Antagonists of the TMEM16A Calcium-activated Chloride Channel Modulate Airway Smooth Muscle Tone and Intracellular Calcium

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ABSTRACT

Background: Perioperative bronchospasm refractory to β agonists continues to challenge anesthesiologists and intensivists. The TMEM16A calcium-activated chloride channel modulates airway smooth muscle (ASM) contraction. The authors hypothesized that TMEM16A antagonists would relax ASM contraction by modulating membrane potential and calcium flux.

Methods: Human ASM, guinea pig tracheal rings, or mouse peripheral airways were contracted with acetylcholine or leukotriene D4 and then treated with the TMEM16A antagonists: benzbromarone, T16Ainh-A01, N-((4-methoxy)-2-naphthyl)-5-nitroantranilic acid, or B25. In separate studies, guinea pig tracheal rings were contracted with acetylcholine and then exposed to increasing concentrations of isoproterenol (0.01 nM to 10 μM) ± benzbromarone. Plasma membrane potential and intracellular calcium concentrations were measured in human ASM cells.

Results: Benzbromarone was the most potent TMEM16A antagonist tested for relaxing an acetylcholine-induced contraction in guinea pig tracheal rings (n = 6). Further studies were carried out to investigate the clinical utility of benzbromarone. In human ASM, benzbromarone relaxed either an acetylcholine- or a leukotriene D4-induced contraction (n = 8). Benzbromarone was also effective in relaxing peripheral airways (n = 9) and potentiating relaxation by β agonists (n = 5 to 10). In cellular mechanistic studies, benzbromarone hyperpolarized human ASM cells (n = 9 to 12) and attenuated intracellular calcium flux from both the plasma membrane and the sarcoplasmic reticulum (n = 6 to 12).

Conclusion: TMEM16A antagonists work synergistically with β agonists and through a novel pathway of interrupting ion flux at both the plasma membrane and sarcoplasmic reticulum to acutely relax human ASM. (Anesthesiology 2015; 123:569-81)

Asthma is a very common respiratory disease, affecting up to 10% of adults and 30% of children in the Western world.1 These patients frequently present for surgical procedures requiring anesthesia, and instrumentation of the airway is a potent stimulus for acute bronchoconstriction. The pathogenesis of asthma involves chronic airway inflammation, increased mucus secretion, irritable airway nerves, and a hyperresponsiveness of airway smooth muscle (ASM) that can lead to acute bronchospasm that greatly impairs the ability to provide adequate ventilation. The incidence of intraoperative bronchospasm has been reported to be up to 20% in active asthmatics,2,3 and poorly controlled asthma is a major risk factor.4 Although β agonist therapy is the accepted standard for the treatment of bronchospasm, chronic β agonist use is associated with tachyphylaxis and
increased mortality.5,6 Perioperative bronchospasm refractory to β agonists is a challenge for anesthesiologists. Because relaxation of ASM is an important goal in the treatment of bronchospasm and no new drugs have been developed in several decades to facilitate acute ASM relaxation, there is an obvious need for novel bronchodilators that work synergistically with β agonists.

The modulation of chloride flux is a promising new approach to achieve ASM relaxation. Chloride flux is important for ASM plasma membrane depolarization after activation of calcium-activated chloride channels, which enhances influx of extracellular calcium through voltage sensitive plasma membrane channels. Calcium-activated chloride channels have also been localized to the sarcoplasmic reticulum (SR) where they may play a key role in the release and refill of calcium by modulating charge balance across the SR membrane.7 Calcium-activated chloride current was identified in ASM as an important component of contraction several decades ago.8 However, the identity of the specific calcium-activated chloride channels remained elusive. In 2008, three separate laboratories discovered the molecular identity of this channel as the TMEM16 or anoctamin family of proteins.9–11 The discovery of this family of proteins has revolutionized the understanding of calcium regulation of chloride flux in many cell types.12 Our laboratory has shown that TMEM16A is expressed in ASM, and others have demonstrated that blockade modulates ASM contraction.13

With the molecular discovery of TMEM16A, many groups have sought to identify potent and selective TMEM16A antagonists and agonists. By using high-throughput screening methods, four different groups have identified TMEM16A antagonists: benzbrromarone,14 T16Ainh-A01,15 N-((4-methoxy)-2-naphthyl)-5-nitroanilinic acid (MONNA),16 and B25.17 These groups investigated antagonist activity at TMEM16A with IC50 of less than 10 μM. Affinity for other chloride channels such as TMEM16B, cystic fibrosis transmembrane conductance regulator (CFTR), bestrophin, and CLC chloride channel family (CLC) were investigated with TMEM16A antagonists, but the investigator was not blinded. Vehicle tissues were randomized to receive vehicle or TMEM16A antagonists, and vehicle was exchanged every 15 min for 1 h during equilibration of tracheal rings at 1 g resting tension. All baths received 10 μM indomethacin to block the synthesis of endogenous prostanoids, 10 μM N-vanillylnonanamide (capsaicin analog) to activate and subsequently block the effects of nonadrenergic, noncholinergic nerves, 1 μM tetrodotoxin to block neuronal effects, and 10 μM of pyrilamine to block the effects of endogenous histamine release. Histamine and neuronal effects can affect intrinsic tone and confound results when studying an acetylcholine contraction. Preliminary contractile challenges of each ring consisted of two cycles of acetylcholine dose responses (100 nM to 100 μM) to define the EC50 of acetylcholine for each tracheal ring. Rings were contracted with an EC50 concentration of acetylcholine, and force was allowed to plateau. For all organ bath experiments, tissues were randomized to receive vehicle or TMEM16A antagonists, but the investigator was not blinded. Vehicle (dimethyl sulfoxide [DMSO]) or TMEM16A blockers were then added to organ bath in cumulatively increasing concentrations at 30-min intervals (10, 50, and 100 μM). The amount of contractile force remaining at 30 min after each concentration was expressed as the percent of initial force immediately before antagonist addition.

