-AR agonist, inhibits spontaneous action potentials and hyperpolarizes the membrane to mediate UBSM relaxation. Recent studies show that agonist-induced stimulation of β-ARs causes activation of different K\(^+\) channels leading to membrane hyperpolarization and relaxation in various smooth muscle tissues (11, 45). In guinea pig UBSM, isoproterenol, a non-selective β-AR agonist, inhibits spontaneous action potentials and hyperpolarizes the membrane through PKA activation (35). In addition, relaxation of guinea pig UBSM in response to isoproterenol is mediated mainly by activation of the BK channels (27, 42). Our previous studies indicate that isoproterenol-induced BK channel activation in UBSM involves increased Ca\(^{2+}\) entry through L-type Cav channels and Ca\(^{2+}\) spark activity (42). The latter effect appears to be mediated by PKA-induced phosphorylation of phospholamban, which, when in a phosphorylated state, activates the SR Ca\(^{2+}\)-pump, elevates SR Ca\(^{2+}\) load and thus RyRs and Ca\(^{2+}\) spark activity.

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In rat and human UBSM, mRNA that codes for the three known β-ARs subtypes, β1-, β2-, and β3-ARs, has been detected (14, 24, 25, 34, 37, 43, 44). Increasing evidence suggests that the β-AR relaxation of UBSM is mediated mainly by β3-ARs (8, 15, 45, 48). However, the contribution of each of the three separate β-ARs to the BK channel activation in UBSM is unknown. A recent study on human myometrium reports that stimulation of β3-AR with 50–100 μM BRL 37344, a β3-AR-specific agonist, may cause activation of the BK channels and thus smooth muscle relaxation (10). In the urinary bladder, β3-AR stimulation may lead to BK channel activation, suggesting a functional link to facilitate UBSM relaxation.

To test this hypothesis, we employed functional studies on UBSM contractility and patch-clamp electrophysiology using BRL 37344 to stimulate the β3-ARs. We found that β3-AR-induced relaxation of UBSM is mediated by STOCs activation and membrane potential hyperpolarization. To further reveal the cellular mechanism of possible functional coupling between β3-ARs and the BK channels, we applied a variety of patch-clamp protocols and pharmacological tools to elucidate the different regulatory pathways at the level of BK channel Ca\(^{2+}\) signaling.

**METHODS**

**Animal studies and UBSM tissue harvesting.** All animal studies were carried out in accordance with guidelines of the Animal Welfare Act and the Association for Assessment and Accreditation of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of the University of South Carolina (Animal Use Protocol No. 1482). In the present study we used 77 adult Sprague-Dawley rats (54 males and 23 females), 3–5 mo old, with an average weight of 321.9 ± 7.6 g. Rats were euthanized with CO\(_2\), followed by exsanguination. The entire urinary bladder was removed and placed in ice-cold nominally Ca\(^{2+}\)-free dissection solution (see Solutions and Drugs for composition). The bladder was then pinned to the bottom of a Sylgard-coated petri dish containing dissection solution. After the surrounding adipose and connective tissue were removed, the bladder was cut open with a longitudinal incision beginning from the urethral orifice. The entire mucosal layer of the bladder, including the urothelium, was removed, and the bladder was pinned with the serosal side up for dissection.

**Contractility studies.** Up to eight small UBSM strips (1–2 mm wide and 5–6 mm long) were excised free from each bladder and transferred to a small petri dish containing dissection solution. Individual strips were placed in thermostatically controlled (37°C) tissue baths (5-ml volume). One end of the strip was attached to a stationary metal hook, whereas the other end was connected to a force-displacement transducer for isometric tension recording. The force generation by the muscle strips was recorded using a MyoMed myograph and MyoViewer data acquisition system (both from MedAssociates, St. Albans, VT). The UBSM strips were suspended under initial 10 mN tension. These procedures were carried out in a nominally Ca\(^{2+}\)-free dissection solution. Five minutes later, the bath solution was replaced with a Ca\(^{2+}\)-containing physiological saline solution (see Solutions and Drugs for composition). An equilibration period of at least 45–60 min followed, during which the bath solution was changed every 15 min. Most of the strips exhibited spontaneous phasic contractions during the equilibration period. Only strips that had stable controls and contracted spontaneously for at least 30–45 min were used for the experiments.

