European Journal of Pharmacology 745 (2014) 135–143

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

Pharmacological characterization of the interaction between aclidinium bromide and formoterol fumarate on human isolated bronchi

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form
13 October 2014
Accepted 15 October 2014
Available online 22 October 2014

Chemical compounds studied in this article:
Aclidinium bromide
Formoterol fumarate

Keywords:
LAMA
LABA
Human bronchi
Synergistic interaction
COPD

ABSTRACT

Long-acting muscarinic receptor antagonists (LAMAs) and long-acting β_2-adrenoceptor agonists (LABAs) cause airway smooth muscle (ASM) relaxation via different signal transduction pathways, but there are limited data concerning the interaction between these two drug classes on human bronchi. The aim of this study was to investigate the potential synergistic interaction between aclidinium bromide and formoterol fumarate on the relaxation of human ASM. We evaluated the influence of aclidinium bromide and formoterol fumarate on the contractile response induced by acetylcholine or electrical field stimulation (EFS) on human isolated airways (segmental bronchi and bronchioles). We analyzed the potential synergistic interaction between the compounds when administered in combination by using Bliss independence (BI) theory. Both aclidinium bromide and formoterol fumarate completely relaxed segmental bronchi pre-contracted with acetylcholine (E_{max}: 97.5 ± 2.6% and 96.4 ± 1.1%; pEC_{50} 8.5 ± 0.1 and 8.8 ± 0.1; respectively). Formoterol fumarate, but not aclidinium bromide, abolished the contraction induced by acetylcholine in bronchioles (E_{max}: 68.1 ± 4.5% and 99.0 ± 5.6%; pEC_{50} 7.9 ± 0.3 and 8.4 ± 0.3; respectively). The BI analysis indicated synergistic interaction at low concentrations in segmental bronchi (+18.4 ± 2.7%; $P<0.05$ versus expected effect) and from low to high concentrations in bronchioles (+19.7 ± 0.9%; $P<0.05$ versus expected effect). Low concentrations of both drugs produced a synergistic relaxant interaction on isolated bronchi stimulated with EFS that was sustained for 6 h post-treatment (+55.1 ± 9.4%; $P<0.05$ versus expected effect). These results suggest that combining aclidinium bromide plus formoterol fumarate provides synergistic benefit on ASM relaxation of both medium and small human airways, which may have major implications for the use of this combination in the clinic.

1. Introduction

Bronchodilators are crucial for the management of symptoms of chronic obstructive pulmonary disease (COPD) and asthma (GINA, 2012; GOLD, 2014). There are currently two classes of long-acting bronchodilators with different pharmacological mechanisms: muscarinic receptor antagonists agents and β_2-adrenoceptor agonists. Long-acting muscarinic receptor antagonist (LAMAs) and long-acting β_2-adrenoceptor agonists (LABAs) are used for regular treatment of COPD, whereas short-acting muscarinic receptor antagonists agents and

Abbreviations: β_2-AR, β_2-adrenoreceptor; ASM, airway smooth muscle; BI, Bliss independence; COPD, chronic obstructive pulmonary disease; CRC, concentration-response curve; E, effect; EFS, electrical field stimulation; EC_n, effective concentration for n% of maximal effect; E_{max}, maximal effect; FEV_1, forced expiratory volume in 1 s; GINA, Global Initiative for Asthma; GOLD, Global Initiative for Chronic Obstructive Lung Disease; KH, Krebs-Henseleit; LAMA, long-acting antimuscarinic; LABA, long-acting β_2-adrenoceptor agonist; pEC_{50}, the negative logarithm of EC_{50}; PCLS, precision-cut lung slices

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http://dx.doi.org/10.1016/j.ejphar.2014.10.025
0014-2999/

short-acting β_2-adrenoceptor agonists are used as rescue medications for acute treatment of airways obstruction.

Combining a LAMA with a LABA might be a valuable therapeutic approach for maintenance treatment of patients with stable-moderate COPD whose symptoms are not adequately controlled with monotherapy using the LAMA tiotropium bromide (Rodrigo et al., 2012). Furthermore, it has been suggested that fixed-dose combination therapy with two classes of bronchodilator in the same inhaler would simplify treatment regimens and improve patient adherance (Cazzola and Matera, 2008).

A twice-daily fixed-dose combination of the LAMA aclidinium bromide and the LABA formoterol fumarate is currently under clinical development for the treatment of COPD. The Phase III ACLIFORM COPD study showed that aclidinium bromide/formoterol fumarate 400/6 μg and 400/12 μg significantly improved 1-h post-dose forced expiratory volume in 1 s (FEV_1) versus aclidinium bromide monotherapy and trough FEV_1 versus formoterol fumarate monotherapy at Week 24 (Singh et al., 2014). Both parameters were significantly improved versus placebo and the findings of a second Phase III study – AUGMENT COPD – were similar (D'Urzo et al., 2014).

Although these studies suggest that combining aclidinium bromide with formoterol fumarate may be clinically useful, the full extent of the interaction between these compounds is not yet well understood. As LABAs and LAMAs both cause airways smooth muscle (ASM) relaxation via different signal transduction pathways, combining a LABA plus a LAMA might prove beneficial for a number of reasons (Cazzola and Molimard, 2010). For example, LABAs decrease the release of acetylcholine through modulation of cholinergic neurotransmission by acting on prejunctional β_2-adrenoreceptors (β_2-ARs) leading to the activation of calcium-activated potassium channels that hyperpolarize the cell membrane, which amplifies the ASM relaxation induced by the LAMA. Furthermore, LAMAs antagonize the bronchoconstrictor effects of acetylcholine, whose release can be modified by the LABA, which may amplify the bronchodilation induced by the LABA through the direct stimulation of ASM β_2-ARs (Cazzola and Molimard, 2010; Cazzola et al., 2013). In addition, crosstalk between G_q-coupled M_3 receptors and G_s-coupled β_2-ARs may influence the β-agonist-induced relaxation, possibly by activation of protein kinase C (PKC) and subsequent phosphorylation of β_2-AR and/or G_s protein (Cazzola et al., 2013).