Separate studies were performed to determine whether the treatment with benzbrromarone could potentiate a β agonist-induced relaxation of an acetylcholine contraction.

**Materials and Methods**

All materials were obtained from Sigma-Aldrich® (USA) unless otherwise specified. Tetrodotoxin was purchased from Calbiochem, EMD Biosciences (USA). MK571, T16Ainh-A01, Eact, and albuterol were purchased from Tocris (USA). Membrane potential dye (fluorescent imaging plate reader [FLIPR] blue reagent) was obtained from Molecular Devices (USA). Fura-2 AM and mag-fluo-4 AM were obtained from Molecular Probes (USA). B25 (5-[(2,6-difluorobenzyl)oxy]-2-(2-naphthyl)benzofuran-3-carboxylic acid) was synthesized as suggested by Kumar et al.13 MONNA was a gift from Dr. C. Justin Lee from Korea Institute of Science and Technology, South Korea.

**Guinea Pig Tracheal Rings**

Animal protocols were approved by the Columbia University Animal Care and Use Committee. Male Hartley guinea pigs weighing approximately 400 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). Trachea were removed and transferred to ice-cold Krebs-Henseleit (KH) buffer in the following composition: 118 mM NaCl, 5.6 mM KCl, 0.5 mM CaCl2, 0.24 mM MgSO4, 1.3 mM NaH2PO4, 25 mM NaHCO3, 5.6 mM glucose, pH 7.4. Epithelial-denuded tracheal rings were prepared using a dissecting microscope. Epithelium was removed by gently passing cotton fibers through the lumen of the tracheal rings. The rings were suspended in 4 mL water-jacketed organ baths (37°C; Radnoti Glass Technology, USA) and connected to Grass FT03 force transducers (Grass Telefactor, USA) using silk sutures. The transducers were coupled to the computer with Biopac hardware, and data were collected using Acqknowledge 7.3.3 software (BioPac Systems, USA). KH buffer was bubbled with a gas mixture of 95% O2 and 5% CO2, and buffer was exchanged every 15 min for 1 h during equilibration of tracheal rings at 1 g resting tension. All baths received 10 μM indomethacin to block the synthesis of endogenous prostanoids, 10 μM N-vanillylnonanamide (capsaicin analog) to activate and subsequently block the effects of nonadrenergic, noncholinergic nerves, 1 μM tetrodotoxin to block neuronal effects, and 10 μM of pyrilamine to block the effects of endogenous histamine release. Histamine and neuronal effects can affect intrinsic tone and confound results when studying an acetylcholine contraction. Preliminary contractile challenges of each ring consisted of two cycles of acetylcholine dose responses (100 nM to 100 μM) to define the EC50 of acetylcholine for each tracheal ring. Rings were contracted with an EC50 concentration of acetylcholine, and force was allowed to plateau. For all organ bath experiments, tissues were randomized to receive vehicle or TMEM16A antagonists, but the investigator was not blinded. Vehicle (dimethyl sulfoxide [DMSO]) or TMEM16A blockers were then added to organ bath in cumulatively increasing concentrations at 30-min intervals (10, 50, and 100 μM). The amount of contractile force remaining at 30 min after each concentration was expressed as the percent of initial force immediately before antagonist addition.
Rings were contracted with an EC₅₀ concentration of acetylcholine, and relaxation was induced with ½ log increments of isoproterenol (0.01 nM to 10 μM) in the presence of vehicle (0.1% DMSO), 10 or 25 μM benzbromarone added simultaneously with the 0.5 nM concentration of isoproterenol.

Human Tracheal Strips

Studies using deidentified human tissue were reviewed by the Columbia University Institutional Review Board and deemed not human subjects research. Tissues were obtained from healthy, normal organ donor discarded surgical waste subsequent to lung transplant surgery. Tissues were collected and placed in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, USA) and bubbled overnight in 5% CO₂ and 95% O₂ at 4°C. The following morning, the smooth muscle tissue was carefully dissected under a dissecting microscope, and the epithelium was removed. The strips were anchored in the organ bath as described in Guinea Pig Tracheal Rings using KH buffer of the same composition. The strips were allowed to equilibrate for 1 h with buffer exchanges every 15 min and were treated with 1 μM tetrodotoxin and 10 μM pyrilamine. MK571 (10 μM) was added to baths contracted with acetylcholine but not leukotriene D₄ (LTD₄) to block endogenous leukotriene release. Endogenous leukotriene release contributes to the intrinsic tone of human ASM and may confound results when studying an acetylcholine contraction.²⁰ Preliminary contractile challenges of each human ASM strip consisted of two cycles of acetylcholine dose responses (100 nM to 1 mM) to define the EC₅₀ of acetylcholine for each strip. If tissue was not stored properly, stored too long, cut in a way that is not optimal for our contraction studies, or did not contract properly, the experiment was terminated before treatment with drugs or vehicle. Tissue was contracted with an EC₅₀ dose of acetylcholine or 20nM LTD₄. When a plateau in contractile force was reached, the strips were exposed to 0.3 μM methacholine, then exposed to 10 μM benzbromarone, and washed. The area of the airway lumen was calculated from each image using a custom-written script in Video Savant (IO Industries Inc., Canada) that distinguishes the lumen from the surrounding tissue. The lumen area was normalized to the area before stimulation, and the changes in lumen area were plotted against time.