**UBSM single cell isolation.** Small UBSM strips (1–2 mm wide and 5–7 mm long) were excised from the bladder wall. Several muscle strips were placed in a vial containing 2 ml of dissection solution supplemented with 1 mg/ml bovine serum albumin, 1 mg/ml papain (Worthington, Lakewood, NJ), and 1 mg/ml dithioerythritol and incubated for 30–32 min at 37°C. After that, the tissue strips were transferred to 2 ml of dissection solution containing 1 mg/ml BSA, 1 mg/ml collagenase (type II from Sigma), and 100 μM CaCl\(_2\) for 9–14 min at 37°C. After incubation, the digested tissue was washed several times in dissection solution and then was gently triturated to yield single smooth muscle cells. Several drops of the solution containing the dissociated cells were then placed in a recording chamber. Cells were allowed to adhere to the glass bottom of the chamber for about 20 min. Most cells were elongated and had a bright, shiny appearance when examined with a phase-contrast microscope. The average UBSM cell capacitance was 27.7 ± 1.2 pF (n = 71 cells). Cells were used for patch-clamp recordings within 3–4 h after isolation.

**Electrophysiological (patch-clamp) recordings.** The amphotericin-perforated, whole cell configuration of the patch-clamp technique was employed (16, 22). Whole cell currents were filtered using an eight-pole Bessel filter (model 900CT/98BL. Frequency Devices) and recorded using an Axopatch 200B amplifier, Digidata 1440A, and pClAMP software (Molecular Devices, Sunnyvale, CA). Patch-clamp pipettes were pulled from borosilicate glass (Sutter Instruments, Novato, CA) using a Narishige PP-830 vertical puller coated with sticky dental wax to reduce capacitance and polished with a Micro Forge MF-830 fire polisher (Narishige) to give a fine tip resistance of ~4–6 MΩ. STOCs were recorded while the UBSM cells were held at a holding potential (V\(_h\)) of −40 mV, a potential similar to the resting membrane potential of intact UBSM preparations (1, 41). To determine the mean amplitude and frequency of the transient BK currents, analysis was performed off-line using Clampfit of the pClamp software version 10.1. The threshold for the STOCs was set at 12 pA, which is more than three times the single-channel amplitude at −40 mV. STOCs were recorded over 10-min periods in the absence (control) and presence of BRL 37344. In a separate series of experiments, voltage-step protocols were used to elicit steady-state K\(^+\) outward current. UBSM cells were held at −70 mV and then depolarized from −40 mV to +80 mV at 20 mV steps of 200-ms duration. The steady-state K\(^+\) outward currents were recorded in the presence of ryanodine (30 μM) and thapsigargin (100 nM), which indirectly block STOCs. Nifedipine (1 μM) was also used to block the L-type Ca\(^{2+}\) channels. For the voltage-step protocols, 4–6 controls were recorded and the data were averaged. Only cells with stable control currents in response to depolarization steps within at least 15 min were used to study BRL 37344 effects. After BRL 37344 administration, voltage-step protocols were applied every 1–2 min for at least a 15-min period, and steady-state K\(^+\) outward currents were recorded. The average steady-state K\(^+\) current value during the last 50 ms of the 200-ms depolarization step was used to plot current-voltage relationships.

In another series of experiments, single BK channel activity was recorded from UBSM cells in whole cell mode as previously described (42). The large amplitude and low open probability (P\(_o\)) of the BK channel in UBSM cells permits the measurement of single BK channel currents with the use of the amphotericin-perforated, whole cell configuration of the patch-clamp technique when the cells' native environment and signal transduction pathways were preserved. To observe single BK channel currents, Ca\(^{2+}\) sparks, and hence STOCs were prevented by blocking RyRs and SR Ca\(^{2+}\)-pump with ryanodine (30 μM) and thapsigargin (100 nM), respectively. The L-type Ca\(^{2+}\) channels were inhibited with nifedipine (1 μM), and the cells were clamped at 0 mV, a potential at which L-type Ca\(^{2+}\) and voltage-gated K\(^+\) channels are largely inactivated (46). Under these conditions, single UBSM BK channels were identified by their characteristic large, single-channel conductance, voltage dependence, and sensitivity to IBTX (42). Single BK channel P\(_o\) was calculated from continuous recordings of 7- to 10-min intervals in the absence (control) and presence of BRL 37344. Because the total number of BK channels (N) for each individual cell is unknown, the cell NP, was normalized to
the cell capacitance (measured as \( N P_{\text{app}} \)). In some cells, at the end of the recordings, IBTX (200 nM) was applied to confirm that the recorded currents were through BK channels. UBSM cell membrane potential was recorded using the current-clamp mode of the patch-clamp technique. All patch-clamp experiments were carried out at room temperature (22-23°C).