Although there is clear scientific rationale for combining LAMAs and a LABAs in the treatment of COPD, to date there are limited pharmacologic data investigating the interaction between these drugs at the level of human bronchi. Therefore, the aim of this study was to investigate the pharmacological interaction between aclidinium bromide and formoterol fumarate on the relaxation of human segmental bronchi. In addition, small airways were evaluated in order to better understand the interaction between these drugs on ASM found at different anatomical levels of human respiratory tract.

2. Material and methods

2.1. Ethical approval and informed consent

Ethical approval and informed consent were obtained from the Istituto Regina Elena – Istituto San Gallicano (Rome, Italy) and they were consistent with the 2009 National Committee of Bioethics, National Committee of Bio-safety, Biotechnology and Sciences (Italy) recommendations on the collection of biological samples for research purposes, the 2010 Italian ethical and legal recommendations concerning the biobank, and the research biorepository (Istituto Nazionale dei Tumore – Independent Ethics Committee, 2010), and the Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze per la Vita (Raccolta di campioni biologici a fini di reicerca, consenso informato, 2009; available at: http://www.governo.it/bioetica/gruppo_misto/Consenso_Informato_allegato_Petrini_2009.pdf).

2.2. Preparation of tissues

Regions of macroscopically normal lungs were taken from uninvolved areas resected from 23 patients (14 male, 9 female; aged 63.1 ± 2.2 years) undergoing lobectomy for lung cancer, but without a history of chronic airway disease.

Tissue samples were immediately placed into oxygenated Krebs-Henseleit (KH) buffer solution (NaCl 119.0 mM, KCl 5.4 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25.0 mM, glucose 11.7 mM; pH 7.4) containing the cyclooxygenase inhibitor indomethacin (5.0 μM), and transported at 4 °C from the Regina Elena National Cancer Institute (Rome, Italy) to the Laboratory of Respiratory Pharmacology in the Medical School of the University of Rome "Tor Vergata" (Rome, Italy). None of the patients had been chronically treated with theophylline, β_2-agonists, or glucocorticosteroids. Serum immunoglobulin E levels determined on the day of surgery were in the normal range. Preoperative lung function parameters were generally normal and there were no signs of respiratory infections.

In the laboratory, airways were dissected from connective and alveolar tissues and refrigerated overnight in KH buffer solution. The next morning, bronchi were cut into rings (medium airways, segmental bronchi; thickness: 1–2 mm; diameter: 4–6 mm) and transferred into a 10 ml High Tech 8 Channels Manual Compact Organ Bath system (Panlab Harvard Apparatus, Spain) containing KH buffer solution (37 °C) and continuously aerated with O_2/CO_2 (95:5%).

Precision-cut lung slices (PCLSs) were sectioned (small airways, bronchioles; thickness: < 500 μm; diameter: 0.93 ± 0.08 mm) using a Vibroslice Microtome equipped with ceramic blades (Campden Instruments, UK). Slices were processed without the complications related to the use of confounding agarose gel to inflate the lung or complex parenchymal sections that have numerous contracting elements (Calzetta et al., 2014b; van Lunteren and Moyer, 2001; Wohlsen et al., 2001). PCLSs were mounted into a Visual Imaging and Patching Chamber connected to a Proportional Integral Derivative Temperature Controller with dual thermistor feedback CI7800 (Campden Instruments, UK), containing KH buffer solution (37 °C) and continuously aerated with O_2/CO_2 (95:5%).

2.3. Preparation of drugs

Test compounds were maintained under dry conditions and prepared daily. Stock solutions used in this study were: acetylcholine (Sigma-Aldrich, Italy), papaverine (Sigma-Aldrich, Italy), indomethacin (Sigma-Aldrich, Italy), formoterol fumarate (a kind gift from Almirall, Spain), and aclidinium bromide (a kind gift from Almirall, Spain). All products were dissolved in distilled water, except aclidinium bromide, which was dissolved in 1N HCl 1% (v/v). Indomethacin was dissolved in pure ethanol and diluted in KH buffer solution prior to use. The maximal concentration of ethanol used to prepare drug solutions (0.02%) did not influence isolated tissue responses as reported elsewhere (Freas et al., 1989; Hatake and Wakabayashi, 2000). Appropriate dilutions were obtained in freshly prepared medium. Stock solutions were stored at −80 °C until use.

2.4. Measurement of bronchial smooth muscle contraction

Each bronchial ring was connected to an isometric force transducer (Fort25; WPI, UK) and was allowed to equilibrate for 90 min before being flushed with fresh KH buffer solution every 10 min. The signal was amplified by a Powerlab 8/36 and Octal Bridge Amp system (AD instruments, UK) and recorded and

analyzed with LabChart 7 interface software (AD instruments, UK). Passive tension was determined by gentle stretching of the tissue (0.5–1.0 g) during equilibration. The transducer measured the isometric change in tension and tissue responsiveness was assessed using acetylcholine (100 μM) or electrical-field stimulation (EFS) at 10 Hz. When the contractile response reached a plateau, the bronchial rings were washed three times and allowed to equilibrate further.

Videomicrometry of small airway preparations was assessed using a stereo microscope (SZR-10 Zenith, Italy) and a digital Optikam-B5 managed by OptikaView 7 Software (Optika Microscopes, Italy). The small airways were continuously flushed with KH buffer solution for 90 min to allow them to equilibrate and to ensure that the luminal area was stable. The luminal area was measured using image processing and analysis software Image J (National Institutes of Health, Bethesda, USA).

2.5. Study design

2.5.1. Study 1: evaluation of the interaction between aclidinium bromide and formoterol fumarate on the relaxation of human segmental bronchi and small airways pre-contracted with acetylcholine

Following equilibration of the tissues, bronchial rings/PCLSs were submaximally contracted with acetylcholine (70% of maximal contraction [EC_{70}]). Experiments were carried out in an isolated organ bath system or in a videomicrometry system to evaluate the relaxation associated with ASM strength and the related increase in intraluminal bronchial area.

