Intracellular interactions of umeclidinium and vilanterol in human airway smooth muscle

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Background: Intracellular mechanisms of action of umeclidinium (UMEC), a long-acting muscarinic receptor antagonist, and vilanterol (VI), a long-acting β2-adrenoceptor (β2R) agonist, were investigated in target cells: human airway smooth-muscle cells (ASMCs).

Materials and methods: ASMCs from tracheas of healthy lung-transplant donors were treated with VI, UMEC, UMEC and VI combined, or control compounds (salmeterol, propranolol, ICI 118.551, or methacholine [MCh]). Cyclic adenosine monophosphate (cAMP) was measured using an enzyme-linked immunosorbent assay, intracellular free calcium ([Ca2+]i) using a fluorescence assay, and regulator of G-protein signaling 2 (RGS2) messenger RNA using real-time quantitative polymerase chain reaction.

Results: VI and salmeterol (10-12–10-4 M) induced cAMP production from ASMCs in a concentration-dependent manner, which was greater for VI at all concentrations. β2R antagonism by propranolol or ICI 118.551 (10-12–10-4 M) resulted in concentration-dependent inhibition of VI-induced cAMP production, and ICI 118.551 was more potent. MCh (5×10-6 M, 30 minutes) attenuated VI-induced cAMP production (P<0.05), whereas pretreatment with UMEC (10-6 M, 1 hour) restored the magnitude of VI-induced cAMP production. ASMC stimulation with MCh (10-11–5×10-8 M) resulted in a concentration-dependent increase in [Ca2+]i, which was attenuated with UMEC pretreatment. Reduction of MCh-induced [Ca2+]i release was greater with UMEC + VI versus UMEC. UMEC enhanced VI-induced RGS2 messenger RNA expression.

Conclusion: These data indicate that UMEC reverses cholinergic inhibition of VI-induced cAMP production, and is a more potent muscarinic receptor antagonist when in combination with VI versus either alone.

Keywords: COPD pharmacology, cough/mechanisms/pharmacology, drug reactions

Introduction
Bronchodilation is central to the pharmacological treatment of stable chronic obstructive pulmonary disease (COPD),1 and is mediated either directly by smooth-muscle (SM) relaxation via stimulation of the β2-adrenoceptor (β2R) or indirectly by antagonism of the muscarinic receptor (MR) subtypes M2 and M3 in airway SM cells (ASMCs).2,3 Antagonism of the presynaptic M2 subtype leads to an increase in acetylcholine release from the vagal nerve ending, whereas antagonism of the postsynaptic M3 subtype increases the β2R-induced stimulation of adenyl cyclase activity and subsequent airway SM relaxation.4 Antagonism of the M3 subtype inhibits bronchial contractility.5

The long-acting MR antagonist (LAMA) umeclidinium (UMEC) and the combination of UMEC with the long-acting β2R agonist (LABA) vilanterol (UMEC/VI) are approved maintenance treatments for COPD in the US, Canada, the EU, and several other countries.6–11 In a previous study, once-daily UMEC/VI 62.5/25 μg was shown to improve lung function...
compared with UMEC 62.5 μg, VI 25 μg, and placebo. Significant improvements in trough forced expiratory volume in 1 second (FEV₁) were observed for UMEC/VI 62.5/25 μg compared with UMEC 62.5 μg (52 mL, P=0.004) and VI 25 μg (95 mL, P<0.001), in patients with moderate–severe COPD. In another 24-week trial, in patients with moderate–very severe COPD, an improvement of 90 mL (P=0.006) was observed for UMEC 62.5 μg added to VI 25 μg compared with VI 25 μg alone. Recently, the results of a trial demonstrated that the LAMA/LABA combination indacaterol/glycopyrronium was effective in preventing COPD exacerbations in patients with a history of exacerbations in the previous year.

