

# Effects of Formoterol on Contraction and Ca<sup>2+</sup> Signaling of Mouse Airway Smooth Muscle Cells

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Formoterol, a long-acting  $\beta_2$ -receptor agonist, is used to relieve bronchial constriction. However, formoterol is often a racemic formulation, and contains both (R,R)- and (S,S)-enantiomers. Because the activity of each isomer is poorly defined, the mechanisms by which formoterol relaxes smooth muscle cells (SMCs) of intrapulmonary airways are not well understood. Consequently, we compared the effects of (S,S)-, (R,R)-, and racemic formoterol, as well as (R)-albuterol, on the contraction and Ca<sup>2+</sup> signaling of airway SMCs in mouse lung slices with phase-contrast and confocal microscopy. Small airways were contracted with methacholine and the associated SMCs displayed sustained Ca<sup>2+</sup> oscillations and an increase in Ca<sup>2+</sup> sensitivity. These contracted airways displayed a substantial, concentration-dependent relaxation in response to (R,R)-formoterol. Racemic formoterol had a similar potency as (R,R)-formoterol for relaxing airways. By contrast, (S,S)-formoterol only induced a small relaxation. In conjunction with relaxation, (R,R)- and racemic formoterol stopped and decreased the methacholine-induced Ca<sup>2+</sup> oscillations and Ca<sup>2+</sup> sensitivity of the SMCs, respectively, whereas (S,S)-formoterol only decreased the Ca<sup>2+</sup> sensitivity. In these studies, (R,R)- and racemic formoterol had a similar, but much greater, potency than (R)-albuterol for relaxing mice airways. This action was quickly initiated at high concentrations by decreasing the frequency of Ca<sup>2+</sup> oscillations, but was more usually mediated at lower concentrations by decreasing the Ca<sup>2+</sup> sensitivity of the SMCs.

**Keywords:** Ca<sup>2+</sup> sensitivity; Ca<sup>2+</sup> oscillations; confocal microscopy; lung slices; methacholine

$\beta_2$ -Receptor agonists have been in use for many years to relieve bronchial constriction associated with asthma or chronic obstructive pulmonary disease. Formoterol is a long-acting  $\beta_2$ -receptor agonist (>12-h duration of action), often used, in combination with glucocorticosteroids, in the maintenance therapy of asthma, whereas short-acting  $\beta_2$ -receptor agonists (SABAs) (3- to 6-h duration of action) are essentially used as rescue medications to relieve symptoms and treat exacerbations (1–3). However, formoterol has a rapid onset of action, whether given alone or in combination with glucocorticosteroids, and appears to be as effective as SABAs in relieving acute asthma exacerbations (1, 4–6). Formoterol occurs in two formulations: racemic formoterol (50% of (R,R)- and (S,S)-enantiomers) and purified (R,R)-formoterol. Clinical and animal data suggest that (R,R)- and racemic formoterol have a similar potency to promote airway relaxation (7–9). However, others studies suggest that (S,S)-formoterol may not be biologically inactive as pre-

## CLINICAL RELEVANCE

The efficacy and mechanisms of short- or long-acting  $\beta_2$ -adrenergic agonists are important for evaluating treatments designed to induce bronchodilation to facilitate the management of asthma and chronic obstructive pulmonary disease.

viously thought, but may reduce the relaxant efficacy of the racemic mixture (10). In addition, (S,S)-formoterol may promote inflammation (11).

It has been well established that smooth muscle cell (SMC) contraction usually requires an increase in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and this has been observed to occur as a series of Ca<sup>2+</sup> oscillations in airway SMCs of many species (12–17). The frequency of the Ca<sup>2+</sup> oscillations within SMCs is a key parameter that correlates with the contractility of SMCs. Agents that elevate cAMP, such as SABAs (i.e., isoproterenol [ISO] and albuterol) or forskolin (FSK), have been shown to slow down the frequency of the Ca<sup>2+</sup> oscillations, and this has been correlated with airway relaxation (18, 19).

A second mechanism that determines the extent of airway contraction is the Ca<sup>2+</sup> sensitivity of the SMC contractile apparatus (20, 21). As a result,  $\beta_2$ -receptor agonists may also induce airway relaxation by reducing the Ca<sup>2+</sup> sensitivity of airway SMCs. We have previously shown that (R)-albuterol was more effective than racemic albuterol in decreasing the Ca<sup>2+</sup> sensitivity of airway SMCs, and this was correlated with a stronger efficacy for airway relaxation (19).

Because it is unknown if formoterol induces airway relaxation by altering the Ca<sup>2+</sup> oscillations and Ca<sup>2+</sup> sensitivity of airway SMCs, the aim of this study was to characterize and compare the effects of (R,R)-, (S,S)-, and racemic formoterol on the contraction and Ca<sup>2+</sup> signaling of airway SMCs. Although previous studies have used tissues or cells isolated from the trachea to investigate the effects of  $\beta_2$ -receptor agonists, a simultaneous correlation of the contractile responses with cellular changes in Ca<sup>2+</sup> was not possible in these preparations. In addition, it is unclear if the responses of tracheal SMCs reflect those of the small airway. Consequently, we have compared the effects of isomers of formoterol and (R)-albuterol on small intrapulmonary airways in mouse lung slices where contraction and Ca<sup>2+</sup> signaling are more easily studied.

## MATERIALS AND METHODS

### Animals and Reagents

Male BALB/C mice, 7 to 10 weeks old, were purchased from Charles River Breeding Labs (Needham, MA). Mice were killed by intraperitoneal injection of pentobarbital sodium (nembutal), as approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Hanks' balanced salt solution (HBSS) without phenol red was supplemented with 25 mM Hepes (sHBSS [pH 7.4]). (R)-albuterol, (R,R)-, (S,S)-, and racemic formoterol were donated by Sepracor Inc. (Marlborough, MA). Cell-permeant,

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caged-IP<sub>3</sub> was obtained from Alexis Biochemicals (San Diego, CA). Other reagents were obtained from Invitrogen Corp. (Carlsbad, CA), Calbiochem (La Jolla, CA), or Sigma-Aldrich Corp. (St. Louis, MO).

### Preparation of Mouse Lung Slices

Lung slices were prepared by the methods previously described (14). In brief, the lungs were inflated with warm 2% agarose in sHBSS. Subsequently, a small bolus of air was injected to flush the agarose-sHBSS out of the airways and into the distal alveolar space. The inflated lungs were cooled with cold sHBSS to gel the agarose. A lung lobe was sectioned into roughly 130- $\mu$ m-thick lung slices with a vibratome in cold sHBSS. Lung slices were maintained in Dulbecco's modified Eagle's Medium supplemented with antibiotics and antimycotics at 37°C in 10% CO<sub>2</sub>. Lung slices were used within 3 days. Similarly sized airways (150–210  $\mu$ m in diameter) were used in all experiments.

### Measurement of the Contractile Response of Airways

Lung slices were mounted in a custom-made chamber and perfused by a gravity-fed perfusion system (14). Lung slices were observed with phase-contrast microscopy on an inverted microscope with a 10 $\times$  objective, and images were recorded using a charge-coupled device camera and image acquisition software (Video Savant; IO Industries, London, ON, Canada). Digital images were recorded in time lapse (0.5 Hz), stored on a hard drive, and analyzed using custom-written scripts with Video Savant. The area of the airway lumen was calculated with respect to time by pixel summing. Area values were normalized to the initial lumen area.