After approximately 15 min at plateau, semi-logarithmic concentration-response curves (CRCs) to aclidinium bromide and/or formoterol fumarate, alone or in combination, were constructed at a range of isoeffective concentrations from 0.1 nM to 10 μM for each compound. Each CRC was obtained by the cumulative addition of aclidinium bromide and/or formoterol fumarate at 5–15 min intervals to reach a stable level of relaxation before the next concentration was administered.

A cumulative concentration of vehicle was used as a time control. At the end of the experiments, papaverine (100 μM) was added to the bronchial rings and PCLSs to determine the maximal relaxant response (E_{max}) achievable for each isolated bronchus.

2.5.2. Study 2: an investigation of the time course of the interaction between aclidinium bromide and formoterol fumarate on the relaxation of human isolated segmental bronchi contracted by EFS

Contractile responses were induced by EFS, delivered by using bipolar platinum electrodes (2Biological Instruments, Italy), 10 mm apart, connected to a 3165 Multiplexing Pulse Booster stimulator (Ugo Basile, Italy).

Trains of 10 Hz EFS were delivered (biphasic pulse with a constant current of 10 V, 0.5 ms, 10 s) with one pulse every 5–10 min in order to simulate vagus nerve firing as previously described (Cazzola et al. 2011).

The concentration of aclidinum bromide and formoterol fumarate required to induce 20% relaxation (EC_{20}) was determined in bronchial rings contracted by EFS. The EC_{20} of each compound was then used to assess the time cover of the relaxant effect in isolated bronchi contracted by EFS at 10 Hz for up to 12 h.

In further experiments, after initiating EFS, bronchi were treated with aclidinium bromide and formoterol fumarate for 60 min, either alone or in combination, with the EC_{20} for each drug. Tissue samples were then washed three times before being flushed with KH buffer solution (30 mL/h) for 12 h. Papaverine (100 μM) was then added to the bronchial rings to determine the E_{max} achievable for each isolated bronchus.

2.6. Data analysis

2.6.1. Study 1

The extent of relaxation of isolated bronchial rings and PCLSs was expressed as a percentage of the E_{max} (contractile tone reduction and luminal area enhancement, respectively) induced by papaverine (100 μM) on the plateau response induced by acetylcholine EC_{70}.

Appropriate curve-fitting to a sigmoidal model was used to calculate the effect (E), the E_{max}, the dose inducing 50% E_{max} (EC_{50}), and the EC_{70}. The equation used was log (agonist; antagonist) versus response, Variable slope, expressed as $Y=Bottom+(Top-Bottom)/\{1+10[(\log EC_{50}-X)*HillSlope]\}$. For statistical analysis of the potency, the pEC_{50} value was used where $pEC_{50}=-\log EC_{50}$ as described elsewhere (Motulsky and Christopoulos, 2004).

2.6.2. Study 2

The contractile response to EFS was expressed as a percentage of the effect induced by control EFS preceding treatment with aclidinium bromide or formoterol fumarate. Polynomial curves were constructed by fitting models of biological data using non-linear regression as described elsewhere (Motulsky and Christopoulos, 2004). The E_{max} (% reduction of the EFS contractile tension) and the onset of action (time to evoke half maximal relaxation) were identified. One in every seven bronchial rings was used as a time control.

2.6.3. Interaction analysis

The interaction of aclidinium bromide and formoterol fumarate on the relaxation of human isolated bronchi was evaluated using the Bliss independence (BI) theory, that provides outcomes for the expected additive response for the combination. Different specimens from every donor received LAMA, LABA and the combination separately and all treatments have been performed by randomization schedule. The main assumption of BI theory is that two or more agents act independently of each other with the mode and, possibly, the site of action of the compounds differing from each other.

BI theory for two agents is expressed by the equation:

$$E(x,y)=E_x+E_y-(E_x*E_y)$$

where E is the fractional effect, and x and y are the doses of two compounds in a combination experiment. If the combination effect is higher than the expected value, the interaction is synergistic; if it is lower than the expected value, the interaction is antagonistic; otherwise, the effect is considered additive with no interaction as described elsewhere (Boik et al., 2008; Boucher and Tam, 2006; Goldoni and Johansson, 2007; Greco et al., 1995; Lee, 2010; Meletiadis et al., 2003).

In Studies 1 and 2, 'x' and 'y' in the BI equation are aclidinium bromide and formoterol fumarate, respectively. The BI equation was used to establish the expected relaxant effect induced by the interaction of aclidinium bromide plus formoterol fumarate at isoeffective concentrations.

2.6.4. Statistical analysis

Values have been presented as mean ± standard error of the mean of 3 bronchi obtained from different donors. Statistical significance was assessed by a t test or one-way analysis of variance (ANOVA) with Bonferroni's and Dunnet's multiple comparison tests when necessary. Statistical significance was defined as $P<0.05$. Data analyses were performed using Prism 5 software (GraphPad Software Inc, CA, USA).

3. Results

3.1. Study 1

3.1.1. Contractile tone of isolated segmental bronchi

Both aclidinium bromide and formoterol fumarate induced potent concentration-dependent relaxation of human isolated bronchi submaximally pre-contracted with acetylcholine (aclidinium bromide pEC_{50}: 8.51 ± 0.13; formoterol fumarate pEC_{50}: 8.76 ± 0.05), and the potency of these drugs was not significantly different ($P > 0.05$). Aclidinium bromide and formoterol fumarate completely abolished the contractile tone induced by acetylcholine EC_{70} (acetylcholine EC_{70}: 9.21 ± 2.50 μM; aclidinium bromide E_{max}: $97.49 \pm 2.57\%$; formoterol fumarate E_{max}: $96.44 \pm 1.13\%$) (Fig. 1A). Vehicle had no effect on bronchial tone.

There was a significant difference ($P < 0.05$) between the slope of the CRCs obtained with aclidinium bromide (0.74 ± 0.13) and formoterol fumarate (1.18 ± 0.12). Therefore, a study of the correlation between the drug concentrations and E, expressed as percentage of E_{max}, was carried out to identify the isoeffective relaxant concentrations for each drug (Fig. 1B).