The mechanisms of action of LAMAs and LABAs alone are reasonably well understood, although the precise interactions between the two signaling pathways – β₂R agonism and MR antagonism – are yet to be fully elucidated. Cyclic adenosine monophosphate (cAMP) is a key intracellular mediator in ASMCs for the activation of protein kinase A (PKA) (Figure 1). Stimulation of β₂R increases the activity of adenyl cyclase, resulting in increased production of intracellular cAMP. cAMP inhibits the release of intracellular free calcium ion ([Ca²⁺]), reduces membrane [Ca²⁺] entry, and removes Ca²⁺, which in turn leads to SM relaxation. Conversely, cholinergic activation of M₂ receptors inhibits adenyl cyclase and cAMP formation, and activation of M₁ receptors results in an increase in inositol trisphosphate formation and [Ca²⁺] release, leading to muscle contraction. These M₂- and M₁-receptor effects may be suppressed by MR antagonists, such as UMEC. In addition, stimulation of β₂R increases the synthesis of regulator of regulator of G-protein signaling 2 (RGS2), a protein that downregulates signaling from multiple Gₛ-coupled receptors (including M₁ and M₃ receptors), and thus may enhance the therapeutic activity of a LAMA.

Although the pharmacological profiles of both UMEC monotherapy and VI monotherapy have been reported previously, these experiments were carried out on Chinese hamster ovary (CHO) cells. Here, we present results of the first study of either UMEC or VI on target cells – human ASMCs. This is the first report of pharmacological data for UMEC and VI acting together at the target cells. We investigated the ability of UMEC (with or without VI) to induce cAMP production in the presence and absence of cholinergic activation. We tested our hypothesis that UMEC would attenuate methacholine (MCh)-induced [Ca²⁺] release (with or without VI), and that both UMEC and VI would induce RGS2.

Figure 1 Proposed mechanisms of action of the long-acting MR agonist UMeC and the long-acting β₂R agonist VI.
Abbreviations: AC, adenylyl cyclase; ACh, acetylcholine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; β₂R, β₂-adrenoreceptor; cAMP, cyclic adenosine monophosphate; CRE, cAMP-responsive element; CREB, CRE binding protein; G₁, inhibitory G proteins; G₂, stimulatory G protein; IP₃, inositol 1,4,5-trisphosphate; M₁, muscarinic receptor 1; M₂, muscarinic receptor 2; M₃, muscarinic receptor 3; MR, muscarinic receptor; PLC, phospholipase C; RGS2, regulator of G-protein signaling 2; UMeC, umeclidinium; VI, vilanterol.
Materials and methods

ASMC isolation and cell culture

ASMCs were isolated from healthy lung-transplant donor tracheas (n=8) and bronchoscopic biopsies (n=7) of healthy nonsmokers. All donors provided written informed consent. The study was approved by the Institution Ethics Committee (Royal Brompton and Harefield, and National Heart and Lung Institute, Imperial College London, London, UK [03-105]). Human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents.

ASMCs isolated from tracheas were transferred to a 25 cm² culture flask. Cells were grown to confluence using Dulbecco’s Modified Eagle’s Medium (Thermo Fisher Scientific, Paisley, UK) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1.5 μg/mL amphotericin B. For ASMCs isolated from bronchial biopsies, the biopsies were cut into small pieces (<1 mm²) and transferred to six-well culture plates. Once confluent, cells were harvested and split into larger flasks at each passage. Cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide, and medium were changed every 2–3 days. ASMCs were identified by the characteristic “hill-and-valley” morphology and expression of calponin, SM α-actin, and myosin heavy chains. By this definition, ASMCs comprised >95% of harvested cells.

After serum starvation, cells at passage five were plated into serum-deficient culture medium, and after 24 hours were treated with a range of concentrations of VI and UMEC (alone or in combination) or one of four control compounds: salmeterol (a β₂R agonist), propranolol (a pan-β-receptor antagonist), ICI 118.551 (a β₂R-specific antagonist), or MCh (an MR agonist).

cAMP-production experiments

The manufacturer’s protocol for the cAMP enzyme-linked immunosorbent-assay kit (Cayman Chemical Company, Ann Arbor, MI, US) was employed. Absorbance at 405–420 nm was measured using a colorimetric 96-well plate reader. Data are expressed as pmol/mL cAMP production into the culture medium. Background cAMP (ie, the concentration of cAMP in the absence of an agonist) was subtracted from the values of cAMP measured in the experiments.

cAMP production with β₂R agonism

ASMCs were plated (4×10⁴ cells/well) and treated with VI or salmeterol (both at 10⁻⁶ M). cAMP concentration in the resultant cell medium was analyzed at the following time points post-treatment: 10, 20, 30, 45, 60, and 90 minutes. In a separate repeat experiment, ASMCs were treated with VI or salmeterol at concentrations of 10⁻¹²–10⁻⁶ M for 60 minutes.

cAMP production with β₂R antagonism

ASMCs were plated (4×10⁴ cells/well) and pretreated with propranolol (10⁻⁶ M) or ICI 118.551 (10⁻⁶ M) for 30 minutes, followed by stimulation with VI (10⁻¹²–10⁻⁶ M) for 60 minutes.