### Measurement of Intracellular Ca<sup>2+</sup>

Lung slices (~12–15 slices) were incubated with Oregon Green 488 BAPTA-1-AM (20  $\mu$ M, 2 ml), sulfobromophthalein (100  $\mu$ M), and 0.05% pluronic F-127 for 40 minutes at 30°C. For de-esterification, the slices were washed for 40 minutes in sHBSS containing 100  $\mu$ M sulfobromophthalein at 30°C. Imaging of loaded slices was performed using a video-rate confocal microscope, as previously described (14, 22). Fluorescence images were saved to a hard disk using Video Savant software at 15 frames per second. Changes in fluorescence (F) were analyzed by selecting a region of interest (5  $\times$  5 pixels) in a single SMC and were normalized to the initial level of fluorescence (F<sub>0</sub>). Final fluorescence values were expressed as a ratio (F/F<sub>0</sub>). Additionally, a line-scan analysis of the images was performed by extracting a row of pixels from each image and placing them sequentially, as a time sequence, in a single image.

### Flash Photolysis of Caged-IP<sub>3</sub>

Flash photolysis of cell-permeant caged-IP<sub>3</sub> was used to increase intracellular IP<sub>3</sub> concentration. Slices were initially loaded with Oregon Green 488 BAPTA-1-AM, as described previously here. Subsequently, lung slices were incubated at room temperature with 2  $\mu$ M caged-IP<sub>3</sub> for 1 hour in sHBSS containing 0.1% pluronic F-127 and 100  $\mu$ M sulfobromophthalein, followed by de-esterification for 30 minutes in the sHBSS containing 100  $\mu$ M sulfobromophthalein. The details of the flash photolysis setup have been previously described (18, 23). Briefly, a flash of ultraviolet (UV) light was produced from a mercury arc lamp with an electronic shutter and a band-pass filter (330 nm). The intensity of the flash was regulated by neutral density filters. The size of the area illuminated was adjusted to be a small spot with an iris diaphragm.

### Preparation of Ca<sup>2+</sup>-Permeabilized Lung Slices

The SMCs of lung slices were made permeable to Ca<sup>2+</sup> by exposure to 20 mM caffeine and 50  $\mu$ M ryanodine in sHBSS for at least 4 minutes. In this procedure, caffeine activates or opens the ryanodine receptors of the sarcoplasmic reticulum, and this allows ryanodine to lock the ryanodine receptor in an open state. This results in the depletion of the intracellular Ca<sup>2+</sup> store (the sarcoplasmic reticulum), which, in turn, increases the Ca<sup>2+</sup> permeability of the plasma membrane by the activation of store-operated Ca<sup>2+</sup> channels (21). Under these conditions, the [Ca<sup>2+</sup>]<sub>i</sub> is determined by the extracellular Ca<sup>2+</sup> concentration, which was set at 1.3 mM (sHBSS). Importantly, this Ca<sup>2+</sup> permeabilization was irreversible when caffeine and ryanodine were removed.

### Statistical Analysis

Statistical analysis of the data showed no difference between the individual mice. Data are expressed as means ( $\pm$ SD) of three or more experiments per mouse for *n* mice. Statistical analysis was assessed by ANOVA. Comparison of paired data was done using a paired samples *t* test. A *P* value of less than 0.05 was considered statistically different.

## RESULTS

### Response of Airways to Formoterol Enantiomers

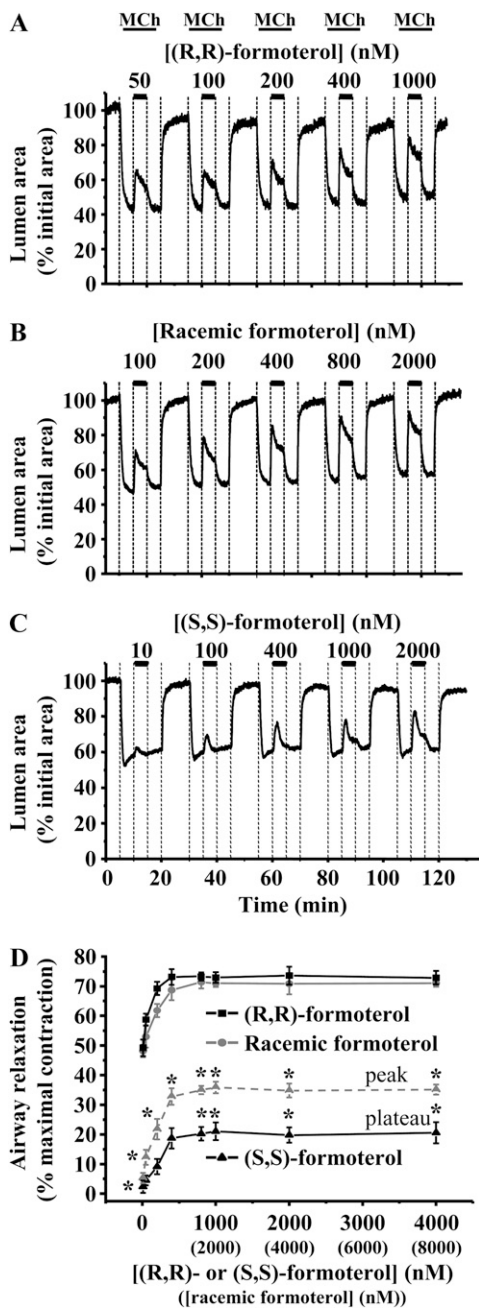
The response of contracted airways to formoterol enantiomers was investigated by exposing airways from mice to 400 nM methacholine (MCh) to induce contraction, followed by a range of formoterol concentrations (Figure 1). A MCh concentration of 400 nM was used, because this induced a midrange contraction. The concentrations of the formoterol enantiomers tested ranged from 0.5 to 4,000 nM, but all data points are not always shown in figures.

At concentrations above 1 nM, (R,R)- and racemic formoterol induced a sustained relaxation of MCh-contracted airways in a concentration-dependent manner (Figures 1–3). However, the initial relaxation was often followed by a small recontraction of the airway, which subsequently stabilized, and the airway remained at a constant relaxed state for the following 30 minutes (Figure 2). Therefore, we quantified the relaxation response at the beginning of this plateau phase (4 min after addition of formoterol). The relaxant effect of (R,R)- or racemic formoterol (containing an equal concentration of (R,R)-formoterol) was reversible, and the airways recontracted to their initial levels after formoterol removal (Figures 1A, 1B, and 2). (R,R)- and racemic formoterol induced a similar airway relaxation at all concentrations tested (Figure 1D). The effective concentration range of relaxation was relatively narrow. A substantial relaxation of approximately 50% was induced by a concentration of 50 nM (R,R)-formoterol or 100 nM racemic formoterol, whereas a maximal relaxation was induced by 800 nM (R,R)-formoterol or 1,600 nM racemic formoterol. Maximal relaxation was 73.29 ( $\pm$ 1.375)% (% maximal contraction; *n* = 7 mice) and 71.36 ( $\pm$ 2.13)% (*n* = 6 mice) for (R,R)- and racemic formoterol, respectively.