Aclidinium bromide plus formoterol fumarate induced a significant synergistic relaxant response in human isolated bronchi submaximally pre-contracted with acetylcholine, compared with the expected response predicted by BI theory ($P < 0.05$) (Fig. 1C). The synergistic interaction was observed at low concentrations (aclidinium bromide 1.15–2.15 nM; formoterol fumarate 0.84–1.20 nM) and induced an increased relaxant response of $+18.37 \pm 2.72\%$, compared with the expected additive response predicted by BI theory (Fig. 1D).

These results were confirmed by the analysis of interaction for low (EC_{20} and EC_{30}) and high concentrations (EC_{80}), where low concentrations of aclidinium bromide and formoterol fumarate, which alone would have achieved 30% relaxation of bronchi, together induced a significant relaxation of $69.31 \pm 2.59\%$ ($P < 0.05$) (Fig. 3A), whereas no synergistic interaction was detected at high concentrations (expected response versus observed response: $P > 0.05$, data not shown).

3.1.2. Videomicrometry of bronchioles

Both aclidinium bromide and formoterol fumarate induced a potent concentration-dependent relaxation of human PCLSs submaximally pre-contracted with acetylcholine (acetylcholine EC_{70}: 3.20 ± 0.72 μM; aclidinium bromide pEC_{50}: 7.93 ± 0.26; formoterol fumarate pEC_{50}: 8.37 ± 0.28) and the potency of these drugs was not significantly different ($P > 0.05$). Formoterol fumarate, but not aclidinium bromide, completely abolished the bronchial contraction induced by acetylcholine EC_{70} (aclidinium bromide E_{max}: $68.07 \pm 4.47\%$; formoterol fumarate E_{max}: $98.99 \pm 5.61\%$; $P < 0.05$) (Fig. 1C). Vehicle did not significantly modify the bronchial tone ($P > 0.05$).

There was a difference between the slope of the CRCs obtained with aclidinium bromide (0.78 ± 0.37) and formoterol fumarate (0.41 ± 0.1). Consequently, a study of the correlation between the drug doses and E, expressed as percentage of E_{max}, was carried out to identify the isoeffective relaxant concentrations for both aclidinium bromide and formoterol fumarate (Fig. 1D).

Aclidinium bromide plus formoterol fumarate induced a significant ($P < 0.05$) synergistic relaxant response in human PCLSs submaximally pre-contracted with acetylcholine compared with the expected response predicted by BI theory (Fig. 2C). In particular, the interaction between aclidinium bromide plus formoterol fumarate was synergistic across the full concentration range used (aclidinium bromide: 3.2 nM–1.0 μM, formoterol fumarate 1.8–63.0 nM) and an increased relaxant response of $+19.67 \pm 0.85\%$ was induced compared with the expected additive response predicted by BI theory (Fig. 2D).

The interaction analysis indicated that low concentrations of aclidinium bromide plus formoterol fumarate induced a significant ($P < 0.05$) luminal area enhancement of $69.89 \pm 2.28\%$, whereas the individual compounds would have elicited a luminal area enhancement of 30% (Fig. 3B).

3.2. Study 2

The EC_{20} of aclidinium bromide and formoterol fumarate in bronchial rings contracted by EFS was 1.60 ± 0.32 nM and 1.60 ± 0.27 nM, respectively.

As expected, neither aclidinium bromide nor formoterol fumarate administered alone was able to reduce the contractile response to EFS by 50% at EC_{20}. However, the isoeffective mixture of aclidinium bromide plus formoterol fumarate at EC_{20} produced a maximal relaxation of $69.74 \pm 6.35\%$ and the relaxant effect increased to $82.36 \pm 2.54\%$ at the 198th minute of the experiment. The onset of action of the relaxant effect for aclidinium bromide plus formoterol fumarate was 15.5 ± 3.5 min that remained stable up to the twelfth hour of the study ($74.34 \pm 7.88\%$, Fig. 4A).

From 0–6 h, the average observed relaxant effect was $68.49 \pm 2.21\%$, which was significantly higher than that predicted by BI theory ($38.55 \pm 1.94\%$; $P < 0.001$).

Low concentrations of aclidinium bromide plus formoterol fumarate produced a significant ($P < 0.001$) synergistic relaxant interaction on EFS-induced contraction of human isolated bronchi that was sustained for 6 h post-treatment (Fig. 4B). The maximal increased relaxant response was $+55.12 \pm 9.37\%$, which was greater than the expected additive response predicted by BI theory, and achieved at the 84th minute of the study.

4. Discussion

The combination of a LABA plus a LAMA has been suggested as a rational approach to treating patients with moderate to severe COPD (Cazzola and Molimard, 2010; Tashkin and Ferguson, 2013). Unfortunately, to date relatively few studies have examined the interaction between LABAs and LAMAs, although some have documented benefits on lung function and other outcomes compared with the individual drug classes used alone (Tashkin and Ferguson, 2013). There remains a paucity of preclinical data investigating the interaction between LAMAs and LABAs and it is unclear whether additive or synergistic interactions occur when these drug classes are combined.

In this study we have demonstrated that aclidinium bromide and formoterol fumarate administered alone both induced potent, significant, and long lasting relaxation of human isolated segmental bronchi. However, when administered together and at low concentrations, these drugs showed synergistic inhibition of ASM tone induced by either EFS or contraction with a cholinergic agonist. This extends and supports observations previously reported in animals, where a favorable interaction between a LABA and a LAMA has been documented in the control of airway changes induced by different challenges in guinea-pigs (Rossoni et al., 2007).

Both aclidinium bromide and formoterol fumarate caused time-dependent inhibition of the contractile response elicited by EFS, confirming the long duration of action of these compounds in human isolated bronchi. Intriguingly, the combination produced a synergistic interaction that induced a considerably faster onset of action on the reduction of the EFS-induced contractile tone, and this relaxant effect was maintained for up to 6 h after administration of the drugs.