Effect of UMEC on cAMP production

ASMCs were plated (4×10⁴ cells/well) and pretreated with UMEC (10⁻⁸ M) for 1 hour, followed by MCh (5×10⁻⁷ M) for 30 min and VI over a range of concentrations (10⁻¹²–10⁻⁶ M) for a further 1 hour.

Calcium assay

ASMCs were plated (8×10³ cells/well) and pre-treated for 2 minutes with UMEC (10⁻¹²–10⁻⁶ M), then stimulated with MCh (5×10⁻⁶ M) prior to measurement of [Ca²⁺], [Ca²⁺], was measured using a Fluo-4 no-wash calcium assay. ASMC media were replaced with 100 μL Fluo-4 loading dye (Fluo-4 NW Calcium Assay Kits, Invitrogen, Paisley, UK) according to the manufacturer’s protocol. After 45 minutes’ incubation at 37°C, excitation/emission 494/516 nm was measured on a 96-well fluorescent plate reader (BMG GmbH, Ortenberg, Germany). Basal fluorescence was measured for 5 seconds, and after treatment with MCh, fluorescence was measured for a further 55 seconds. The following MCh concentrations were used: 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 5×10⁻⁷, 10⁻⁶, and 5×10⁻⁶ M. Fluorescence was normalized to the maximal increase in fluorescence from baseline (=1) in response to MCh at 5×10⁻⁶ M. ASMCs were then pretreated with UMEC (10⁻¹⁰ M) for 2 minutes before measurement of [Ca²⁺], or pretreated for 2 minutes with UMEC (10⁻¹²–10⁻⁶ M) alone or in combination with VI (10⁻¹⁰, 10⁻⁹, 10⁻⁸ M) and then stimulated with MCh, before [Ca²⁺], was determined.

RGS2 messenger RNA

ASMCs were plated (1.6x10⁵ cells/well) and treated with VI or UMEC alone (both at 10⁻⁸ M) or in combination (both at 10⁻⁸ M) over a 4-hour time course. Total RNA was isolated from cells using an RNeasy minikit (Qiagen, Crawley, UK) and converted to complementary DNA using random primers and avian myeloblastosis virus reverse transcriptase (Promega, Southampton, UK). Messenger RNA (mRNA) expression was determined by real-time quantitative polymerase
chain reaction (Rotor-Gene 3000; Corbett Research, St Neots, UK) using SYBR Green PCR Mix Reagent (Qiagen) and normalized to 18S RNA expression.

Statistics
GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA, US) software was used for statistical analysis. Concentration-dependent responses were examined using one-way-analysis of variance (ANOVA) (Kruskal–Wallis test) followed by Dunn’s multiple comparison test. Mann–Whitney test comparison compared results between the groups. Negative logarithm of the inhibition constant (pKᵢ) data were determined from the inhibitor concentration causing a 50% reduction in response (IC₅₀) using the Cheng–Prusoff correction.²⁵ Data in all figures are plotted as mean ± standard error of the mean. Each replicate was performed on a separate sample of cells from a different donor. P<0.05 was taken as statistically significant.

Results

**cAMP production following β₂R agonism**
Both VI (10⁻⁶ M) and salmeterol (10⁻⁶ M) induced cAMP production over a 90-minute time course; however, the magnitude of induction was greater with VI compared with salmeterol (Figure 2A). The greatest difference occurred at 30 minutes poststimulation, where VI induced a cAMP production of 21.65±1.86 pmol/mL compared with 3.45±2.09 pmol/mL for salmeterol.

The time to 50% of the maximal recorded cAMP concentration for VI and salmeterol was 20 versus 38 minutes, respectively. cAMP production in response to a range of concentrations (10⁻¹²–10⁻⁶ M) of VI or salmeterol at 60 minutes poststimulation was subsequently examined. cAMP production was found to be concentration-dependent for both VI and salmeterol (Figure 2B), and greater for VI versus salmeterol at all concentrations. The maximal cAMP production for VI (10⁻⁷ M) and salmeterol (10⁻⁷ M) was 12.03±2.18 pmol/mL and 4.46±1.16 pmol/mL (P<0.01, Kruskal–Wallis, n=7), respectively (Figure 2B). The concentration of VI and salmeterol required to stimulate half the maximal response (EC₅₀) was 3.5×10⁻¹⁰±1.1×10⁻¹⁰ M and 2.1×10⁻⁸±1.3×10⁻⁸ M, respectively.