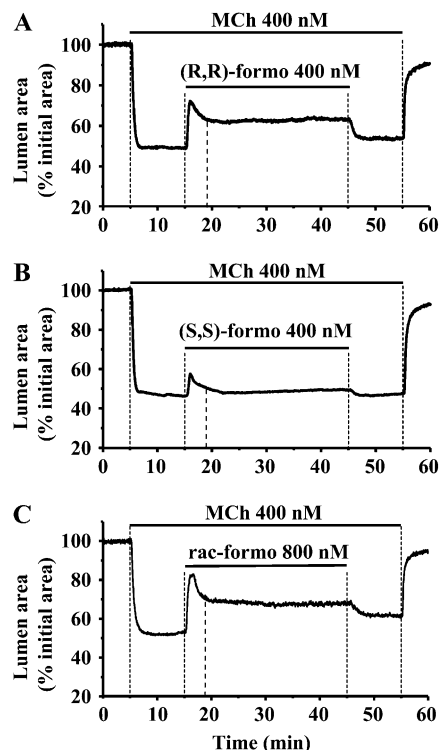
In a similar series of experiments, (S,S)-formoterol was found to induce a small relaxation of MCh-contracted airways (Figure 1C). In addition, (S,S)-formoterol had mainly a transient relaxation effect at low concentrations (<400 nM) (Figures 1C and 2B). The relaxation induced by (S,S)-formoterol (quantified at either the peak relaxation or during the plateau phase) was significantly smaller than that induced by (R,R)- or racemic formoterol at all concentrations tested (*P* < 0.05) (Figure 1D). (S,S)-formoterol induced a maximal relaxation of 21.03 ( $\pm$ 2.97)% (plateau; *n* = 6 mice) at 1,000 nM.

### Comparison of (R)-Albuterol and (R,R)-Formoterol on Airway Contraction

Because long-acting  $\beta_2$ -receptor agonists have been considered as replacements for SABAs, we compared the effectiveness of (R,R)-formoterol and (R)-albuterol on the same MCh-contracted airways (Figure 3A). We found that, although both agonists (at equal concentrations) relaxed the airways with a similar timing, (R,R)-formoterol induced a significantly larger relaxation than (R)-albuterol at all concentrations tested (Figures 3A and 3B). At 1,000 nM, (R,R)-formoterol induced a relaxation of 70.05 ( $\pm$ 2.77)% (% maximal contraction; *n* = 4 mice), which was more than double the relaxation induced by the same concentration of (R)-albuterol (25.5  $\pm$  2.56%). This action was not influenced by the sequence of drug application (Figure 3A). In an additional series of experiments, the exposure of an airway



**Figure 1.** The relaxant effect of (R,R)-, racemic, and (S,S)-formoterol on contracted airways. Airways were contracted with 400 nM methacholine (MCh) and exposed to a series of formoterol concentrations: (A) (R,R)-formoterol (50–1,000 nM), (B) racemic formoterol (100–2,000 nM), and (C) (S,S)-formoterol (10–2,000 nM). (D) The mean concentration-dependent relaxation response of airways contracted by MCh (400 nM) to (R,R)-, racemic, and (S,S)-formoterol. The maximal relaxation induced by each formoterol isomer was quantified during the plateau phase (solid lines). The transient response induced by (S,S)-formoterol was also quantified at peak relaxation (dashed line). The relaxant effect of (R,R)-formoterol and racemic formoterol (containing an equal concentration of (R,R)-formoterol) was not significantly different. However, both (R,R)- and racemic formoterol induced a significantly larger relaxation than (S,S)-formoterol. Mean (±SD) values are presented; a minimum of four experiments was performed for each concentration for each mouse (\*P < 0.05; n = 7 mice for (R,R)-formoterol, n = 6 for racemic formoterol and (S,S)-formoterol).



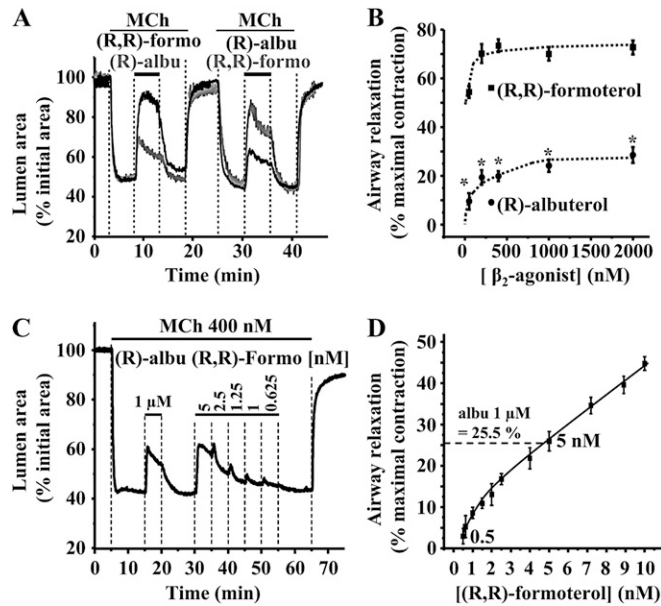
**Figure 2.** Duration of the relaxant effect of (R,R)-, racemic, and (S,S)-formoterol on contracted airways. Airways were contracted with 400 nM MCh and exposed for 30 minutes to 400 nM of (A) (R,R)-formoterol, (B) (S,S)-formoterol, and (C) 800 nM racemic formoterol. Each formulation of formoterol induced an initial strong and fast relaxation, followed by a small recontraction of the airway that subsequently stabilized after approximately 4 minutes (dashed line) of exposure to formoterol, and remained constant for the next 30 minutes.

several times (6 times) to (R)-albuterol (1 μM) did not change the subsequent airway relaxation induced by 500 nM or 1 μM (R,R)-formoterol. Similar results were also obtained if the airway was first exposed to formoterol (6 times) followed by albuterol. These results emphasize that the sequence of drug application was irrelevant, and that, more importantly, the desensitization of the tissue to either agonist does not readily occur.

Our comparison of formoterol with albuterol at equal concentrations suggests that formoterol has greater efficacy. However, an alternative approach to examine the mechanisms by which these two β<sub>2</sub>-receptor agonists induce relaxation is to compare their action during an equivalent relaxation. Consequently, we determined the concentration of (R,R)-formoterol required to match the relaxation induced by 1 μM albuterol (Figures 3C and 3D). With the same MCh-contracted airways, we found that 5 nM (R,R)-formoterol induced a relaxation approximately equivalent to the relaxation induced by 1 μM (R)-albuterol (Figures 3C and 3D). We will subsequently use, in this study, these concentrations of formoterol and albuterol to address the underlying mechanism of formoterol action as compared with albuterol.