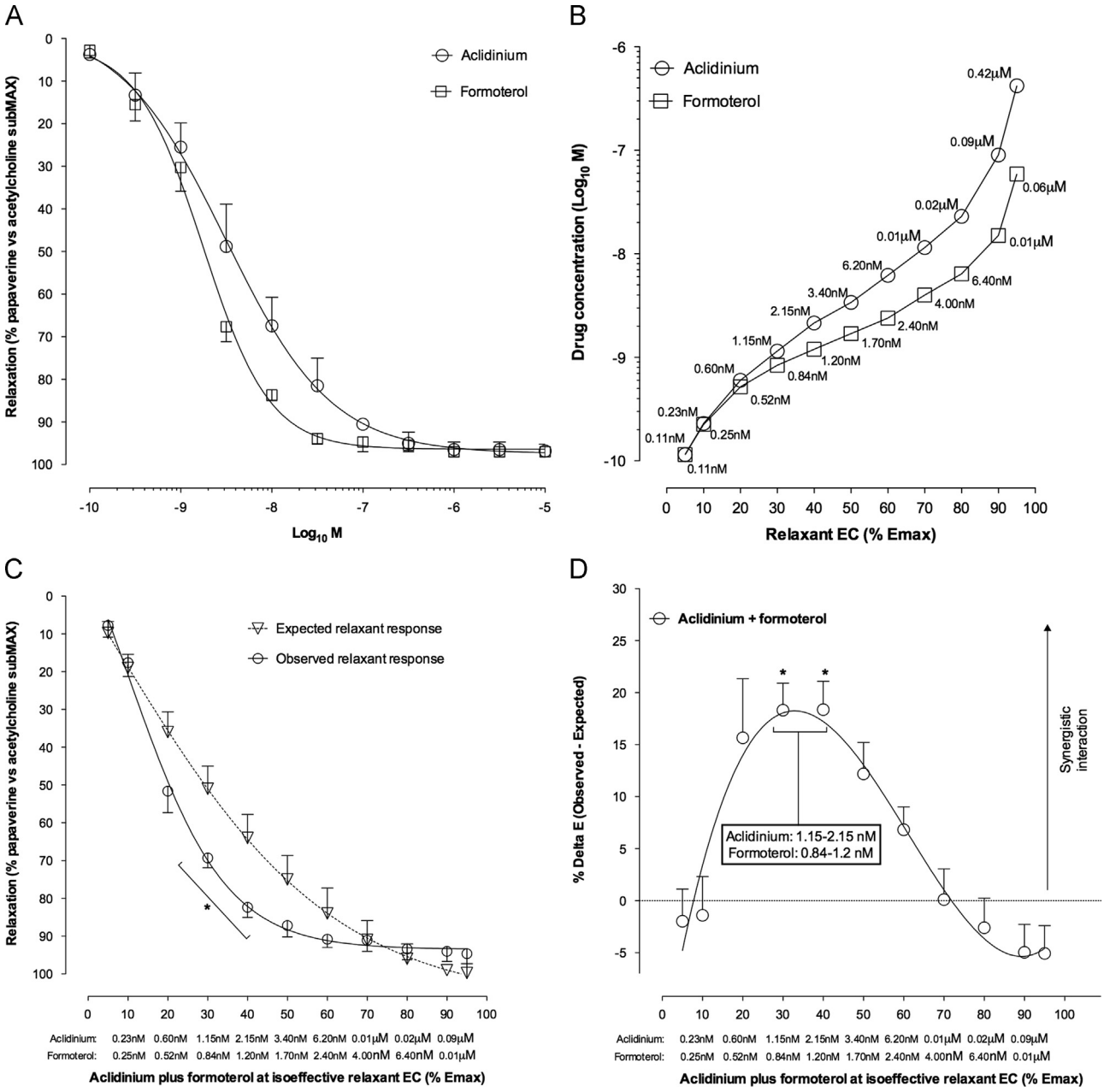

Fig. 1. Influence of aclidinium bromide and formoterol fumarate on the contractile tone of human segmental bronchi (A) submaximally pre-contracted with acetylcholine (EC_{70}) and concentrations inducing isoeffective relaxation (B). Expected and observed relaxant response induced by aclidinium bromide plus formoterol fumarate in human segmental bronchi (C) submaximally pre-contracted with acetylcholine (EC_{70}) and delta effect between observed and expected relaxant response (D). *$P < 0.05$ versus expected relaxant response as predicted by the Bliss independence theory. EC, effective concentration; EC_{70}, effective concentration for 70% of maximal effect; E_{max}, maximal effect; subMAX, submaximal.

Aclidinium bromide and formoterol fumarate also demonstrated a potent relaxant effect in small human airways, and formoterol fumarate – but not aclidinium bromide – completely relaxed bronchioles. In addition, the combination was particularly effective at inhibiting the contractile response of human bronchioles, with a wider extent of synergistic interaction from low to high concentrations compared with observations in larger isolated bronchi.

These data provide evidence that the concomitant administration of aclidinium bromide and formoterol fumarate at low concentrations leads to improved relaxation of both medium and small airways when compared with either drug administered alone. These findings are consistent with those seen in a Phase III clinical study evaluating the bronchodilator effect of a fixed-dose combination of aclidinium bromide/formoterol fumarate in a multi-dose dry powder inhaler (Singh et al., 2014). These data are also consistent with observations with other combinations of β_2-adrenoceptor agonists and antimuscarinic compounds, providing a good basis for explaining the improved clinical data reported with fixed-dose combinations of a LABA and a LAMA (Tashkin and Ferguson, 2013).