**cAMP production following β₂R antagonism**
Propranolol effectively inhibited VI-induced cAMP production, causing a substantial decrease in its potency while having a limited effect on maximal response (as expected for a competitive antagonist) (Figure 3A). In contrast, ICI 118.551 effectively inhibited VI-induced cAMP production over all concentrations examined (Figure 3A). The EC₅₀
for VI in the presence of propranolol and ICI 118.551 was 7×10⁻⁸±3.4×10⁻⁸ M and 6×10⁻¹⁰±3.5×10⁻¹⁰ M, respectively. Further analysis across a range of ICs showed that both propranolol and ICI 118.551 inhibited VI-induced cAMP accumulation in a concentration-dependent manner (Figure 3B). The pKᵢ for ICI 118.551 was greater than that of propranolol (pKi±SEM: 13.91±0.68 versus 11.44±0.26, respectively).

**Effect of cholinergic stimulation in presence and absence of UMEC on VI-induced cAMP production**

For all concentrations of VI, pretreatment of ASMCs with MCh (5×10⁻⁶ M) for 30 minutes attenuated VI-induced cAMP production (Figure 4A). At the lowest concentration of VI (10⁻¹² M), cAMP production was reduced from 6.95±1.56 pmol/mL to 4.25±1.37 pmol/mL, and at the highest concentration of VI (10⁻⁶ M) cAMP production was reduced from 12.09±1.8 pmol/mL to 9.14±1.34 pmol/mL.

Pretreatment with UMEC (10⁻⁸ M) for 1 hour restored the magnitude of the VI-induced cAMP production at all concentrations of VI in the presence of MCh (Figure 4B). The EC₅₀ and the maximum effect (Eₘₐₓ) elicited by the agonist for VI alone was 7.6×10⁻¹⁰ M and 12.1±1.8 pmol/mL, respectively; for UMEC + VI, EC₅₀ and Eₘₐₓ were 2.6×10⁻¹⁰ M and 14.2±3.8 pmol/mL, respectively; for UMEC + VI + MCh, EC₅₀ and Eₘₐₓ were 2×10⁻¹⁰ M and 12.4±2.04 pmol/mL, respectively. In the absence of MCh, pretreatment with UMEC did not enhance VI-induced cAMP production, and importantly did not result in a reduction in cAMP production. Neither UMEC nor MCh alone induced a detectable difference in cAMP production (data not shown).

**Effect of M₂- and M₃-receptor antagonism (UMEC) on MCh-induced Ca²⁺ release**

Stimulation of ASMCs with MCh (10⁻¹¹–5×10⁻⁶ M) resulted in a concentration-dependent increase in [Ca²⁺]ᵢ (P<0.001, Kruskal–Wallis, Figure 5A). UMEC attenuated MCh-induced [Ca²⁺]ᵢ release in a concentration-dependent manner, with a maximum suppressive effect of 50% at a concentration of 10⁻⁶ M (Figure 5B). Attenuation of [Ca²⁺]ᵢ release was observed over the full range of MCh concentrations used (Figure 5C), reaching significance at 5×10⁻⁷ M (P<0.05) and 5×10⁻⁶ M (P<0.01).
Effect of β₂R activation (VI) on M₂- and M₃-receptor antagonist (UMEC)-mediated attenuation of MCh-activated Ca²⁺ release

Following the observation of the suppressive effect of M₂- and M₃-receptor antagonism on MCh-activated [Ca²⁺], release, potential cross talk between the muscarinic and β₂R-signaling pathways on attenuation of [Ca²⁺] was investigated. UMEC (10⁻¹²–10⁻⁸ M) in combination with VI (10⁻⁸, 10⁻⁶ M) reduced the [Ca²⁺] release elicited by MCh to a greater extent than UMEC alone (Figure 6A and B). The inhibitory effect of UMEC alone at 10⁻⁸ M was achieved with a 100-fold reduction in concentration of UMEC (10⁻¹² M) in the presence of even the lowest concentration of VI (10⁻⁶ M). The greatest enhancement in the suppressive effect of UMEC was observed at 10⁻⁶ M VI (P<0.01) (Figure 6B).