Cultured Human Airway Smooth Muscle Cells

Immortalized human ASM cell lines modified to stably express human telomerase reverse transcriptase were a kind gift from Dr. William Gerthoffer (University of South Alabama, USA) and were prepared as described previously²¹ and grown in DMEM/F12 media (GIBCO), with 10% fetal bovine serum and antibiotics.

For calcium studies with acetylcholine, human ASM cells stably transfected to express the human M3 muscarinic receptor were used because this receptor is not highly expressed in our cell line. Production of the lentiviral vector and transduction of cells was described previously.²²

FLIPR Membrane Potentiometric Dye Assay

Cells were prepared as described previously.²³ Briefly, immortalized human ASM cells were cultured on black-walled 96-well plates to 100% confluence and were washed four times with warmed (37°C) assay buffer. A stock solution (100% dye) of FLIPR blue dye was prepared by reconstitution of one vial (125 mg) with 100 ml of assay buffer. Cells loaded with 50% working stock over 45 min at 37°C in a humidified cell culture incubator (95% air/5% CO₂). Fluorescence was measured with a FlexStation 3 microplate.
reader (Molecular Devices) using an excitation wavelength of 530 nm and emission wavelength of 565 nm. Baseline fluorescence was measured every 2 s for 1 min, and then for 2 min after injection of the vehicle or drug of interest as indicated and the maximal change in fluorescence was recorded.

**Fura-2 Calcium Assay**
Immortalized human ASM cells were cultured on black-walled 96-well plates to 100% confluence and washed four times with warmed modified (37°C) HBSS buffer (137.93 mM NaCl, 5.33 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 2.38 mM Hepes, 5.5 mM glucose, pH to 7.4). Cells were loaded with 5 μM fura-2 AM for 45 min and washed an additional three times with buffer. Cells were then pretreated with vehicle (0.1% DMSO) or benzbro- marone (10, 50, and 100 μM) for 10 min after which they were placed in a FlexStation 3 microplate reader (Molecular Devices). Baseline fluorescence was measured, and histamine or bradykinin (10 μM final concentration) was pipetted into the wells of the plate. Fluorescence was read every 4 s for 400 s using excitation wavelengths of 340 and 380 nm, an emission wavelength of 510 nm, and a cutoff filter of 495 nm. Fluorescence values were reported as F/F₀ according to the calculation: \[ \Delta F = (340 \text{ nm})f/(380 \text{ nm})f - (340 \text{ nm}0)/(380 \text{ nm}0) \]. For studies in 0 mM external calcium, the cells were loaded as mentioned earlier. After being loaded, the cells were washed four times with calcium-free HBSS (supplemented with 200 μM ethylene glycol tetraacetic acid). The remainder of the study was carried out in calcium-free HBSS.

**Simultaneous Fura-2 and Mag Fluo-4 Calcium Assay**
Simultaneous measurement of SR and cytosolic calcium were adapted from previous studies for the FlexStation 3 microplate reader (Molecular Devices) and ASM cells. Immortalized human ASM cells were cultured on black-walled 96-well plates to 100% confluence and washed four times with fluorescence buffer (FB; 145 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 0.5 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 5 mM glucose, pH 7.4, 37°C). Cells were incubated with 5 μM mag-fluo-4 AM with 0.1% pluronic in warmed modified (37°C) HBSS buffer. After 30 min, the cells were then pretreated with vehicle (0.1% DMSO) or benzbromarone (10, 50, and 100 μM) for 40 min. Cells were washed three times and incubated with 5 μM mag-fluo-4 AM with 0.1% pluronic for 30 min. Then cells were then washed with 5 μM fura-2 AM for 45 min and washed an additional three times with buffer. Cells were then pretreated with vehicle (0.1% DMSO) or benzbromarone (10, 50, and 100 μM) for 10 min after which they were placed in a FlexStation 3 microplate reader (Molecular Devices). Baseline fluorescence was measured, and bradykinin (10 μM) or acetylcholine (100 μM) was pipetted into the wells of the plate. Fluorescence was read every 4 s using excitation wavelengths of 340, 380, and 465 nm simultaneously with emission wavelength of 510 nm. For studies with acetylcholine, M3-overexpressing human ASM cells were used.