The BI theory used in our study to analyze the drug interaction is generally applied to investigate combined effects of drugs and it has been extensively validated experimentally using *in vitro* human airway smooth muscle preparations (Calzetta et al., 2013; Rogliani et al., 2013). The main assumption of the BI theory is that two or more agents act independently from one another in terms of site of action of the drugs in the mixture (Goldoni and Johansson, 2007; Greco et al., 1995). The validity of the BI model

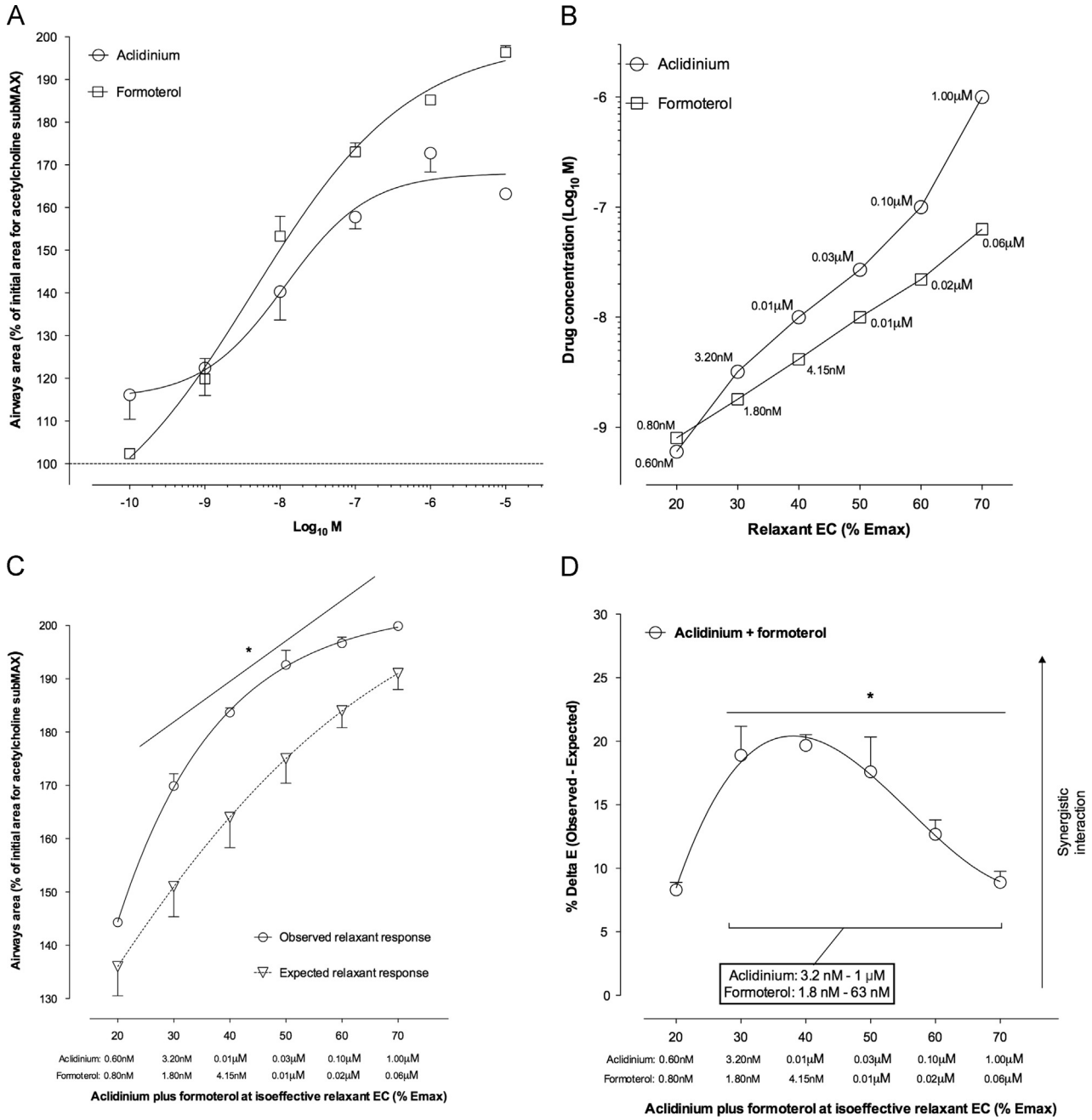

Fig. 2. Influence of aclidinium bromide and formoterol fumarate on the luminal area of human bronchioles (A) submaximally pre-contracted with acetylcholine (EC_{70}) and concentrations inducing isoeffective relaxation (B). Expected and observed relaxant response induced by aclidinium bromide plus formoterol fumarate of human bronchioles (C) submaximally pre-contracted with acetylcholine (EC_{70}) and delta effect between observed and expected relaxant response (D). $^{*}P < 0.05$ versus expected relaxant response as predicted by the Bliss independence theory. EC, effective concentration; EC_{70}, effective concentration for 70% of maximal effect; E_{max}, maximal effect; subMAX, submaximal.

might be questioned for drugs with very steep CRCs because, in such cases, this mathematical model tends to overestimate the synergy. As the potential overestimation occurs for Hill Slope >2, we can consider the BI criterion adequate for evaluating the synergistic interaction in our study as the slope of the CRCs for aclidinium bromide and formoterol fumarate were always <1.2 (Goldoni and Johansson, 2007). Therefore, the BI method applied to our experiments permitted an accurate statistical analysis with high biological plausibility that would not be possible by other pharmacological interaction analysis methods such as the Berenbaum approach and the dose-equivalence approach (Berenbaum, 1989; Goldoni and Johansson, 2007; Greco et al., 1995; Lee, 2010; Tallarida, 2001). The BI approach also has the advantage of allowing analysis of single combination points, as carried out in our study for investigating low concentration interactions at EC_{20} and EC_{30} (Goldoni and Johansson, 2007).

Our findings support the scientific rationale for combining aclidinium bromide and formoterol fumarate at low concentrations in order to optimize airway smooth muscle relaxation. Obviously, it is now of interest to identify the most favorable dosage of this drug combination to achieve optimal bronchodilation in patients with COPD via this synergistic interaction. This would also potentially allow improved bronchodilation while reducing the possibility of adverse events.