RGS2 marker for possible β₂R and MR interaction

VI induced expression of RGS2 mRNA, with maximal expression reached at approximately 60 minutes. This was maintained to 2 hours postinduction, but expression then declined to 50% of the maximum value at 4 hours (Figure 7). UMEC alone had no observed effect on RGS2 mRNA expression. However, the presence of UMEC-enhanced VI-induced RGS2 mRNA expression across all time points, reaching significance at 4 hours compared with VI alone (8.5±1.3-fold increase compared with baseline, respectively; P<0.05).

Discussion

We investigated the intracellular effects of UMEC and VI at their sites of action in healthy human ASMCs to provide an insight into the possible mechanisms underlying improvement in lung function observed in patients with COPD receiving UMEC/VI treatment compared with its components UMEC and VI. Overall, results demonstrated that VI is a more effective inducer of cAMP production than salmeterol over the range of time points and concentrations studied, thus supporting previous research conducted in CHO cells. Antagonism of β₂R suppressed the ability of VI to induce cAMP expression. We also demonstrated that MCh stimulation of ASMCs resulted in a concentration-dependent increase in [Ca²⁺] which was attenuated by pretreatment with UMEC. A major

Figure 4 UMEC antagonism of muscarinic receptors reverses MCh-induced attenuation of VI-mediated cAMP production.

Notes: (A) cAMP production in human ASMCs with VI for 1 hour following 30-minute pretreatment with MCh (5×10⁻⁶ M; n=8; *P<0.05, **P<0.01, ***P<0.001 VI versus VI+MCh). (B) VI concentration–response curves for cAMP production at 1 hour in human ASMCs (n=8; #P<0.05, ##P<0.01 VI+MCh versus VI+MCh+UMEC; ¶P<0.05 VI+MCh versus VI+UMEC).

Abbreviations: ASMCs, airway smooth-muscle cells; cAMP, cyclic adenosine monophosphate; MCh, methacholine; UMEC, umeclidinium; VI, vilanterol.
The finding of this study was that attenuation of VI-induced cAMP production by MCh was counteracted by UMEC. Further, although pretreatment of ASMCs with VI alone had no effect on inhibiting MCh-induced $\text{Ca}^{2+}$ release, pretreatment with UMEC in the presence of VI improved both the potency of UMEC (between 10- and 100-fold) and increased maximal inhibition. VI-induced RGS2 mRNA expression was also enhanced by UMEC, though UMEC pretreatment with UMEC in the presence of VI improved both the potency of UMEC (between 10- and 100-fold) and increased maximal inhibition. VI-induced RGS2 mRNA expression was also enhanced by UMEC, though UMEC had no effect on inhibiting MCh-induced $\text{Ca}^{2+}$ release.

Figure 5: The antagonism with UMEC suppressed MCh-induced $\text{Ca}^{2+}$ release.

Notes: (A) Concentration–response curve for the release of $\text{Ca}^{2+}$ in response to MCh. (B) Concentration–response curve for the release of $\text{Ca}^{2+}$ in human ASMCs pretreated with 10-8 M UMEC for 2 minutes, followed by one-way ANOVA and Dunnett's multiple comparison test. (C) Concentration–response curve for the release of $\text{Ca}^{2+}$ in human ASMCs in response to MCh, after pretreatment with UMEC for 2 minutes. *P < 0.05, **P < 0.01 MCh versus UMEC (10-8 M).

Abbreviations: ANOVA, analysis of variance; ASMC, airway smooth muscle cell; MCh, methacholine; Mr, muscarinic receptor; UMEC, umeclidinium.
alone did not affect RGS2 expression. Together, these data show that UMEC is a more potent inhibitor of the effects of MCh when in combination with VI versus UMEC monotherapy, and suggest that dual bronchodilators may work together to elicit bronchodilation via the upregulation of RGS2 and the augmentation of cAMP production.

VI is a LABA with a fast onset of activity and a long duration of action, resulting in bronchodilation for at least 24 hours. In human lung slices, VI has been shown to induce rapid reversal of cholinergic bronchoconstriction in vitro, reaching maximal bronchodilation in less than 15 minutes. This was quicker than salmeterol, supporting the results presented here, where VI induced more rapid cAMP production than salmeterol (Figure 2A).