**Solutions and drugs.** The nominally \( \text{Ca}^{2+} \)-free dissection solution had the following composition (in mM): 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 glucose, 10 HEPES, and 2 MgCl\(_2\), pH 7.3, adjusted with NaOH. The \( \text{Ca}^{2+} \)-containing physiological salt solution was prepared daily and contained (in mM): 119 NaCl, 4.7 KCl, 24 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), and 11 glucose, and was aerated with 95% O\(_2\)-5% CO\(_2\) to obtain pH 7.4. The extracellular (bath) solution used in the electrophysiological experiments contained (in mM): 134 NaCl, 6 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl\(_2\), 10 HEPES, and 0.05 EGTA, pH adjusted to 7.2 with NaOH and supplemented with freshly dissolved (every 1-2 h) 200 \( \mu \text{g/ml} \) amphotericin-B. Atropine, nifdefpine, and tetraethylammonium (TEA) were purchased from Acros Organics; BRL 37344, dithioerythritol, albumin, thapsigargin, and amphotericin-B were from Fisher Scientific.

**Data analysis and statistics.** UBSM contraction data were analyzed using MiniAnalysis (Synaptosoft). This software has allowed us to analyze the changes in all four major parameters of the UBSM phasic and tonic contractions (tone)-phasic contraction amplitude, phasic contractions frequency, net muscle force, and muscle tone. Data were further analyzed with GraphPad Prism (version 4) and presented using CorelDraw Graphic Suite X3 (Corel) software. Cumulative concentration response curves for BRL 37344 were obtained by adding increasing concentrations of the drugs directly to the tissue baths every 10 min. A 3-min period from the 7th to the 10th min after exposure to each drug concentration was taken as the analysis period. To compare the phasic contractions parameters, data were normalized to the spontaneous contractions and expressed as percentages. Net muscle force (muscle force integral) was determined by integrating muscle force (muscle force integral) was determined by integrating.

**RESULTS**

**Stimulation of \( \beta_3 \)-ARs with BRL 37344 leads to inhibition of UBSM spontaneous contractility: role of the BK channel and \( \beta_3 \)-adrenoceptors.** In isolated rat UBSM strips, stimulation of \( \beta_3 \)-ARs with BRL 37344 inhibits tonic contractions induced by membrane depolarization due to increased external K\(^+\) (8, 13, 28, 38). High external K\(^+\) (15–50 mM KCl) restrains the function of the K\(^+\) channels, including the BK channel, thus masking the native mechanism of \( \beta_3 \)-AR-induced relaxation of the bladder. In fact, the effects of \( \beta_3 \)-AR stimulation with BRL 37344 on UBSM spontaneous phasic and tonic contractions in a physiological external K\(^+\) solution have, to our knowledge, never been studied.

Here, we sought to investigate whether a pharmacological stimulation of \( \beta_3 \)-ARs with BRL 37344 leads to inhibition of the physiologically relevant spontaneous phasic and tonic contractions that normally decreased during the bladder-filling phase (for a review, see Ref. 1), and to further investigate the underlying mechanism. It is well known that UBSM contractions are modulated by neurotransmitters, released from autonomic nerves located in the bladder wall. To minimize any possible effects caused by neurotransmitter release, all experiments were performed in the presence of 1 \( \mu \text{M} \) tetrodotoxin, a neuronal Na\(^+\) channel blocker. Because BRL 37344 was also reported to have some antisauccinic effects in rats, we also applied 1 \( \mu \text{M} \) atropine, a non-selective muscarinic receptor antagonist.

In isolated rat UBSM strips, a selective stimulation of \( \beta_3 \)-ARs with BRL 37344 (100 nM-100 \( \mu \text{M} \)) caused a concentration-dependent decrease in spontaneous phasic contractions and muscle tone (Fig. 1). Figure 1 also shows the cumulative concentration response curves for BRL 37344 effects on the spontaneous phasic contractions amplitude, frequency, net muscle force, and muscle tone in rat UBSM strips. As illustrated, BRL 37344 (100 nM-100 \( \mu \text{M} \)) inhibited the phasic contraction’s amplitude and net muscle force, and significantly reduced the muscle tone in isolated UBSM strips. In UBSM, a phasic contraction reflects an elevation of Ca\(^{2+}\) entry via L-type Cav channels caused by a single action potential or a burst of action potentials (17). The amplitude of a phasic contraction depends on the increase in Ca\(^{2+}\) entry caused by membrane depolarization during an action potential, whereas the duration of a phasic contraction depends on the single action potential duration or on a whole burst of action potentials (17, 18). The frequency of phasic contractions reflects mechanisms that temporarily cause action potentials to cease, such as an increase in K\(^+\) channel conductance (4, 17, 40–42).