**Results**

**TMEM16A Antagonists Relax an Acetylcholine Contraction in Guinea Pig Tracheal Rings**
Four different TMEM16A antagonists (benzbromarone, T16Ainh-A01, B25, and MONNA) were analyzed for their ability to relax an acetylcholine-induced contraction in guinea pig tracheal rings. Rings were treated with vehicle (0.1% DMSO) or cumulative doses of TMEM16A antagonist (10, 50, and 100 μM) in 30-min intervals. Benzbromarone (10 μM) significantly relaxed an acetylcholine contraction (70.9 ± 6.7% of initial plateau contractile force remaining at 30 min, \( ^{**}P < 0.001, n = 6 \)), whereas T16Ainh-A01, MONNA, and B25 failed to show significant relaxation at 10 μM (fig. 1). Benzbromarone (50 μM), T16Ainh-A01, and MONNA relaxed an acetylcholine contraction (−10.7 ± 3.1, 51.8 ± 4.6, and 73.3 ± 8.0% of initial force remaining at 30 min, respectively, \( ^{**}P < 0.01, P < 0.001 \). B25 failed to show significant relaxation at 10 μM (fig. 1).

![Fig. 1.](image)
***P < 0.001, n = 6). All four TMEM16A antagonists relaxed an acetylcholine contraction at 100 μM (benzbromarone: −40.5 ± 8.2%, T16Ainh-A01: 35.1 ± 4.7%, MONNA: 21.8 ± 9.3%, and B25: 21.8 ± 9.3%, ***P < 0.001, n = 6, two-way ANOVA). Benzbromarone treatment resulted in significantly increased relaxation when compared with all three other TMEM16A antagonists at the 50 and 100 μM concentrations (P < 0.001 comparing benzbromarone with other three TMEM16A antagonists).

**Benzbromarone Relaxes Both an Acetylcholine and Leukotriene D4-induced Contraction in Human Airway Smooth Muscle**

Because benzbromarone was found to be the most effective TMEM16A antagonist in guinea pig tracheal ring organ bath studies, benzbromarone was further tested in human ASM. Human ASM was contracted with acetylcholine EC50 and then treated with vehicle (0.1% DMSO) or cumulative concentrations of benzbromarone in 30-min intervals. Vehicle treatment resulted in no change of contractile force, whereas increasing concentrations of benzbromarone caused a relaxation. When muscle was contracted with acetylcholine EC50, treatment with 10 and 50 μM benzbromarone resulted in a significant relaxation (10 μM: 68.5 ± 10.5%, 50 μM: 39.9 ± 13.3%, and 100 μM: 8.4 ± 12.5% of initial plateau contractile force remaining at 30 min, *P < 0.05, **P < 0.01, ***P < 0.001, n = 8; fig. 3). Therefore, the relaxation by benzbromarone is not specific to an acetylcholine contraction.

**Benzbromarone Relaxes Mouse Peripheral Airways**

As we had successfully shown that benzbromarone was effective in guinea pig tracheal muscle and human ASM from large central airways, we next sought to investigate whether it was effective in small distal peripheral airways. Mouse peripheral airways were contracted with methacholine and then treated with benzbromarone (fig. 4, A and B). Benzbromarone (10 μM) relaxed 72.4 ± 6.2% of a methacholine contraction in mouse peripheral lung slices (fig. 4C). The contraction of a peripheral airway in lung slice by methacholine and its subsequent relaxation by benzbromarone is shown in real time in an accompanying video (Supplemental Digital Content 1, http://links.lww.com/ALN/B172).

**Benzbromarone Potentiates Relaxation of β Agonists**

Next we sought to determine whether benzbromarone could act synergistically with β agonists, the first-line agents used to treat acute bronchospasm. Treatment with 10 μM benzbromarone decreased the isoproterenol EC50 for relaxation from 5.5 to 2.1 nM (n = 9, P < 0.01, two-way ANOVA), which represents a 2.6-fold potentiation of the relaxation effect of the β agonist. Treatment with 25 μM benzbromarone decreased the isoproterenol EC50 for relaxation from 5.5 to 0.7 nM (n = 9, P < 0.01, two-way ANOVA), which represents a 7.9-fold potentiation (fig. 5A). These findings indicate that benzbromarone is working synergistically with isoproterenol to relax an acetylcholine-induced contraction of ASM.

To further demonstrate that benzbromarone is working synergistically with isoproterenol, the percent of remaining...
contractile force was calculated after 1 nM of isoproterenol alone (a subtherapeutic dose in the organ bath model), after 10 μM benzbromarone alone, or after the combination of 1 nM isoproterenol/10 μM benzbromarone. The percent of the remaining contractile force with 1 nM isoproterenol alone was 88.9 ± 3.3% and with 10 μM benzbromarone alone was 79.6 ± 6.7%, whereas the percent remaining contractile force with the combination was 49.8 ± 8.0%.

Fig. 4. Benzbromarone (Benzb) relaxes methacholine (MCh) contraction in mouse peripheral airways. (A) Representative images of an airway in a lung slice at rest and after exposure to 0.3 μM MCh, 10 μM Benzb + MCh, and subsequent Benzb washout with MCh. The images were taken at the times indicated in numbers under the trace in B. (B) Changes in airway lumen area in response to MCh and Benzb superfused at the times indicated by the lines on top of the graph. The airway contracted in response to MCh, relaxed after addition of Benzb, recontraction after Benzb washout with MCh, and relaxed after MCh removal. (C) Summary of the changes in airway contraction in response to MCh, Benzb, and Benzb washout with MCh (n = 9 airways from two animals, ***P < 0.001 and **P < 0.01).
μproterenol. Benzb 10 μM treatment decreased the isoproterenol EC50 for relaxation from 5.5 to 2.1 nM (n = 9, P < 0.05, two-way ANOVA), which represents a 2.7-fold shift in relaxation (fig. 5C). Therefore, this relationship is also observed in human tissue with a β2 selective agonist commonly used for intraoperative bronchospasm.