While MR activation attenuated VI-induced cAMP production, pretreatment with UMEC restored the magnitude of the VI-induced cAMP production. This indicates that blocking of MR with UMEC prevents interaction with β2R, thus potentially enabling VI in the presence of MCh to elicit a biological response. VI-induced cAMP production was blocked by a specific β2R antagonist (ICI 118.551), indicating that VI acts through the β2R on ASMCs. This corresponds with a recent in vitro study, which demonstrated the high selectivity for β2R versus β1R of VI. Clinically, this finding would suggest that any transfer of VI from the lung into the circulatory system is unlikely to have negative side effects.

Figure 6

**β2R agonist VI augments UMEC-mediated attenuation of MCh-induced release in [Ca2+]i.**

**Notes:** (A) Concentration-response curves for Ca2+ release in ASMCs pretreated with UMEC alone or in combination with VI for 2 minutes, followed by stimulation with MCh (n=6–8). (B) Ca2+ release following treatment with MCh (5×10-6 M), with VI alone for 2 minutes, UMEC alone, and a combination of UMEC with a selected range of VI concentrations. **P<0.01 UMEC 10-12 M versus UMEC + VI 10-6 M (n=6–8).**

**Abbreviations:** ASMCs, airway smooth-muscle cells; β2R, β2-adrenoreceptor; MCh, methacholine; UMEC, umeclidinium; VI, vilanterol.

Figure 7

**β2R-agonist-induced RGS2 expression is enhanced by MR antagonism.**

**Notes:** Time course for the expression of RGS2 messenger RNA in response to VI (10-8 M), UMEC (10-8 M), or UMEC/VI (both at 10-8 M) (n=6–8). **P<0.05 VI versus VI + UMEC, paired t-test.

**Abbreviations:** β2R, β2-adrenoreceptor; RGS2, regulator of G-protein signaling; MR, muscarinic receptor; UMEC, umeclidinium; VI, vilanterol.
effects, eg, in the heart (cardiac myocytes), where the $\beta_R$ predominates.\textsuperscript{28,29} This suggestion is supported by a previous randomized trial, in which no dose-related effects on blood pressure or pulse rate with inhaled VI were reported.\textsuperscript{30}

The therapeutic benefit of such LAMAs as UMEC lies in their ability to counteract bronchoconstriction in response to vagal reflexes triggered by stimulation of airway sensory nerve endings and the subsequent release of acetylcholine (Figure 1).\textsuperscript{31,32} Studies in COPD also report heightened levels of acetylcholine release in patients with COPD; this is correlated with disease severity and an increase in $M_3$ receptor expression and signaling, which enhance the bronchoconstrictive drive. The major mechanism by which MR antagonists, such as UMEC, affect bronchodilation is through blocking the action of acetylcholine at the $M_2$ and $M_3$ receptors on ASMCs, inhibiting bronchoconstriction and thereby leading to SM relaxation.

A key finding in this study was that the reduction in [Ca\textsuperscript{2+}] release elicited by MCh was greater with the UMEC–VI combination compared with UMEC alone. These data provide strong evidence for an interaction among the two signaling pathways, $\beta_2$R agonism, and MR antagonism, and that $\beta_2$R agonists have an effect on MR agonist-induced [Ca\textsuperscript{2+}] increase, but only in the presence of UMEC. Of note, the reduction in [Ca\textsuperscript{2+}] release was observed down to $10^{-12}$ M of UMEC.