We have previously shown that nonselective stimulation of all three \( \beta_3 \)-ARs subtypes with isoproterenol leads to BK channel activation (42). Therefore, it was of interest to investigate whether the BK channel is also involved in the \( \beta_3 \)-AR-mediated relaxation of UBSM spontaneous contractility. Blocking the BK channel with 200 nM IBTX increased the spontaneous phasic contraction amplitude and the overall UBSM contractility (Fig. 1). In the presence of 200 nM IBTX, the concentration response curves for BRL 37344 (100 nM-100 \( \mu \text{M} \)) were shifted to the right. The effects were statistically significant at BRL concentrations > 10 \( \mu \text{M} \) (Fig. 1).

Because the BRL 37344 concentration response experiments are extended experiments conducted within a 50-min time frame, time control experiments were performed in parallel for the spontaneous and IBTX-induced phasic contractions in which no BRL 37344 was added. The data for the time controls summarized in Table 1 indicate that both spontaneous and IBTX-induced contractions remained stable during the time course for the BRL 37344 concentration response experiments. Therefore, BRL 37344 indeed had inhibitory effects on the contraction parameters as illustrated in Fig. 1, and they were not due to rundown of the UBSM preparations.

In another experimental series, we first applied a single concentration of BRL 37344 (100 \( \mu \text{M} \)) to achieve maximum inhibition on the spontaneous UBSM contractions. In the presence of 100 \( \mu \text{M} \) BRL 37344, application of IBTX (200 nM) caused not only complete recovery of all contraction parameters (amplitude, force, frequency, and tone) to the level of spontaneous contractility but further significantly increased contraction amplitude and frequency above the level of spon-
aneous contractions (Fig. 2). This experimental series indicates that pharmacological blockade of the BK channel overcomes the inhibitory effect of BRL 37344 on spontaneous contractions, suggesting a functional interaction between β3-ARs and the BK channels. These experiments clearly indicate the key role of the BK channel in UBSM relaxation mediated by the β3-ARs.

To confirm that the effect of BRL 37344 is mediated via β3-ARs, we used the β3-AR-specific antagonist SR59230A. In this experimental series, UBSM strips were first exposed to 100 μM BRL 37344 to achieve a maximal relaxation, and then the drug was washed out. After recovery of spontaneous contractility, the strips were pretreated with 10 μM SR59230A for 30 min and 100 μM BRL 37344 was applied again. In the presence of 10 μM SR59230A, the inhibitory effect of BRL 37344 was significantly reduced, indicating that BRL 37344 effects are mediated via β3-ARs (Table 2).

To confirm that stimulation of β3-ARs with BRL 37344 leads to PKA activation, we used the PKA-specific inhibitor H89. This inhibitor was selected because of its membrane permeability. In this experimental series, we tested two concentrations of BRL 37344, 10 μM and 100 μM. UBSM strips were first probed by a given BRL 37344 concentration and after a maximal relaxation was achieved, the drug was washed out. The UBSM strips were then pretreated with 10 μM H89 for 30 min, and the same concentration of BRL 37344 was applied. In the presence of 10 μM H89, the tone of UBSM strips was significantly inhibited at both concentrations used, indicating that at least the BRL 37344 reduction of tone is mediated by PKA (Table 3).

To further reveal the cellular mechanism of BK channel activation upon β3-AR stimulation, we performed perforated whole cell, patch-clamp experiments on isolated UBSM cells under various experimental conditions. The perforated mode of

Table 1. Time controls, without BRL 37344 added, were measured 50 min after the initial control (taken to be 100%) for the spontaneous contractions and 50 min after the IBTX application for the IBTX-induced contractions, respectively

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude (%)</th>
<th>Frequency (%)</th>
<th>Force (%)</th>
<th>Tone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous contractions</td>
<td>102.7 ± 6.4%</td>
<td>98.9 ± 8.1%</td>
<td>110.0 ± 7.0%</td>
<td>100.1 ± 1.9%</td>
</tr>
<tr>
<td>IBTX-induced contractions</td>
<td>91.4 ± 5.4%</td>
<td>104.9 ± 1.8%</td>
<td>91.0 ± 4.8%</td>
<td>96.8 ± 1.8%</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of separate urinary bladder smooth muscle (UBSM) strips; N, the number of individual rats. The 50-min time period is equivalent to the time period for the BRL 37344 concentration-response curves. TTX (1 μM) was present throughout the experiments.
the patch-clamp technique maintains the native environment of the cell, preserving the signal transduction pathways intact. In all electrophysiological experiments below, we used a single BRL 37344 concentration of 100 μM. At this concentration, BRL 37344 reached a maximal effect on UBSM contractility (Figs. 1–2), and, at this concentration, BRL 37344 has previously been shown to increase BK channel activity in human myometrium (10).