**Benzbromarone Hyperpolarizes Human Airway Smooth Muscle Cells**

To elucidate the contributing cellular mechanisms involved in the ASM relaxation induced by benzbromarone, human ASM cells in culture were used. FLIPR membrane potentiometric dye was used to assess whether benzbromarone changed membrane potential. Potassium gluconate (40 mM) was used as a positive control as a depolarizing agent, and 10 μM NS1619 (K+ channel opener) was used as a positive control as a hyperpolarizing agent. Benzbromarone of (50 μM) caused a hyperpolarization reflected by a decrease in relative fluorescent unit (RFU) 115.3 ± 4.4 (***p < 0.001, n = 8 to 12; fig. 6).

**Benzbromarone Attenuates an Increase in Intracellular Calcium Induced by Histamine or Bradykinin**

To further elucidate the cellular mechanisms involved in relaxation induced by benzbromarone, the effects on Gq-coupled increases in intracellular calcium were investigated. Human ASM cells were loaded with the calcium indicator fura-2 AM and were pretreated with either vehicle or benzbromarone before the addition of histamine or bradykinin, agonists for receptors coupled to Gq. Figure 7A is a representative tracing demonstrating a dose-dependent attenuation by benzbromarone of the increase in intracellular calcium by histamine in the presence of 2 mM extracellular calcium. Treatment with 10 μM benzbromarone attenuated the peak in fluorescence from 0.83 ± 0.04 in control to 0.56 ± 0.07 RFU ratio (F/Fo) or 68.2 ± 8.4% of control (fig. 7B). The peak in fluorescence was attenuated dose-dependently, such that the peak calcium increase was 0.20 ± 0.05 (24.7 ± 5.5% control) with 50 μM benzbromarone and 0.02 ± 0.003 (or 2.3 ± 0.4% control) with (fig. 5B). These results suggest a synergistic relationship, as the percent relaxation of the combination of the two drugs (50.2%) is greater than the sum of each treated separately (11.1 + 20.4 = 31.5%).

For a further clinical correlation of this conclusion from guinea pig studies, studies with human ASM and albuterol were carried out. Benzbromarone (25 μM) decreased the EC50 of albuterol from 62.9 to 23.2 nM (n = 5, P < 0.05, two-way ANOVA), which represents a 2.7-fold shift in relaxation (fig. 5C). Therefore, this relationship is also observed in human tissue with a β2 selective agonist commonly used for intraoperative bronchospasm.

**Fig. 5. Benzbromarone (Benzb) treatment decreases the EC50 dose of isoproterenol (iso) or albuterol required for relaxation.** (A) When guinea pig tracheal rings were contracted with acetylcholine (Ach) EC50 and then treated with cumulatively increasing concentrations of albuterol, a dose-dependent relaxation was observed. At the 0.5 nM concentration of isoproterenol, vehicle (0.1% dimethyl sulfoxide [DMSO]), 10 μM Benzb, or 25 μM Benzb was added. The EC50 for relaxation with vehicle-treated guinea pig tracheal rings was 5.5 nM isoproterenol. Benzb 10 μM treatment decreased the isoproterenol EC50 for relaxation from 5.5 to 2.1 nM (n = 9, P < 0.01). Benzb 25 μM treatment decreased the isoproterenol EC50 for relaxation from 5.5 to 0.7 nM (n = 9 rings from four animals, P < 0.01 two-way ANOVA). (B) Treatment with a combination of isoproterenol 1 nM and Benzb 10 μM (n = 12) shows a significant decrease in airway smooth muscle force compared with treatments with either compound alone (relaxation not normalized to DMSO vehicle control, isoproterenol study carried out in the presence of DMSO vehicle of Benzb). *P < 0.05 compared with vehicle, $P < 0.001 compared with isoproterenol 1 nM alone (n = 10), and #P < 0.05 compared with Benzb 10 μM alone (n = 9). ns = not significant. (C) When human airway smooth muscle was contracted with Ach EC50 and then treated with cumulatively increasing concentrations of albuterol, (fig. 5B). These results suggest a synergistic relationship, as the percent relaxation of the combination of the two drugs (50.2%) is greater than the sum of each treated separately (11.1 + 20.4 = 31.5%).

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For a further clinical correlation of this conclusion from guinea pig studies, studies with human ASM and albuterol were carried out. Benzbromarone (25 μM) decreased the EC50 of albuterol from 62.9 to 23.2 nM (n = 5, P < 0.05, two-way ANOVA), which represents a 2.7-fold shift in relaxation (fig. 5C). Therefore, this relationship is also observed in human tissue with a β2 selective agonist commonly used for intraoperative bronchospasm.
Fig. 6. Benz bromarone (Benzb) hyperpolarizes, whereas Eact depolarizes human airway smooth muscle cells. (A) Representative tracings of continuous fluorescence recordings of membrane potential using fluorescent imaging plate reader potentiometric indicator. Eact and potassium gluconate (K-gluc) depolarize, whereas NS1619 (K⁺-channel opener) and Benzb hyperpolarize human airway smooth muscle cells. K-gluc and NS1619 were used as controls because they are known to depolarize and hyperpolarize, respectively. (B) Quantitative change in relative fluorescent units (RFU) showing a decrease in RFU with NS1619 (n = 12) and Benzb (n = 9) and an increase with K-gluc (n = 10) and Eact (n = 9; ***P < 0.001). dmso = dimethyl sulfoxide.