**Effects of Formoterol Enantiomers on Ca<sup>2+</sup> Oscillations**

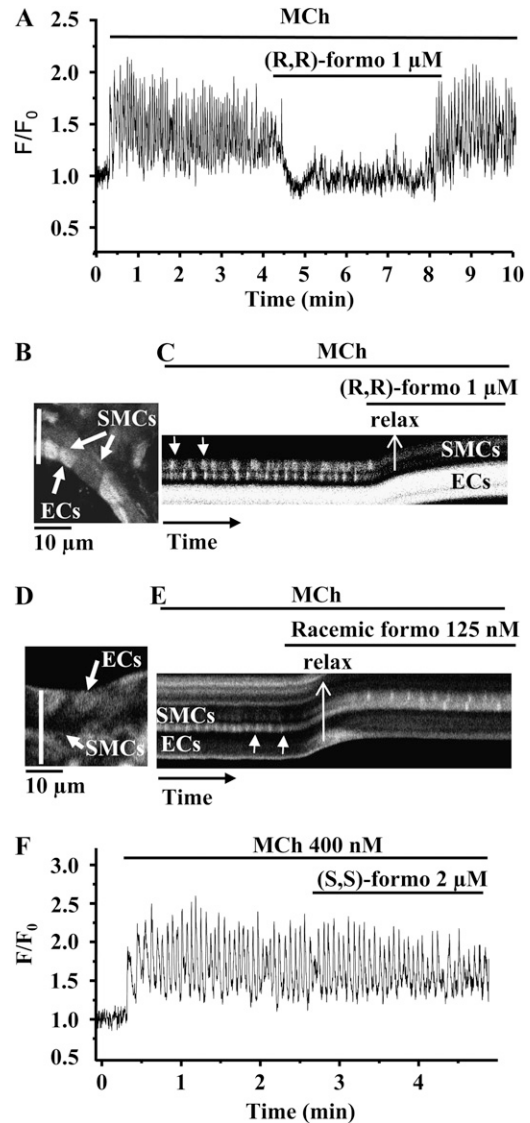
In response to 400 nM MCh, mouse airway SMCs displayed an increase of [Ca<sup>2+</sup>]<sub>i</sub> that rapidly developed (within 1 min) into sustained rhythmic Ca<sup>2+</sup> oscillations (14.08 ± 1.86 oscillations per min; six mice; Figures 4 and Figures 5). The subsequent addition of either (R,R)- or racemic formoterol, at high concentrations of approximately 1 μM quickly stopped (Figures



**Figure 3.** Comparison of airway relaxation induced by (R)-albuterol and (R,R)-formoterol. (A) Representative experiments showing the effect of (R,R)-formoterol (1  $\mu$ M) and (R)-albuterol (1  $\mu$ M) on the same MCh-contracted airway. (R,R)-formoterol induced a larger relaxation than the subsequent exposure to (R)-albuterol (*black line*). Similar results were obtained if the application order of (R)-albuterol and (R,R)-formoterol was reversed (*gray line*). (B) The mean concentration-dependent relaxation induced by (R)-albuterol and (R,R)-formoterol (50–2,000 nM) in airways contracted with MCh (400 nM). Relaxation induced by (R,R)-formoterol was significantly greater than that induced by (R)-albuterol ( $*P < 0.05$ ;  $n = 4$  mice for (R,R)-formoterol and (R)-albuterol). Mean ( $\pm$ SD) values are presented; a minimum of three experiments was performed for each concentration for each mouse. (C) Representative experiment showing the relaxant effect of (R)-albuterol (1  $\mu$ M) followed by 5, 2.5, 1.25, 1, and 0.625 nM of (R,R)-formoterol on the same MCh-contracted airway. (D) The mean concentration-dependent relaxation to low concentrations of (R,R)-formoterol (0.5–10 nM). The maximal relaxation induced by 5 nM (R,R)-formoterol was 26.65 ( $\pm$ 2.35)% (% maximal contraction;  $n = 5$  mice; a minimum of three experiments was performed for each concentration for each mouse), which was equivalent to the relaxation induced by 1  $\mu$ M (R)-albuterol.

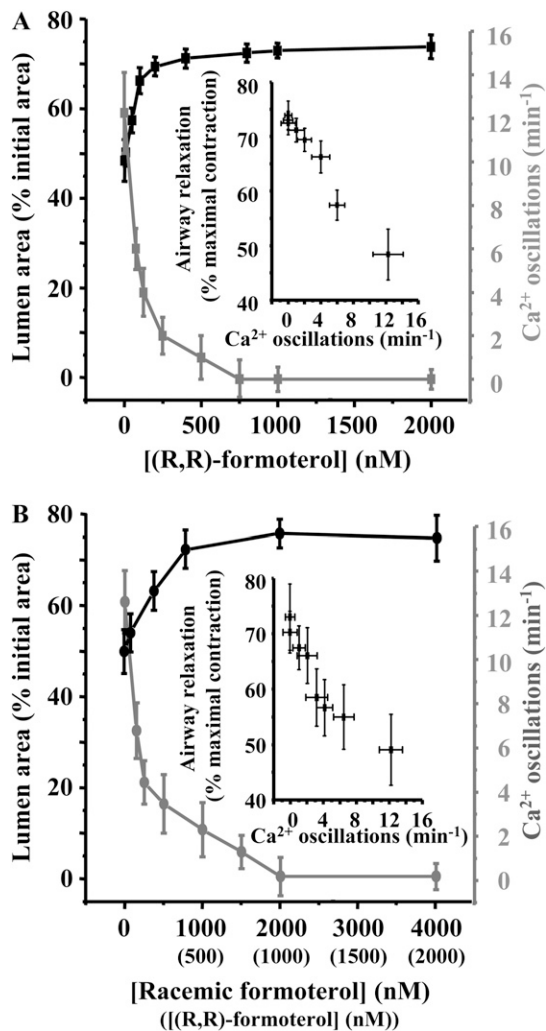
4A–4C), or, at lower concentrations, slowed (Figure 4E), the  $Ca^{2+}$  oscillations. This inhibition of the  $Ca^{2+}$  oscillations was accompanied by airway relaxation (Figures 4C and 4E). Upon the removal of formoterol, the  $Ca^{2+}$  oscillations resumed with a frequency equal to the original rate (Figure 4A), and the airway recontracted (Figures 1 and 2). By contrast, (S,S)-formoterol (2  $\mu$ M) had no effect on the frequency of the  $Ca^{2+}$  oscillations (Figure 4F).