Table 2. Inhibitory effect of 100 μM BRL37344 on UBSM spontaneous contractility in the absence and presence of 10 μM SR59230A

<table>
<thead>
<tr>
<th>Control No</th>
<th>+10 μM SR59230A</th>
<th>%Spontaneous Contractions</th>
<th>Contraction amplitude (pA)</th>
<th>Contraction frequency (Hz)</th>
<th>Net muscle tone (%Tone)</th>
<th>Tone (mV)</th>
<th>n, N, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control No</td>
<td>+10 μM SR59230A</td>
<td>%Spontaneous Contractions</td>
<td>Contraction amplitude (pA)</td>
<td>Contraction frequency (Hz)</td>
<td>Net muscle tone (%Tone)</td>
<td>Tone (mV)</td>
<td>n, N, P</td>
</tr>
<tr>
<td>Control No</td>
<td>+10 μM SR59230A</td>
<td>%Spontaneous Contractions</td>
<td>Contraction amplitude (pA)</td>
<td>Contraction frequency (Hz)</td>
<td>Net muscle tone (%Tone)</td>
<td>Tone (mV)</td>
<td>n, N, P</td>
</tr>
<tr>
<td>Control No</td>
<td>+10 μM SR59230A</td>
<td>%Spontaneous Contractions</td>
<td>Contraction amplitude (pA)</td>
<td>Contraction frequency (Hz)</td>
<td>Net muscle tone (%Tone)</td>
<td>Tone (mV)</td>
<td>n, N, P</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of separate UBSM strips; N, the number of individual rats. Spontaneous contractions were taken as 100%. TTX (1 μM) and atropine (1 μM) were present throughout the experiments.

Stimulation of β3-ARs with BRL 37344 causes activation of STOCs in single, isolated UBSM cells. In UBSM, Ca2+ sparks activate the closely located BK channels, thus causing STOCs (20, 42). Therefore, STOCs are indicative for Ca2+ sparks. To examine the effect of BRL 37344 on STOCs, STOCs were recorded from single, isolated UBSM cells that were voltage-clamped at −40 mV (see METHODS). BRL 37344 (100 μM) caused a sustained increase in STOC frequency by 46.0 ± 20.1% vs. control (n = 5, N = 5; P < 0.05; Fig. 3), whereas the STOC amplitude was unaffected (92.5 ± 6.4% vs. control, n = 5, N = 5; P > 0.05; Fig. 3). These results support the idea that in UBSM, stimulation of β3-ARs can activate BK channels through STOCs activation. STOCs activation is likely to move the UBSM resting membrane potential away from the threshold of action potential activation and significantly inhibit the action potentials and the associated phasic contractions.

Under conditions of blocked Ca2+ sources for BK channel activation, stimulation of β3-ARs with BRL 37344 does not change single BK channel activity. The observed elevation of STOCs frequency could be explained by a BRL 37344-induced increase in Ca2+ spark frequency. An alternative hypothesis would be an elevation of the BK channel P0 caused by direct BRL 37344 effects on the channel or by PKA-induced phosphorylation of the channel. To test the effects of BRL 37344 on UBSM BK channels in their native physiological environment, single-channel currents were recorded from isolated UBSM cells in whole cell mode, using the amphotericin-perforated patch-clamp technique (see METHODS). In whole cell mode at Vh = 0 mV, BRL 37344 (100 μM) did not significantly change the single BK channel P0: 0.0022 ± 0.0018 NPo/pF (control) and 0.0019 ± 0.0012 NPo/pF (BRL 37344) (n = 5, N = 3; P > 0.05; Fig. 4). The single BK channel current amplitude was not affected by 100 μM BRL 37344 at 0 mV: 5.17 ± 0.28 pA (control) and 5.55 ± 0.41 pA (BRL 37344) (n = 5, N = 3; P > 0.05; Fig. 4). These results are consistent with the idea that in rat UBSM cells, BRL 37344-induced activation of STOCs is not due to any direct effect on the BK channel or changes in the BK channel P0.
induced steady-state K$^+$ known extra- and intracellular Ca$^{2+}$ sources for BK channel activation were eliminated (see METHODS). Under these conditions, stimulation of the β3-AR with 100 μM BRL 37344 did not significantly change the amplitude and open probability ($P_o$) of single BK channels under conditions when the Ca$^{2+}$ sources for BK channel activation were pharmacologically inhibited. Single BK channel currents were recorded with the whole cell, perforated, patch-clamp technique from single smooth muscle cells at 0 mV holding potential. A: shown is a series of BK channel openings from a single smooth muscle cell. B: summary data illustrating the lack of BRL 37344 (100 μM) effect on the single BK channel amplitude. Values are means ± SE (n = 5, N = 3; P > 0.05).