The results from the current study demonstrate that the LAMA alone, unlike the LABA, was unable to completely relax the tone

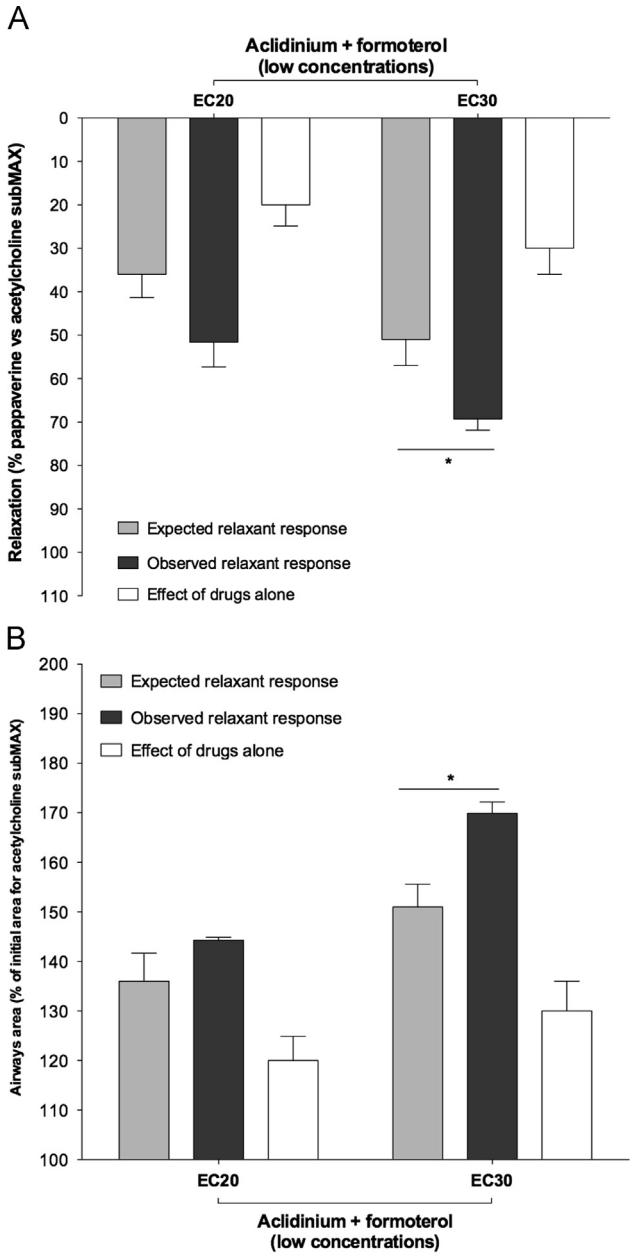

Fig. 3. Analysis of interaction between isoeffective low (EC_{20} and EC_{30}) concentrations of aclidinium bromide plus formoterol fumarate in human segmental bronchi (A) and human bronchioles (B) submaximally pre-contracted with acetylcholine (EC_{70}). *$P < 0.05$ versus expected relaxant response as predicted by the Bliss independence theory. EC_{20}, effective concentration for 20% of maximal effect; EC_{30}, effective concentration for 30% of maximal effect; EC_{70}, effective concentration for 70% of maximal effect; subMAX, submaximal.

induced by acetylcholine in the small airways. This finding is in agreement with the fact that, while the density of β_2-ARs in ASM does not change at different airway levels, the density of muscarinic receptors is greater in larger airways compared with bronchioles (Barnes, 2004). Intriguingly however, the combination of aclidinium bromide plus formoterol fumarate at low concentrations completely prevented the bronchoconstriction induced by acetylcholine, even at the level of small airways. Therefore, the greater effectiveness of a LAMA plus a LABA combination on small airways, compared with the partial effect of LAMA alone, might be of clinical relevance for improving air–trapping related to the obstruction of bronchioles – an important but neglected treatment target in COPD (Sturton et al., 2008).

Since the bronchodilation achieved by aclidinium bromide might be influenced by the extent of M_3 muscarinic receptor activation, we standardized the bronchial contractility at EC_{70}, as reported in previous studies (Calzetta et al., 2013, 2014a; Cazzola et al., 2014; Matera et al., 2009, 2011b; Rogliani et al., 2013). This approach, that allow inducing submaximal airways contraction, permitted also to adequately investigate the effect of the LABA formoterol fumarate on the bronchial tone produced by cholinergic activation. Although we cannot exclude a functional synergistic interaction due to the effect of different drugs on different pathways, such as inhibiting muscarinic tone by LAMA and stimulating relaxant response cAMP-mediated by LABA, we cannot rule out more sophisticated interactions concerning the intracellular cross-talking between muscarinic M_2, M_3 and β_2-ARs. In fact, post-synaptic G_i-coupled M_2 muscarinic receptors inhibit adenylyl cyclase reducing the β_2-ARs mediated cAMP production and alter the hyperpolarazion of Ca^{2+} dependent K^+ channels via $G_{\beta\gamma}$ subunits (Meurs et al., 2013). Furthermore, cross-talk between G_q-coupled M_3 receptors and G_s-coupled β_2-AR may modify the bronchial relaxation by inducing desensitization of β_2-AR and G_s-proteins via PKC induced phosphorylation (Meurs et al., 2013). Overall, these intracellular crass-talking makes the muscarinic and the β-adrenoceptor pathways so linked to justify a potential mechanistic interaction between LAMAs and LABAs. However, although we have demonstrated the presence of synergistic interaction between these compounds, further specific basic research is needed to pharmacologically characterize the nature of this synergism (Cazzola and Molimard, 2010).

The latest update of the Global initiative for chronic Obstructive Lung Disease guidelines recommends the combination of two long-acting bronchodilators with differing modes of action in patients with COPD that is not adequately controlled with monotherapy (GOLD, 2014) and we believe the data obtained from the present study give support to this recommendation. Our results also suggest that the synergistic interaction observed when combining a LABA plus a LAMA might, theoretically, also have the potential to improve the bronchodilator response in a synergistic manner, without the need to increase the dose of either component (Cazzola and Tashkin, 2009). However, there are very few studies in the literature investigating the potential interaction between LABAs and LAMAs and this information has mainly come from the investigation of the combination of tiotropium plus olodaterol on ovalbumin-induced bronchoconstriction in anesthetized guinea-pigs and on acetylcholine-induced bronchoconstriction in anesthetized dogs (Bouyssou et al., 2010, 2011).