Fig. 7. Benz bromarone (Benzb) attenuates an increase in intracellular calcium induced by histamine or bradykinin in cultured human airway smooth muscle cells. (A) Representative fura-2 calcium tracings in the presence of 2 mM extracellular calcium. Human airway smooth muscle cells were pretreated with either vehicle (0.1% dimethyl sulfoxide [DMSO]) or Benzb (10, 50, and 100 μM), and then 10 μM histamine was pipetted into the wells of the plate. An increase in intracellular calcium was attenuated in a concentration-dependent manner by pretreatment with Benzb. (B, C) Average peak fluorescence in 2 mM extracellular calcium. Cells were pretreated with either vehicle (0.1% DMSO; n = 12) or Benzb (10, 50, and 100 μM) and then 10 μM histamine (B) (n = 9) or bradykinin (C) (n = 6). Pretreatment with Benzb (10, 50, and 100 μM) showed an attenuation of increases in intracellular calcium when compared with vehicle control (***P < 0.001). Attenuation was concentration dependent (#P < 0.001 compared with 10 μM, $P < 0.01 compared with 50 μM). (D) Representative tracing (as in fig. 7A) of intracellular calcium concentrations in the absence of extracellular calcium. Increases in intracellular calcium were attenuated by pretreatment with increasing concentrations of Benzb. (E, F) Average peak fluorescence (as in fig. 7, B and C) of intracellular calcium induced by histamine or bradykinin in the absence of extracellular calcium. Pretreatment with Benzb (50 and 100 μM) showed an attenuation of increases in intracellular calcium when compared with vehicle control (***P < 0.001; vehicle and 50 μM Benzb: n = 10 and 100 μM Benzb: n = 7).
100 μM benzbromarone (n = 6 to 12, ***P < 0.001 compared with vehicle control, #P < 0.001 compared with 10 μM, and $P < 0.01 compared with 50 μM). Similar results were observed when cells were challenged with bradykinin, another agonist of a receptor coupled to Gq (fig. 7C). Treatment with 10 μM benzbromarone attenuated the peak in intracellular calcium fluorescence from 0.90 ± 0.06 in control to 0.52 ± 0.04 (57.9 ± 4.4% of control), whereas treatment of 50 μM (0.12 ± 0.03 or 13.8 ± 2.9% control) and 100 μM (0.03 ± 0.007 or 2.9 ± 0.7% control) showed further significant attenuation. Therefore, benzbromarone attenuates the increase in intracellular calcium, which in the presence of 2 mM extracellular calcium is entering the cell cytosol from calcium channels on both the plasma membrane and the SR.

Calcium assays were then repeated in the presence of 0 mM external calcium to investigate whether benzbromarone was affecting intracellular calcium flux involving the SR. As demonstrated in figure 7D (compared with fig. 7A), calcium flux was attenuated by removal of external calcium as seen by smaller peak fluorescence as expected. Calcium flux in response to both histamine and bradykinin was not significantly attenuated by 10 μM benzbromarone but was attenuated by 50 and 100 μM benzbromarone pretreatment (fig. 7, E and F). With pretreatment of 50 μM benzbromarone, the increase in intracellular calcium with histamine was attenuated from 0.37 ± 0.06 in control to 0.05 ± 0.02 or 14.6 ± 6.3% of control (n = 7 to 10, ***P < 0.001). With bradykinin challenge, 50 μM benzbromarone attenuated peak fluorescence from 0.51 ± 0.05 in control to 0.03 ± 0.005 or 5.7 ± 1.1% of control (n = 7 to 10, ***P < 0.001). No further attenuation was seen by treatment with 100 μM. These results suggest that an increase in the component of TMEM16A antagonists’ attenuation of intracellular calcium is because of the blockade of SR calcium release.

**Benzbromarone Attenuates SR Calcium Release**

To further demonstrate that benzbromarone is affecting SR calcium release, we measured SR calcium flux with the calcium indicator mag-fluo-4 AM simultaneously with fura-2 AM. Mag-fluo-4 has been used as an indicator for SR calcium release because it has a low affinity for calcium and preferentially detects calcium in the SR where the calcium levels are higher.26 We first verified that mag-fluo-4 reflects SR calcium levels in ASM cells. For verification studies, we used thapsigargin, an irreversible SR calcium-ATPase inhibitor. SR calcium-ATPase continuously refills SR calcium; therefore, inhibition with thapsigargin causes a slow depletion of SR calcium stores. Cells were loaded with both mag-fluo-4 AM and fura-2 AM in calcium-free buffer, and thapsigargin was added. As seen in figure 8A, thapsigargin caused a decrease of mag-fluo-4 fluorescence, representing a loss of calcium from the SR, while simultaneously observing an increase in fura-2, representing an increase in cytosolic calcium as the calcium exits the SR. Cells were then treated with bradykinin, a Gq-coupled agonist known to induce SR calcium release. In vehicle-treated cells, bradykinin caused the expected decrease in mag-fluo-4 and increase in fura-2, representing calcium leaving the SR and entering the cytoplasm. In cells treated with thapsigargin, as expected no bradykinin effect was seen as cells had been predepleted of SR calcium.