Under conditions of blocked Ca$^{2+}$ sources for BK channel activation, stimulation of β3-ARs with BRL 37344 does not change the steady-state K$^+$ current. To examine further whether stimulation of β3-ARs with BRL 37344 leads to activation of the total steady-state K$^+$ current, we performed experiments on isolated UBSM cells using voltage-step protocols and the whole cell, perforated, patch-clamp technique. From $V_h = -70$ mV, voltage-step depolarizations with 200-ms duration, applied in 20-mV increments from -40 to +80 mV induced steady-state K$^+$ currents (Fig. 5). We have previously shown that the majority of these voltage-step-induced steady-state K$^+$ currents consist of a steady-state BK current component due to their high sensitivity to IBTX (42). As in the previous experimental series on single BK channel activity, all known extra- and intracellular Ca$^{2+}$ sources for BK channel activation were eliminated (see METHODS). Under these conditions, stimulation of β3-ARs with BRL 37344 (100 μM) did not activate the depolarization-induced (-40 to +80 mV) steady-state outward K$^+$ currents (Fig. 5). As illustrated in Fig. 5, the current-voltage relationships for the voltage-step-induced steady-state K$^+$ currents in the presence and absence of 100 μM BRL 37344 were largely unaffected (n = 8, N = 6; P > 0.05). Furthermore, we repeated the same experimental series in the presence of 200 nM IBTX, using the same voltage-step protocol under conditions of blocked Ca$^{2+}$ sources. Again, the current-voltage relationships for the voltage-step-induced, IBTX-insensitive, steady-state K$^+$ currents in the presence and absence of 100 μM BRL 37344 were largely unaffected (n = 4, N = 4; P > 0.05; data not illustrated). These experiments suggest that stimulation of β3-ARs does not activate the steady-state BK current or any other voltage-dependent, steady-state K$^+$ currents directly but rather requires functional Ca$^{2+}$ sources for BK channel activation.

Stimulation of β3-ARs with BRL 37344 causes UBSM cell membrane potential hyperpolarization mediated by the BK channels. In UBSM, the BK channel contributes to the maintenance of the resting membrane potential to some extent (18, 39). The increased STOCs frequency caused by BRL 37344-induced β3-ARs stimulation may lead to membrane potential hyperpolarization. To test this hypothesis, we performed current-clamp experiments using the perforated whole cell, patch-clamp technique. Under current-clamp conditions, stimulation of the β3-AR with 100 μM BRL 37344 hyperpolarized the resting membrane potential from $-26.1 ± 2.1$ mV (control) to $-29.0 ± 2.2$ mV (BRL 37344; n = 18; N = 12; P < 0.0001; Fig. 6). The BRL 37344-induced hyperpolarization was prevented by preliminary application of the BK channel-specific inhibitor, IBTX (200 nM) or by nonspecific BK channel inhibition with 1 mM TEA. In the presence of 200 nM IBTX,
Fig. 5. Stimulation of β3-ARs with BRL 37344 does not increase the voltage-step-induced steady-state K⁺ currents in single UBSM cells under conditions when the Ca²⁺ sources for BK channel activation were pharmacologically inhibited. A: illustration of the voltage-step protocol that was applied to record whole cell, steady-state K⁺ currents using the perforated-patch technique at a V₉₅ of −70 mV. B: current-voltage relationships for the depolarization-induced steady-state K⁺ currents in the presence and absence of 100 μM BRL 37344 were unchanged. Values are means ± SE (n = 8; N = 6; P > 0.05; original recordings illustrating the steady-state K⁺ currents in the presence and absence of 100 μM BRL 37344 (D). Ryanodine (30 μM), thapsigargin (100 nM), and nifedipine (1 μM) were present throughout the experiments.

BRL 37344 (100 μM) did not significantly change the resting membrane potential: −24.0 ± 4.0 mV (IBTX control) and −24.2 ± 4.1 mV (BRL 37344) (n = 9; N = 3; P > 0.05; Fig. 6). In the presence of 1 mM TEA, BRL 37344 (100 μM) also did not significantly change the resting membrane potential: −24.3 ± 4.6 mV (TEA control) and −25.1 ± 4.6 mV (BRL 37344) (n = 8; N = 3; P > 0.05; Fig. 6). These experiments indicate that BRL 37344-induced STOCs activation is associated with membrane potential hyperpolarization leading to an inhibition of UBSM spontaneous contractility.

In another experimental series, the BRL 37344-induced (100 μM) hyperpolarization was eliminated by subsequent application of IBTX (200 nM): control (−26.8 ± 2.3 mV; BRL 37344 (−28.7 ± 2.7 mV; P < 0.05 vs. control); BRL 37344 and IBTX (−27.3 ± 2.3 mV n = 9; N = 8; P < 0.05 vs. BRL 37344; P > 0.05 vs. control). This experimental series confirms that pharmacological blockade of the BK channel overcomes the hyperpolarizing effect of BRL 37344 in isolated UBSM cells, suggesting a functional interaction between β3-ARs and the BK channels at the level of the resting membrane potential.