To the best of our knowledge, this is the first study that has pharmacologically confirmed under controlled experimental settings the synergistic interaction between LABAs and LAMAs in relaxing both medium and small isolated human airways. Although the response of isolated human ASM may not always be identical to that elicited *in vivo*, isolated airway preparations provide a suitable, convenient, and reproducible biological model for the evaluation of drug effects on ASM independent from the potential systemic influences found *in vivo* (Endoh and Hori, 2006; Iglarz et al., 2008). In particular, the configuration of the smooth muscle bundles is largely preserved in human isolated bronchi and therefore the bronchial contraction is directly related to airway narrowing (Calzetta et al., 2011, 2013; Cazzola et al., 2011, 2012; Hewson et al., 2012; Hulsmann and de Jongste, 1993; Matera et al., 2005, 2008, 2009, 2011a, 2011b; Rogliani et al., 2013).

In conclusion, although this study was conducted on airways collected from patients without history of chronic airway diseases, our results suggest that the hypothesis of combining a LABA and a LABA in the treatment of patients with COPD might have a rational by providing synergistic benefit on ASM relaxation of both

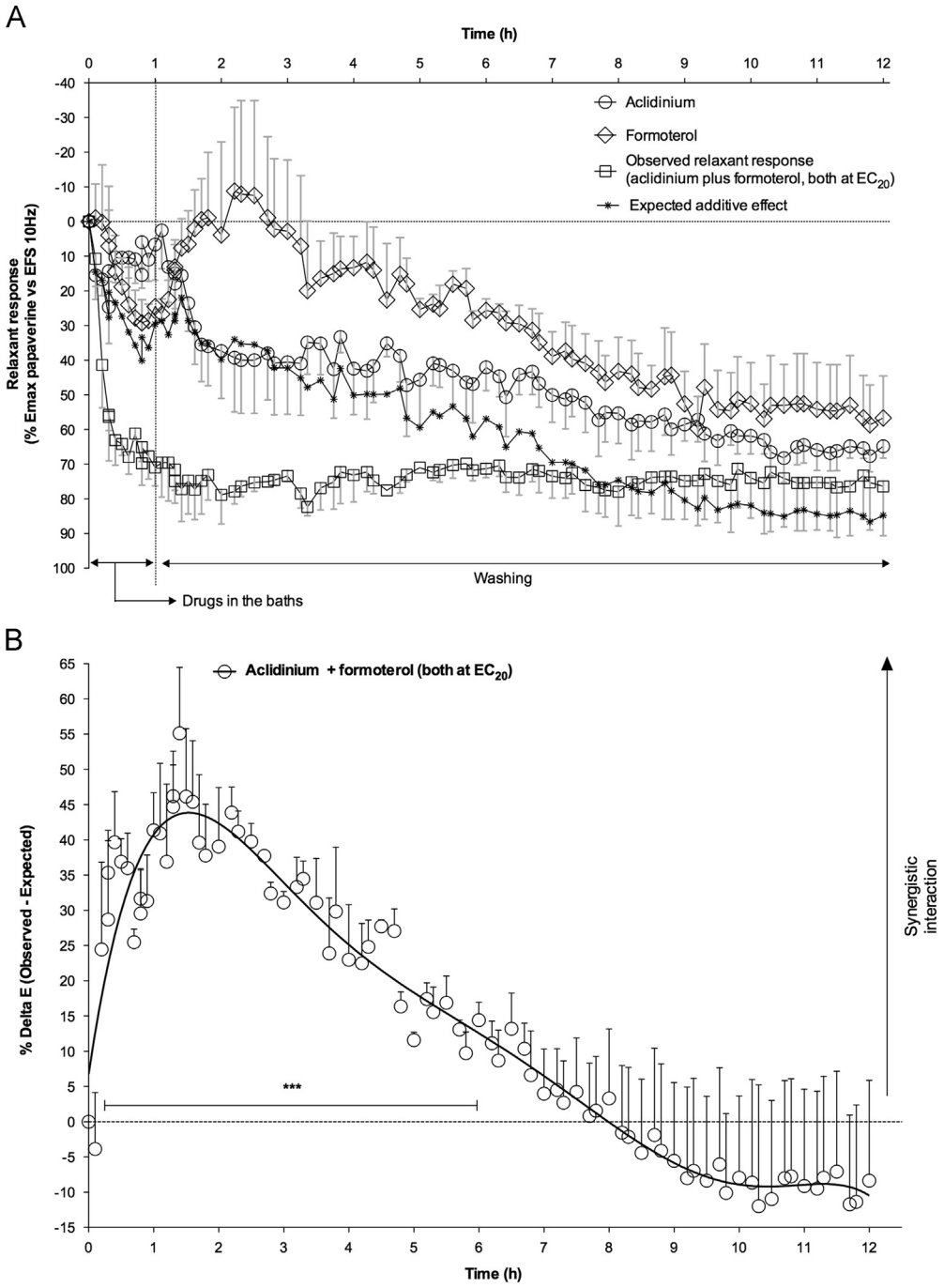

Fig. 4. Duration of action (A) and delta effect between observed and expected relaxant response (B) for aclidinium bromide and formoterol fumarate in human isolated bronchi stimulated with EFS at 10 Hz for mimicking the vagus nerve firing during the 12-h study. ***$P < 0.001$ versus expected relaxant response as predicted by the Bliss independence theory. E, effect; EC_{20}, effective concentration for 20% of maximal effect; EFS, electrical field stimulation.

medium and small human airways, which, in turn, may have major implications for the use of such combinations in the clinic.

Authorship contributions

Participated in research design: Cazzola, Calzetta, Page, Rogliani, Gavaldà, Matera.

Conducted experiments: Calzetta, Facciolo.

Contributed new reagents or analytic tools: Gavaldà.

Performed data analysis: Calzetta.

Wrote or contributed to the writing of the manuscript: Cazzola, Calzetta, Page, Rogliani, Matera.

Acknowledgments

This study was supported by Almirall S.A., Barcelona, Spain. Medical writing assistance, funded by Almirall S.A., was provided by Richard Knight, PhD and Suzanne McAllister, PhD, of Complete Medical Communications.

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