In this study, UMEC was shown to enhance VI-induced RGS2 mRNA expression, while UMEC alone did not affect RGS2 expression. Acetylcholine is known to activate MAPK signaling,\textsuperscript{33} which influences gene transcription. UMEC may enhance the effects of $\beta_2$R activation by inhibiting the activation of MR through reversing the effects of acetylcholine produced by ASMCs.\textsuperscript{34,35} Regulators of G-protein signaling proteins are GTPase-activating proteins that attenuate signaling by heterotrimeric $G_i$-coupled G-protein-coupled receptors, including muscarinic $M_1$, $M_3$, and $M_4$ receptors.\textsuperscript{36,37} RGS2 is a highly potent and relatively selective inhibitor of $G_{s}$ function,\textsuperscript{38} which is coupled to both the $M_2$ and $M_3$ receptors. RGS2 activation/induction is known to block $M_3$ receptor-mediated activation of phospholipase C and PKA, and hence airway SM contraction,\textsuperscript{36,39} and inhibit $M_2$-receptor signaling.\textsuperscript{40} The latter could also reduce the negative impact of cholinergic activation of the $M_3$ receptor on $\beta_2$R-stimulated adenylyl cyclase activity (Figure 1). The $RGS2$ proximal gene promoter contains a conserved cAMP-response element that is critical for CREB binding and RGS2-promoter activation,\textsuperscript{41} and RGS2 gene transcription has been shown to be cAMP-dependent in ASMC.\textsuperscript{19} Moreover, LABAs and glucocorticoids can synergistically enhance RGS2 expression;\textsuperscript{32} evidence from our group shows VI can enhance the anti-inflammatory effects of the inhaled corticosteroid fluticasone furoate. In blood cells from patients with COPD and asthma, VI may act as a steroid-sparing agent (Khorasani et al, personal communication, 2016). Overall, these studies suggest the potential for triple therapy, where the addition of low-dose fluticasone furoate could improve the bronchodilatory effects of UMEC/VI by synergistically increasing VI-induced RGS2 expression. Future studies need to test the effect of adding an inhaled corticosteroid to a LAMA/LABA combination in ASMCs. Data also suggest that the potential synergy between UMEC and VI could allow for reduced therapeutic doses of UMEC/VI compared with corresponding doses of monotherapy, thereby reducing the risk of side effects associated with long-term usage of long-acting bronchodilators.

Limitations of this study include the different cell batches used, which may have introduced variations in the study data. Additionally, experiments in this study were conducted in ASMCs from healthy donor lungs, in order to provide a surrogate for studying tissue of patients affected by COPD or asthma. However, the use of healthy lung donor tissue may be perceived as a possible limitation, and it would be informative for future studies to be conducted in ASMCs from patients with COPD or asthma. A study by Lo et al (personal communication, 2016) showed that salmeterol can reduce the proliferation, myofibroblastic differentiation, and CC-chemokine receptor 7 expression of fibrocytes from healthy subjects and patients with nonsevere asthma, but not from patients with severe asthma. Moreover, fibrocytes from patients with severe asthma had lower baseline surface $\beta_2$R expression, but were not insensitive to the direct effects of exogenous cAMP (8-Br-cAMP). This finding suggests an impairment at the level of $\beta_2$R itself. It is possible that impairments in $\beta_2$R or $\beta_2$R signaling may lead to increased use of bronchodilator therapy in patients with severe COPD or asthma, though further studies would need to test this hypothesis.

With regard to the mechanisms involved in RGS2 expression, further experiments would be useful in confirming our hypothesis that UMEC+VI enhances RGS2 expression compared with VI alone. These experiments include Fluo4 fluorescence studies in $RGS2$-knockout ASMCs to determine whether the absence of RGS2 expression affects [Ca\textsuperscript{2+}] release, and real-time examination of the effect of UMEC, VI and UMEC/VI on [Ca\textsuperscript{2+}] release and RGS2 expression/activity in the presence and absence of $\beta_2$R antagonists over the time frame of the cAMP-monitoring experiments (90 minutes).
This would provide further evidence to determine whether RGS2 expression is directly affected by UMEC and VI. These experiments were beyond the scope of the current investigation, but should be considered for future studies.

In summary, we showed that in human ASMcs: 1) VI induces cAMP production from β2R more effectively than salmeterol, 2) cholinergic activation attenuates VI-induced cAMP production, 3) attenuation of VI-induced cAMP production by MCh is counteracted by UMEC, 4) the combination of UMEC+VI attenuated cholinergic agonist-induced [Ca2+]i release to a greater extent than UMEC alone, and 5) UMEC enhanced VI-induced RGS2 mRNA expression. The mechanism for the enhanced [Ca2+]i release with UMEC+VI compared with UMEC alone could lie in the increase in RGS2 mRNA expression, which inhibits Gαi-coupled receptors. We thus provided some insight into the mechanisms of and rationale for the use of dual (LAMA/LABA) bronchodilators over their monotherapy components.

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Author contributions
All authors contributed toward drafting and revising the paper. NS, MJ, KFC, JHR, SW and PKB designed the research. NS and PKB performed the research. NS, MJ, DAH, JHR, SW and PKB analyzed the data. All authors agree to be accountable for all aspects of the work.

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