In the last experimental series, illustrated in Fig. 7, the BRL 37344-induced (100 μM) hyperpolarization was not observed in the presence of 30 μM ryanodine (control, −24.9 ± 4.0 mV; BRL 37344, −25.3 ± 4.5 mV; n = 7; N = 5; P < 0.05 vs. control). This final experimental series confirms that pharmacological blockade of the RyR overcomes the hyperpolarizing effect of BRL 37344, indicating that the RyRs are required to facilitate the functional link between β3-ARs and the BK channels.

Fig. 6. In isolated UBSM cells, stimulation of β3-ARs with BRL 37344 causes membrane potential hyperpolarization, which was prevented by blocking the BK channels with 200 nM IBTX or 1 mM TEA. A: original recordings illustrating the BRL 37344 (100 μM) hyperpolarizing effect on the resting membrane potential under control conditions (top trace), and lack of an hyperpolarizing effect in the presence of 200 nM IBTX (middle trace), or 1 mM TEA (bottom trace). Resting membrane potential was recorded in current-clamp mode of the perforated-patch technique. B: summary data illustrating BRL 37344 (100 μM) hyperpolarizing effect under control conditions. Values are means ± SE (n = 11; N = 6; ****P < 0.0001). C: summary data illustrating lack of BRL 37344 (100 μM) hyperpolarizing effect in the presence of 200 nM IBTX (n = 9; N = 3; P > 0.05). D: summary data illustrating lack of BRL 37344 (100 μM) hyperpolarizing effect in the presence of 1 mM TEA (n = 8; N = 3; P > 0.05).
channels control the opening and closing of L-type Ca channels and inhibition of UBSM spontaneous phasic contractions and 3), leading to UBSM cell membrane hyperpolarization (Fig. 6) coupling, which involves activation of STOCs frequency (Fig. 7A). In isolated UBSM cells, stimulation of 3-ARs in controlling the UBSM excitability by utilizing the pharmacological tools, such as ryanodine, thapsigargin, and nifedipine, to dissect the different sources of Ca^{2+} for BK channel activation.

Although cAMP/PKA is the main signal transduction pathway that mediates 3-AR effects, both PKA-dependent and -independent mechanisms have been proposed to operate in UBSM (11, 12, 45, 47, 48). Most of the information for a PKA-independent mechanism, however, comes from indirect evidence on UBSM contractility (12, 47, 48). Uchida et al. (47) have further indicated that in “noncontracted” UBSM (spontaneous contractions), relaxation mediated via 3-ARs is achieved solely by a cAMP-dependent mechanism. We have previously found that forskolin increases single BK channel current, whereas nonspecific activation of all 3-ARs with isoprenaline is not sufficient to increase BK channel current (42). It has been shown that physiological coupling between 3-ARs and BK channels occurs by PKA-mediated phosphorylation of the channel pore-forming α-subunit (36). It has also been reported that 3-ARs are closely associated with the BK channel α-subunit and a PKA-anchoring protein to mediate the 3-AR agonist responses in UBSM (29). Our data do not provide evidence for a similar colocalization and direct coupling between the 3-ARs and BK channels in rat UBSM. Instead, the functional coupling between the 3-ARs and BK channels in UBSM utilizes BK channel ryanodine-sensitive Ca^{2+} signaling mechanisms at the SR level. Our data indicates that BRL 37344-induced hyperpolarization is eliminated in the presence of ryanodine (Fig. 7). BRL 37344, via PKA activation, can act directly on the RyRs and/or indirectly by phospholamban phosphorylation. Our data show that the BRL 37344 relaxant effect on the UBSM tone is inhibited by H89, consistent with a role for PKA. In skeletal and cardiac muscle, PKA-phosphorylated phospholamban activates the SR Ca^{2+}-pump and elevates SR Ca^{2+} load, which increases the $P_o$ of RyRs channels (9). PKA can also increase the $P_o$ of the RyRs channel and thus RyRs activity by direct phosphorylation of the receptor (9). Most likely, the observed BRL 37344-induced increase in STOC activity may use both mechanisms.

**DISCUSSION**

The present study reveals important details of a fundamental regulatory pathway involved in the control of urinary bladder contractility; 3-ARs and BK channels are functionally coupled to promote UBSM relaxation via BK channel activation upon 3-ARs stimulation. These results further uncover the complex Ca^{2+}-dependent mechanism of this physiological coupling, which involves activation of STOCs frequency (Fig. 3), leading to UBSM cell membrane hyperpolarization (Fig. 6) and inhibition of UBSM spontaneous phasic contractions and tone (Figs. 1 and 2).