To characterize the relationship between the frequency of the  $Ca^{2+}$  oscillations of the SMCs and the relaxation of the airway induced by formoterol, MCh-contracted airways (400 nM) were exposed to a range of concentrations (up to 2  $\mu$ M) of (R,R)- or racemic formoterol (Figure 5), and the frequency of the  $Ca^{2+}$  oscillations was measured. At concentrations up to 750 nM, (R,R)-formoterol decreased the frequency of the  $Ca^{2+}$  oscillations induced by MCh, whereas, at concentrations greater than 750 nM, (R,R)-formoterol inhibited the  $Ca^{2+}$  oscillations (Figure 5A). Racemic formoterol also decreased the frequency of  $Ca^{2+}$  oscillations in a concentration-dependent manner (Figures 4E and 5B), and stopped the  $Ca^{2+}$  oscillations at concentrations of greater than 2,000 nM (equal to 1,000 nM (R,R)-



**Figure 4.** The effect of formoterol isomers on the frequency of MCh-induced  $Ca^{2+}$  oscillations. (A) Representative experiment showing that the  $Ca^{2+}$  oscillations induced in an airway smooth muscle cell (SMC) by 400 nM MCh were quickly abolished by exposure to (R,R)-formoterol (1  $\mu$ M). (B) Fluorescence image indicating the location of SMCs and epithelial cells (ECs) from an observed airway. (C) Line scan analysis (for 90 s from *white line* indicated in [B]) showing rhythmic  $Ca^{2+}$  oscillations induced by MCh 400 nM (*arrows*) in two SMCs. These  $Ca^{2+}$  oscillations were inhibited by the addition of 1  $\mu$ M (R,R)-formoterol. The upward shift of the fluorescence trace (C) away from the lumen (in the direction of *arrow*) indicates the onset of the relaxation of the airway. (D) Fluorescence image showing SMCs and ECs of a second airway. (E) Line scan plot (for 150 s, indicated by the *white line* in [D]) showing rhythmic  $Ca^{2+}$  oscillations (*arrows*) induced by 400 nM MCh in two SMCs. The frequency of the  $Ca^{2+}$  oscillations was decreased by a low concentration of racemic formoterol (125 nM) and the airway relaxed. (F) Representative experiment showing that  $Ca^{2+}$  oscillations induced by 400 nM MCh were not altered by (S,S)-formoterol (2  $\mu$ M).

formoterol). No significant differences in the decrease of the frequency of the  $Ca^{2+}$  oscillations induced by (R,R)- and racemic formoterol (at equal concentrations of (R,R)-formoterol) were observed. (S,S)-formoterol had no effect on the  $Ca^{2+}$  oscillations at all concentrations tested (10–4,000 nM).



**Figure 5.** The mean concentration-dependent relaxation (black line and left scale) and the change in the frequency of the Ca<sup>2+</sup> oscillations (gray line and right scale) induced by (A) (R,R)-formoterol (0–2,000 nM) and (B) racemic formoterol (0–4,000 nM) in airways contracted with MCh (400 nM). Numbers in parentheses indicate the equivalent (R,R)-formoterol concentration in racemic formoterol. Insets: the relationship between the reduction in Ca<sup>2+</sup> oscillation frequency in SMCs and airway relaxation. As the concentration of (R,R)-formoterol or racemic formoterol increased, the relaxation increased and the Ca<sup>2+</sup> oscillation frequency decreased ( $n = 4$  mice for (R,R)-formoterol and racemic formoterol). Mean ( $\pm$ SD) values are presented; a minimum of three experiments was performed for each concentration for each mouse.

The formoterol concentration-dependent Ca<sup>2+</sup> oscillation frequency data were plotted against the formoterol concentration-dependent airway size data to obtain the relationship between the Ca<sup>2+</sup> oscillation frequency and airway relaxation (Figure 5, insets). (R,R)-formoterol induced a relaxation of the airway area that was a near linear function of the Ca<sup>2+</sup> oscillation frequency. The relaxation relationship for racemic formoterol was similar. It is interesting to note that, although the Ca<sup>2+</sup> oscillations are fully inhibited (i.e., no Ca<sup>2+</sup> signaling), the airways are not fully relaxed.

#### Effect of Formoterol on the Release of Intracellular Ca<sup>2+</sup>

To investigate the mechanism by which formoterol inhibited the Ca<sup>2+</sup> oscillations, we examined the effect of formoterol on IP<sub>3</sub>-induced Ca<sup>2+</sup> release from internal Ca<sup>2+</sup> stores. In control

studies, airway SMCs were loaded with caged-IP<sub>3</sub> and briefly illuminated with UV light (a 500-ms flash). This single UV flash initiated several Ca<sup>2+</sup> oscillations that propagated as Ca<sup>2+</sup> waves from the illuminated area to the rest of the SMC (data not shown). A flash exposure had no effect on Ca<sup>2+</sup> release of SMCs not loaded with caged-IP<sub>3</sub>.

Similarly, airway SMCs loaded with caged-IP<sub>3</sub> were subsequently contracted with MCh to induce Ca<sup>2+</sup> oscillations. The exposure of these SMCs to (R,R)-formoterol stopped the Ca<sup>2+</sup> oscillations. However, in the continued presence of MCh and (R,R)-formoterol, a UV flash exposure applied to the SMCs to release IP<sub>3</sub> also initiated a short period of Ca<sup>2+</sup> oscillations (Figures 6A and 6B). A transient recontraction of the airway was associated with this brief resumption of the Ca<sup>2+</sup> oscillations. Equivalent results were found with racemic formoterol. These results suggest that formoterol may either limit the production of IP<sub>3</sub> and/or decrease the sensitivity of the IP<sub>3</sub> receptor (IP<sub>3</sub>R) to concentrations of IP<sub>3</sub> within the cell.

#### Comparison of (R)-Albuterol and (R,R)-Formoterol on Ca<sup>2+</sup> Oscillations

As previously reported (19), we found that 1  $\mu$ M (R)-albuterol stopped Ca<sup>2+</sup> oscillations induced by MCh 400 nM during the initial 1–2 minutes of exposure. However, in the continued presence of albuterol, the Ca<sup>2+</sup> oscillations resumed, but at a lower frequency than with MCh alone (Figure 6C). By contrast, we found that “equivalent effective concentrations” of (R,R)-formoterol (<10 nM) had no effect on MCh-induced Ca<sup>2+</sup> oscillations (Figures 5A and 6D). Therefore, although 5 nM formoterol induced a similar relaxation as compared with 1  $\mu$ M (R)-albuterol, this was achieved without a change in the Ca<sup>2+</sup> oscillation frequency.

#### Effects of Formoterol Enantiomers on the Ca<sup>2+</sup> Sensitivity of SMCs

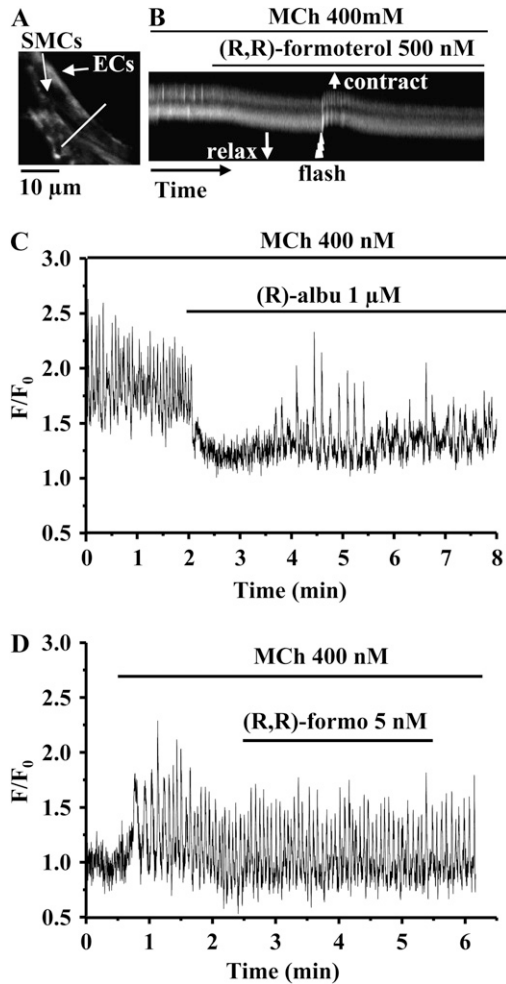
The sensitivity of the contractile apparatus to Ca<sup>2+</sup> provides a second potential mechanism of action for formoterol to relax airways. To investigate this possibility, it is necessary to clamp the SMC [Ca<sup>2+</sup>]<sub>i</sub> at a high level. This was achieved by creating Ca<sup>2+</sup>-permeabilized lung slices by treatment with ryanodine and caffeine. The details of this procedure have been previously published (21).