After verifying the technique, the effect of pretreatment with benzbromarone and MONNA was investigated. As seen in the tracing in figure 8B, benzbromarone attenuated the increase in fura-2 and decrease in mag-fluo-4. Under control conditions with vehicle pretreatment, bradykinin treatment resulted in an increase in fura-2 fluorescence (fig. 8C) and a simultaneous 21.1 ± 2.2% decrease in mag-fluo-4 fluorescence (fig. 8D). Pretreatment with either benzbromarone (10 μM: 12.0 ± 0.8% and 50 μM: 6.2 ± 1.2%) or MONNA (10 μM: 14.5 ± 1.8% and 50 μM: 9.5 ± 2.0%) significantly attenuated this change (fig. 8D), confirming that TMEM16A antagonists were blocking SR calcium release.

Studies were then repeated in human ASM cells overexpressing the M3 muscarinic receptor to investigate whether this mechanism would also apply to relaxation of an acetylcholine contraction as observed in our organ bath studies. Acetylcholine treatment resulted in an increase in fura-2 fluorescence (fig. 8E) and a simultaneous 12.0 ± 1.8% decrease in mag-fluo-4 fluorescence (fig. 8F), and pretreatment with either benzbromarone (10 μM: 5.7 ± 2.6% and 50 μM: 2.0 ± 0.7%) or MONNA (10 μM: 2.3 ± 0.6% and 50 μM: 1.5 ± 0.4%) significantly attenuated this change (fig. 8F). All studies were carried out with simultaneous recording of fura-2 fluorescence (fig. 8, C and E).

**Discussion**

There are several reasons why perioperative bronchospasm is a major concern for anesthesiologists. Although most cases of acute bronchospasm are treated effectively by deepening the anesthetic and using inhaled bronchodilators, there is the potential for catastrophic patient outcomes when it does occur. This is corroborated by evidence derived from the American Society of Anesthesiologists closed-claims database, which shows that although these cases represent only a small number of total cases in the database (2%), when they do occur, 90% of these cases are associated with death or severe brain injury.27 Given the fact that 10 to 30% of people carry the diagnosis of asthma,1 that up to 34% of these patients are poorly controlled,28 and that in this latter group nearly one in five patients experience intraoperative bronchospasm,2,3 the likelihood of encountering this problem in the perioperative period is extremely high. This problem is further compounded by the fact that many patients with chronic bronchospastic disease are tolerant or refractory to β-adrenoceptor agonists.29 All these factors underscore the importance of expanding the anesthesiologist’s pharmacologic armamentarium to include novel compounds that relax acute bronchospasm in the perioperative period and can be used in concert with existing drugs to provide synergistic relaxation.
We believe that antagonists of the TMEM16A receptor of the calcium-activated chloride channel family belong to one such drug class. In this study, we demonstrate that four different TMEM16A antagonists relax acetylcholine-induced contractions in guinea pig tracheal rings and that benzobromarone relaxes both acetylcholine- and LTD4-induced contractions in human ASM. Although previous studies have shown that TMEM16A antagonists can prevent a methacholine contraction with pretreatment, this is the first study to demonstrate that they can relieve a pre-existing contraction to a variety of contractile mediators in both small and large airways and work synergistically with...
β agonists. In addition, we show mechanistic evidence that benz bromarone hyperpolarizes the plasma membrane of human ASM cells and attenuates intracellular calcium flux.

Although Huang et al. demonstrated that pretreatment with benz bromarone attenuated a methacholine-induced contraction in human ASM, and Zhang et al. used an ovalbumin-sensitized model to demonstrate that benz bromarone attenuates methacholine-induced contractions, the experimental approach in both of these studies does not mirror the clinical reality that anesthesiologists encounter in the perioperative period. In clinical scenarios, the need to relieve a preexisting bronchospasm is a more common occurrence, yet both of these studies used an experimental paradigm that gave a pretreatment before a contraction to attenuate the initiation of a contraction. Our study is unique in that we treated with benz bromarone after the ASM was precontracted and in a plateau phase of contraction. We specifically designed the experiments this way to mimic clinical bronchospasm. As such, this study is the first to show that benz bromarone holds promise as a prophylactic treatment to prevent bronchospasm and perhaps more importantly for perioperative physicians, that TMEM16A antagonism is an effective tool to acutely relieve ongoing bronchospasm.

The four different TMEM16A antagonists varied in their dose-dependent ability to relax ASM. This could be due to simply different potencies of each drug, but benz bromarone actually has the highest IC50 of all of the antagonists (9.97 μM) and demonstrated the greatest degree of relaxation. However, it is difficult to directly compare the IC50s of the four TMEM16A antagonists because the original measurements were done in different recombinant models using different high-throughput screening methods.

An alternative explanation for the differences in relaxation efficacies seen between these four TMEM16A antagonists is differences in their specificities for TMEM16A versus other chloride-modulating proteins, which may also contribute to ASM contractile tone. After the initial identification of these four novel TMEM16A antagonists, each group determined the selectivity for TMEM16A versus other chloride channel proteins. For example, 10 μM benz bromarone effectively blocks TMEM16A and TMEM16B, but not CFTR. The effect of benz bromarone on other chloride channels such as bestrophin or the CLC family of chloride transporters was not investigated. MONNA inhibited TMEM16A but not CFTR, CLC, or bestrophins. TMEM16B was not tested in the study, whereas benz bromarone was tested for the lack of effect at the CFTR channel, where no specificity information was given with T16Ainh-A01. It is possible that a component of benz bromarone’s effect is because of blockade of other chloride channels in addition to TMEM16A or another off-target effect that promotes relaxation.