As key regulators of UBSM membrane excitability, BK channels control the opening and closing of L-type CaV channels, thereby affecting UBSM contractility. Our experiments on the spontaneous phasic and tonic contractions showed that stimulation of 3-ARs with BRL 37344 led to concentration-dependent inhibition of phasic contraction amplitude, net muscle force, and muscle tone. BRL 37344 (300 μM) is also effective at inhibiting spontaneous contractions of isolated whole guinea pig bladder (15). In rat UBSM, the potential inhibitory effect on the overall spontaneous contractility upon 3-ARs stimulation with FR165101 and the possible involvement of the BK channel in this process has previously been suggested (47). Here, we have further separated and characterized the 3-AR inhibitory effects on the different contraction parameters. In the presence of IBTX, a rightward shift in the concentration response curves was observed, indicating the importance of the BK channel in the BRL 37344 inhibitory effect on contractility (Fig. 1).

Our current-clamp experiments suggest a prominent role of 3-ARs in controlling the UBSM excitability by utilizing the BK channel (Figs. 6 and 7). The increase in STOCs frequency upon 3-ARs stimulation contributes to membrane hyperpolarization and moves the UBSM resting membrane potential away from the threshold of action potential activation and thus has significant inhibitory effects on action potentials and related phasic contractions. Our experiments on whole cell, K^+-steady-state currents (Fig. 5) and single BK channel recordings (Fig. 4) do not support a mechanism for a direct activation of the BK channels by BRL 37344 in intact cells with blocked sources of Ca^{2+} necessary for BK channel activation. Rather, they support the concept that 3-AR stimulation activates BK channels by increasing STOCs activity in UBSM cells. Collectively, our data indicate that the physiological coupling between the 3-ARs and BK channels, which is responsible for UBSM hyperpolarization and relaxation, requires active sources of Ca^{2+} for BK channel activation.

Although cAMP/PKA is the main signal transduction pathway that mediates 3-AR effects, both PKA-dependent and -independent mechanisms have been proposed to operate in UBSM (11, 12, 45, 47, 48). Most of the information for a PKA-independent mechanism, however, comes from indirect evidence on UBSM contractility (12, 47, 48). Uchida et al. (47) have further indicated that in “noncontracted” UBSM (spontaneous contractions), relaxation mediated via 3-ARs is achieved solely by a cAMP-dependent mechanism. We have previously found that forskolin increases single BK channel current, whereas nonspecific activation of all 3-ARs with isoprenaline is not sufficient to increase BK channel current (42). It has been shown that physiological coupling between 3-ARs and BK channels occurs by PKA-mediated phosphorylation of the channel pore-
physiological protocols by completing the experiments in more physiologically relevant conditions. For example, we utilized a $V_n = 0$ mV, instead of $V_n = +40$ mV. At $V_h = +40$ mV, the BK channel is exceedingly activated (40), and further activation upon pharmacological stimulation is unlikely to occur. Also, we used recording intervals of 7–10 min, which provide a more accurate determination of $P_o$ than recording intervals of ~10 s. Finally, the unjustifiably high Ca$^{2+}$ concentration in the pipette used by Doheny et al. (10) may be a source of contaminating Ca$^{2+}$ activator for the BK channel, whereas our conditions of 0 Ca$^{2+} + 0.05$ mM EGTA (to eliminate any residual Ca$^{2+}$) do not provide this interference. These important experimental details allowed us to identify with high accuracy the intrinsic mechanisms of the functional link between β3-ARs and BK channels in rat UBSM. In addition, Doheny et al. (10) did not record any STOCs in human myometrium. Therefore, tissue and/or species differences in the mechanism of BK channel activation upon β3-AR-stimulation in rat UBSM and human myometrium cannot be ruled out.

Overactive bladder is a condition characterized by symptoms of urgency, frequency, nocturia, and increased UBSM phasic contractions that often follow bladder outlet obstruction due to prostate enlargement (for review, see Refs. 1 and 2). The etiology of overactive bladder is unknown and the current therapies are limited to antimuscarinics that are largely ineffective with numerous undesirable side effects. Therefore, there is a need to develop alternative therapeutic approaches with novel mechanisms of action.

Because the BK channel is a key modulator of UBSM excitability in experimental animals, mutations of this channel may cause overactive bladder. BK channel inhibition with IBTX leads to increased overall UBSM contractility (19, 40), may cause overactive bladder. BK channel inhibition with excitability in experimental animals, mutations of this channel ineffective with numerous undesirable side effects. Therefore, therapies are limited to antimuscarinics that are largely ineffective with numerous undesirable side effects. Therefore, there is a need to develop alternative therapeutic approaches with novel mechanisms of action.

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