In Ca<sup>2+</sup>-permeabilized lung slices, the addition of MCh induced airway contraction (Figure 7) without altering the elevated [Ca<sup>2+</sup>]<sub>i</sub> (data not shown [21]). The subsequent addition of either, racemic, (R,R)-, or (S,S)-formoterol relaxed the contracted airway (Figures 7A and 7B) in a concentration-dependent manner (Figure 7C), but, again, without altering the elevated [Ca<sup>2+</sup>]<sub>i</sub>. The relaxation induced by formoterol in Ca<sup>2+</sup>-permeabilized lung slices was ranked in the following order: racemic formoterol > (R,R)-formoterol  $\gg$  (S,S)-formoterol (Figure 7C). These results demonstrate that all three formoterol preparations decreased SMC Ca<sup>2+</sup> sensitivity.

To compare the mechanism of action of formoterol with that of albuterol, we also examined the response of (R)-albuterol on Ca<sup>2+</sup> sensitivity. In keeping with our previous results, (R)-albuterol also induced relaxation in MCh-contracted airways of Ca<sup>2+</sup>-permeabilized lung slices. However, the extent of this response was significantly less than that induced by equal concentrations of (R,R)-formoterol, but similar to that induced by (S,S)-formoterol (Figure 7C).

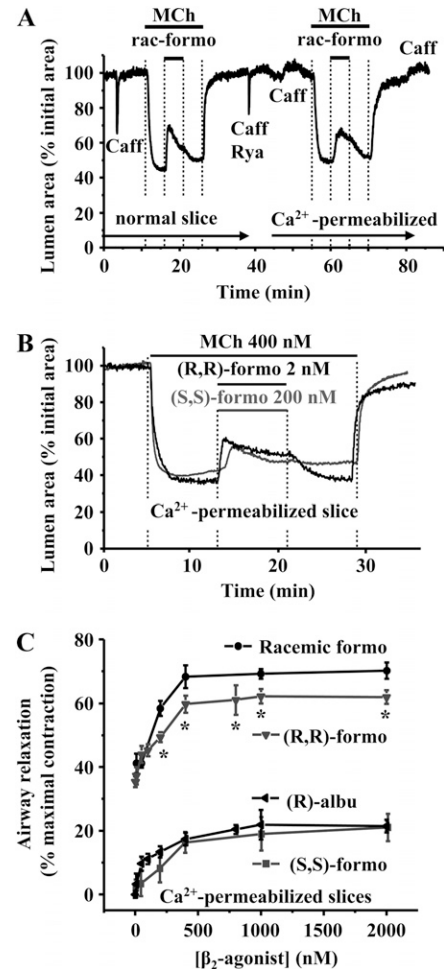
#### Relative Contribution of Ca<sup>2+</sup> Signaling and Sensitivity to Airway Relaxation

To determine the relative contribution of the decrease in the frequency of the Ca<sup>2+</sup> oscillations and Ca<sup>2+</sup> sensitivity induced by formoterol or albuterol in airway relaxation, we compared



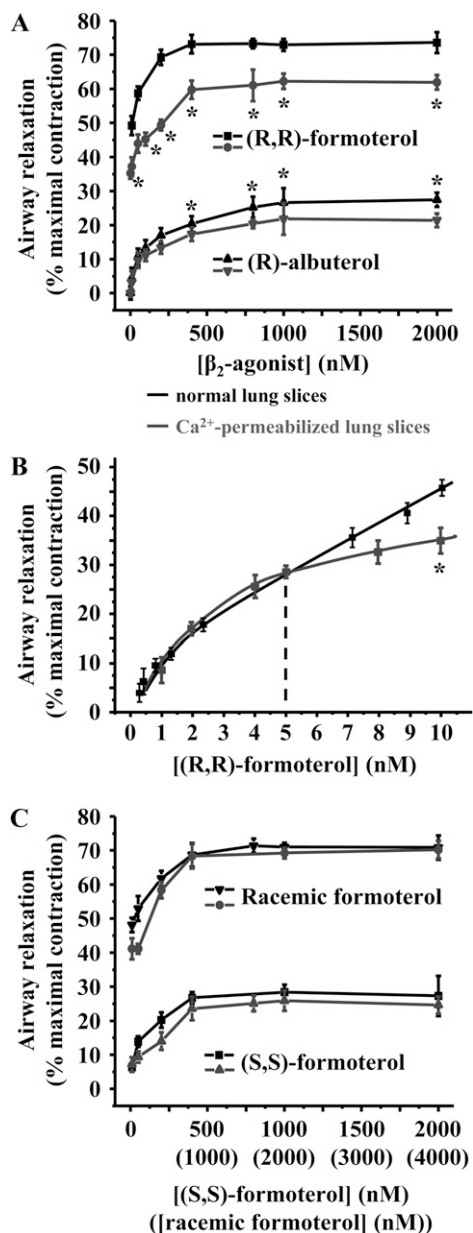
**Figure 6.** The effect of the photolytic release of IP<sub>3</sub> in SMCs exposed to (R,R)-formoterol. (A) Fluorescence image indicating the location of SMCs and ECs from the airway observed. (B) Line scan analysis (for 180 s from the white line indicated in [A]) showing that the rhythmic Ca<sup>2+</sup> oscillations in two SMCs induced by 400 nM MCh were inhibited by 500 nM (R,R)-formoterol, and that this allowed the airway to relax. The subsequent flash photolysis (500 ms, jagged arrow) of caged-iso-Ins(1,4,5)P<sub>3</sub>/PMIP<sub>3</sub> (caged-IP<sub>3</sub>) in presence of MCh and (R,R)-formoterol induced a short period of Ca<sup>2+</sup> oscillations. The upward shift of the fluorescence trace indicates the transient contraction of the airway induced by IP<sub>3</sub> release from caged-IP<sub>3</sub>. As the Ca<sup>2+</sup> oscillations slowed again, the airway relaxed. (C) Representative experiment showing that Ca<sup>2+</sup> oscillations induced in an airway SMC by 400 nM MCh were initially abolished by (R)-albuterol (1 μM), but, after 2 minutes, the Ca<sup>2+</sup> oscillations resumed at a lower frequency. (D) Representative experiment showing that the Ca<sup>2+</sup> oscillations induced by 400 nM MCh were not affected by 5 nM (R,R)-formoterol.