Because benz bromarone was the most effective relaxant in guinea pig studies, it was chosen for translational studies using human ASM. Our present studies also uncovered an important mechanistic distinction regarding TMEM16A-mediated relaxation. Although Huang et al. showed that benz bromarone pretreatment was effective against a methacholine contraction, they failed to show a positive relaxant effect against a KCl contraction, and concluded that benz bromarone’s effect is specific to the G protein–coupled muscarinic receptor. However, we previously showed that pretreatment with either tannic acid or benz bromarone attenuated a substance P-induced contraction in guinea pig tracheal rings, and in addition, the current studies show effectiveness against an LTD4 contraction in human ASM. Therefore, this article clearly establishes the relaxant capacity of TMEM16A blockade against a variety of contractile mediators.

The capacity of TMEM16A antagonists to relieve LTD4–mediated contractile pathways cannot be understated. Leukotrienes, specifically LTD4, are the potent bronchoconstrictors and antileukotriene drugs that have been used to treat asthma since the 1990s. LTD4 binds to the CysLT receptor 1 to mediate mucus secretion and edema in addition to bronchoconstriction. Antileukotriene drugs such as montelukast work through antagonism of the CysLT receptor 1 receptor and have been shown to be clinically effective. If TMEM16A antagonists are also effective against LTD4 contractions (as in this study), they may work synergistically with antileukotriene drugs at downstream effector molecules associated with the G protein–coupled receptor pathway. Indeed, additional studies elaborating benz bromarone’s potential for modulating mucus secretion and edema involved in leukotriene signaling are underway.

In this study, we also demonstrated that benz bromarone is effective in peripheral and central airways. Given the observation that contractile and relaxant pathways may differ at different anatomical locations throughout the tracheobronchial tree, we included an experimental approach to illustrate that TMEM16A antagonism mediates prorelaxant effects on ASM throughout the lung. Our organ bath studies use guinea pig or human samples from large central airways, but we also tested benz bromarone’s effect on mouse peripheral airways. These latter studies are important because peripheral airway constriction is an important (but underrecognized) component of clinical bronchospasm. In the periphery, ASM completely encircles the airway lumen and constriction can cause significant obstruction to airflow and even distal alveolar collapse.

This study also examined the important role of potential synergistic drug effects between airway relaxants. Our study shows that TMEM16A antagonists behave synergistically with the accepted standard agent to treat bronchospasm, β agonists. Benz bromarone significantly potentiated the relaxation induced by isoproterenol and albuterol. As increasing doses of β agonists can lead to tachyphylaxis, drugs that work synergistically to both decrease chronic doses and rescue bronchospasm refractory to β agonists are invaluable. β Agonists work through the G protein–coupled β,β-adrenoreceptor, which activates adenylyl cyclase, increases cAMP, and activates
protein kinase A.\textsuperscript{37,38} Protein kinase A has many downstream targets including myosin light-chain kinase and the SR ryanodine receptor, as well as BK\textsubscript{Ca} channels.\textsuperscript{22} Therefore, β agonists also work through both modulation of intracellular calcium flux (through blockade of the SR ryanodine receptor) and modulation of plasma membrane potential (hyperpolarization through activation of BK\textsubscript{Ca} channels, important potassium channels in ASM). TMEM16A antagonists may be working synergistically by amplifying the blockade of SR calcium release and augmenting the hyperpolarization through blockade of chloride flux at the plasma membrane.

ASM cells have a resting membrane potential of approximately −60 mV, and the opening of a TMEM16A channel should cause depolarization, whereas blockade would cause hyperpolarization. Cellular studies confirmed that benzbromarone hyperpolarized the human ASM cell. The importance of membrane potential in relaxation of ASM has been questioned. When calcium channel blockers failed to show effectiveness for asthma despite efficacy in vascular smooth muscle, drugs targeting changes in membrane potential were abandoned.\textsuperscript{39} Therefore, although hyperpolarization favors relaxation, it may be only one contributing factor in relaxation through TMEM16A blockade. A more important role for TMEM16A may be found intracellularly. It has been proposed that blockade of chloride flux may not only prevent depolarization of the ASM cell at the plasma membrane but may also prevent calcium release from the SR because of the role of chloride in balancing the charge debt created by the movement of calcium across the SR.\textsuperscript{7} In our study, we demonstrate that benzbromarone attenuates calcium flux from both the plasma membrane and the SR. This unique dual action suggests multiple downstream targets of TMEM16A antagonism or suggests that TMEM16A may be present on the SR.

In conclusion, we have identified four different TMEM16A antagonists that demonstrate bronchorelaxant properties. In particular, the TMEM16A antagonist, benzbromarone, has been shown to relax both central and peripheral airways, work synergistically with β agonists, and is effective at relaxing a contraction induced by acetylcholine and LTD\textsubscript{4}. Benzbromarone works in part through membrane hyperpolarization and attenuation of calcium flux at both the plasma membrane and the SR, suggesting a novel role of chloride flux in ASM relaxation.

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Competing Interests

The authors declare no competing interests.

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