(in Figures 8A–8C) the concentration–relaxation data of normal (from Figures 1D, 3B, and 3D) and Ca<sup>2+</sup>-permeabilized lung slices (from Figure 7C). At concentrations equal to or higher than 10 nM, (R,R)-formoterol induced a greater relaxation in normal as compared with Ca<sup>2+</sup>-permeabilized slices (Figure 8A, top). This indicates that the relaxation induced by a decrease in Ca<sup>2+</sup> signaling and sensitivity were additive. Similarly, the relaxation induced by (R)-albuterol in normal airways was greater (small difference, but significant) than in Ca<sup>2+</sup>-permeabilized airways (Figure 8A, bottom). From this analysis, it is also evident that (R,R)-formoterol is significantly more effective at relaxing airways than (R)-albuterol in either normal or Ca<sup>2+</sup>-



**Figure 7.** Effect of formoterol isomers and (R)-albuterol on Ca<sup>2+</sup> sensitivity in Ca<sup>2+</sup>-permeabilized lung slices. (A) A normal airway was contracted with 400 nM MCh. The addition of racemic formoterol (400 nM) induced relaxation of the airway. To convert this lung slice into the Ca<sup>2+</sup>-permeabilized state, the slice was perfused simultaneously with 20 mM caffeine and 50 μM ryanodine, which induced a transient contraction. A second caffeine application did not evoke a contraction, confirming the Ca<sup>2+</sup>-permeabilized status of the lung slice. Exposure to 400 nM MCh induced contraction of the same airway in a similar manner to that induced in the normal airway, indicating that MCh increased Ca<sup>2+</sup> sensitivity. Exposure to racemic formoterol relaxed the contracted airway, indicating a decrease in Ca<sup>2+</sup> sensitivity. (B) Representative experiments showing airway contraction induced by 400 nM MCh, followed by relaxation induced by (R,R)-formoterol (2 nM) (black line) and (S,S)-formoterol (200 nM) (gray line) in a Ca<sup>2+</sup>-permeabilized lung slice. Under these conditions (SMCs with a high clamped intracellular calcium concentration [[Ca<sup>2+</sup>]<sub>i</sub>]), contraction indicates an increase in Ca<sup>2+</sup> sensitivity; relaxation indicates a decrease in Ca<sup>2+</sup> sensitivity. (C) The mean concentration-dependent relaxation of airways contracted by MCh (400 nM) to (R,R)-, racemic, (S,S)-formoterol, and (R)-albuterol in Ca<sup>2+</sup>-permeabilized slices (*n* = 6 mice for (R,R)-formoterol and racemic formoterol; *n* = 5 mice for (S,S)-formoterol and (R)-albuterol). Mean (±SD) values are presented; a minimum of three experiments was performed for each concentration for each mouse.

permeabilized lung slices. One implication of these results is that the decrease in Ca<sup>2+</sup> sensitivity alone induced by formoterol is substantially more effective than the combined effect of (R)-albuterol on the Ca<sup>2+</sup> signaling and Ca<sup>2+</sup> sensitivity. This was confirmed by the fact that low concentrations of (R,R)-formoterol (<10 nM) induced a similar relaxation in both



**Figure 8.** Relative contribution of  $\text{Ca}^{2+}$  signaling and  $\text{Ca}^{2+}$  sensitivity to airway relaxation induced by formoterol isomers and (R)-albuterol. (A) The mean concentration-dependent relaxation of airways (contracted by MCh, 400 nM) induced by (R,R)-formoterol and (R)-albuterol (1–2,000 nM) in normal (black line, data from Figures 1D and 3B) and  $\text{Ca}^{2+}$ -permeabilized lung slices (gray line, data from Figure 7C). Relaxation induced by (R,R)-formoterol and (R)-albuterol in normal and  $\text{Ca}^{2+}$ -permeabilized lung slices was significantly different ( $*P < 0.05$ ). (B) The mean concentration-dependent relaxation of airways (contracted by MCh, 400 nM) induced by low concentrations of (R,R)-formoterol (0.5–10 nM) in normal (black line, data from Figure 3D) and  $\text{Ca}^{2+}$ -permeabilized lung slices (gray line;  $*P < 0.05$ ;  $n = 5$  mice). Mean ( $\pm$ SD) values are presented; a minimum of three experiments was performed for each concentration for each mouse. (C) The mean concentration-dependent relaxation of airways (contracted by MCh, 400 nM) induced by racemic- and (S,S)-formoterol in normal (black line, data from Figure 1D) and  $\text{Ca}^{2+}$ -permeabilized slices (gray line, data from Figure 7C).

normal and  $\text{Ca}^{2+}$ -permeabilized airways (Figure 8B). Whereas (R)-albuterol at 1  $\mu\text{M}$  induced relaxation by a decrease in  $\text{Ca}^{2+}$  signaling and sensitivity (Figure 8A, bottom), 5 nM formoterol induced a similar relaxation by only decreasing the  $\text{Ca}^{2+}$  sensitivity (Figure 8B).

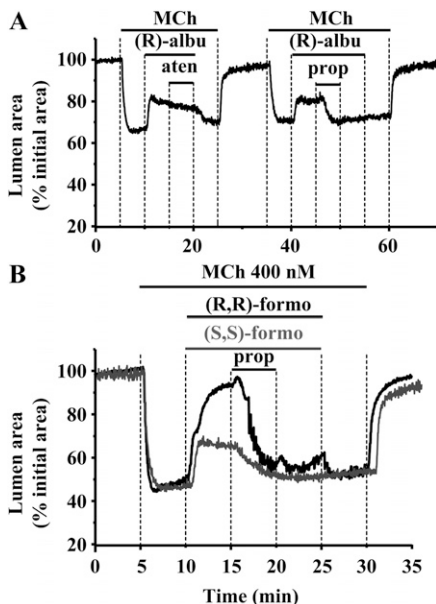
In contrast to (R,R)-formoterol or (R)-albuterol, the relaxation induced by racemic formoterol or (S,S)-formoterol in normal slices, as compared with its effect in  $\text{Ca}^{2+}$ -permeabilized lung slices (Figure 8C), was similar at all concentrations tested. The implication of these results is that the decrease in  $\text{Ca}^{2+}$  sensitivity predominately mediates the relaxant effect. Interestingly, the relaxation induced by (R,R)-formoterol or racemic formoterol in normal slices was similar to the relaxation induced in  $\text{Ca}^{2+}$ -permeabilized lung slices by racemic formoterol (compare Figures 8A and 8C). The implication of this result is that the decrease in the  $\text{Ca}^{2+}$  sensitivity induced by (R,R)- and (S,S)-formoterol is additive to give the maximal response. Importantly, because (S,S)-formoterol does not effect  $\text{Ca}^{2+}$  oscillations even at high concentrations, its mechanism of action appears to be different from that of (R,R)-formoterol.

#### $\beta_2$ -Adrenoceptors and Airway Relaxation Induced by Formoterol and (R)-Albuterol

Albuterol, (R,R)-formoterol, and (S,S)-formoterol are believed to act on  $\beta_2$ -adrenoceptors. However, (R,R)-formoterol, but not (S,S)-formoterol, decreased the frequency of  $\text{Ca}^{2+}$  oscillations. In addition, (R,R)-formoterol is more effective at relaxing airways than similar concentration of (S,S)-formoterol or (R)-albuterol. Consequently, the effects of (R,R)-formoterol, (S,S)-formoterol, or (R)-albuterol may be mediated by mechanisms other than via  $\beta_2$ -adrenoceptors. To explore this possibility, we examined the effect of  $\beta$ -adrenoceptor antagonists on the relaxation responses. We found that atenolol (1 and 10  $\mu\text{M}$ ), a specific  $\beta_1$ -antagonist, failed to modify the relaxation response of airways to formoterol or (R)-albuterol. However, propranolol (1  $\mu\text{M}$ ), a  $\beta_1/\beta_2$ -antagonist, inhibited the effects of both formoterol and albuterol (Figures 9A and 9B). This is consistent with previous data indicating that the  $\beta_2$  receptor primarily mediates the responses to albuterol and formoterol.

#### DISCUSSION

In this study, we characterized the acute effects of (R,R)-, (S,S)-, and racemic formoterol on mouse small airways contracted with MCh. (R,R)- and racemic formoterol were found to have a similar relaxant efficacy; they both induced a fast, large, and sustained relaxation. We also found that (S,S)-formoterol had a relaxant effect, although significantly smaller than (R,R)- or racemic formoterol. Although caution is always required when extrapolating data from mouse airways to human airways, these results are consistent with clinical data that showed no difference between (R,R)-formoterol and racemic formoterol on bronchodilation up to 24 hours for patients with asthma (9). It is not clear if longer exposure would have a similar outcome. However, a recent study of patients with chronic obstructive pulmonary disease showed a similar bronchodilator efficacy for (R,R)- and racemic formoterol after 14 days of treatment (24). Previous studies with guinea pig tracheal strips support the idea that (R,R)- and racemic formoterol have a similar relaxant efficacy, but, in those studies, (S,S)-formoterol had no significant effect on airway contractility (7, 8). It is possible that this difference results from the differences between SMCs from trachea and intrapulmonary airways, between species, or the contractile agonists used. In addition, the method for studying airway relaxation is different. On the other hand, the efficacy of formoterol is high, which makes the detection of small differences difficult.



**Figure 9.** Contribution of the  $\beta_2$ -adrenoceptors to airway relaxation induced by (R)-albuterol or formoterol isomers. (A) An airway was contracted with 400 nM MCh and exposed to 1  $\mu$ M (R)-albuterol to induce relaxation. The subsequent exposure to atenolol (aten; 1  $\mu$ M), a specific  $\beta_1$ -antagonist, failed to modify the response of airways to (R)-albuterol. After washing for 10 minutes with Hanks' balanced salt solutions supplemented with 25 mM Hepes, the same airway was contracted with 400 nM MCh, exposed to (R)-albuterol (1  $\mu$ M) to induce relaxation, and then treated with the  $\beta_1/\beta_2$ -antagonist, propranolol (prop; 1  $\mu$ M). Propranolol inhibited the relaxation induced by (R)-albuterol. (B) Representative experiments showing that 1  $\mu$ M propranolol inhibited the relaxation induced by 1  $\mu$ M (R,R)-formoterol (black line) or 2  $\mu$ M (S,S)-formoterol (gray line).

We also examined if (R,R)- and (S,S)-formoterol act through  $\beta_2$  receptor. The  $\beta_1$ -specific antagonist, atenolol, and a nonselective  $\beta$  receptor blocker, ( $\beta_1/\beta_2$ ) propranolol, were used with our mouse lung slices. Atenolol had no effect on the relaxation induced by (R,R)- or (S,S)-formoterol. However, propranolol inhibited the relaxation induced by (R,R)- or (S,S)-formoterol and (R)-albuterol. This implies the  $\beta_2$  receptor was primarily involved in relaxation induced by all the isomers examined.

Changes in  $\text{Ca}^{2+}$  are believed to be the principle mechanism by which airway SMC contraction is mediated, and data from our previous studies indicate that the frequency of  $\text{Ca}^{2+}$  oscillations within SMCs is a key parameter determining their contractility (14, 16). The frequency of these  $\text{Ca}^{2+}$  oscillations is modified during the relaxation induced by agents that elevate cAMP, such as FSK, ISO (18), and (R)- and racemic-albuterol (19). FSK and ISO slowed the frequency of  $\text{Ca}^{2+}$  oscillations, whereas (R)-albuterol (at a high concentration) briefly arrested the  $\text{Ca}^{2+}$  oscillations induced by MCh during the first 1–2 minutes of exposure. However, the  $\text{Ca}^{2+}$  oscillations usually resumed, but at a slower rate than with MCh alone. By contrast, (R,R)- and racemic formoterol (at concentrations  $>1 \mu\text{M}$ ) completely stopped the  $\text{Ca}^{2+}$  oscillations, and this was correlated with substantial airway relaxation. The  $\text{Ca}^{2+}$  oscillations did not recover even after several minutes of exposure to (R,R)- or racemic formoterol. The strong effect of (R,R)-formoterol on the  $\text{Ca}^{2+}$  oscillations explains, in part, why (R,R)-formoterol has a greater relaxant effect than similar concentrations of albuterol, ISO, or FSK. Because the relaxant effect of FSK was found to be mediated by inhibiting the  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$

release from internal  $\text{Ca}^{2+}$  stores, we tested the possibility that formoterol could affect the  $\text{Ca}^{2+}$  oscillations by a similar mechanism. We found that the  $\text{Ca}^{2+}$  oscillations could be restored by the release of additional  $\text{IP}_3$  from caged- $\text{IP}_3$ . This result suggests that the inherent source of  $\text{IP}_3$  (phospholipase C- $\beta$  activity) has been depleted or inhibited by (R,R)- or racemic formoterol (at concentrations around 1  $\mu\text{M}$ ). Alternatively, the sensitivity of the  $\text{IP}_3\text{R}$  to the endogenous  $\text{IP}_3$  may be greatly reduced. Although it is likely, in view of our previous results with ISO, that the  $\text{IP}_3\text{R}$  sensitivity to  $\text{IP}_3$  is decreased, the continued occurrence of  $\text{Ca}^{2+}$  oscillations in the presence of ISO or (R)-albuterol suggests that this decrease in  $\text{IP}_3\text{R}$  sensitivity was insufficient to render the receptor totally unresponsive to  $\text{IP}_3$ . Thus, a lack of  $\text{IP}_3$  is a more likely explanation for the sustained lack of  $\text{Ca}^{2+}$  oscillations.

Interestingly, (S,S)-formoterol or low concentration of (R,R)-formoterol that induced airway relaxation had no effect on  $\text{Ca}^{2+}$  oscillations; this result suggests that formoterol primarily induces relaxation by a different mechanism. It is well recognized that the contractility of airway SMCs is also regulated by the sensitivity of the contractile apparatus to  $\text{Ca}^{2+}$  or "Ca $^{2+}$  sensitivity" (20, 21). However, the details of this mechanism are poorly understood in airway SMCs. Using  $\text{Ca}^{2+}$ -permeabilized SMCs, in which the  $[\text{Ca}^{2+}]_i$  was clamped at a high constant level, we found that (S,S)-formoterol induced a relaxation similar to that induced in normal airways. Therefore, we concluded that (S,S)-formoterol relaxed airways by altering the  $\text{Ca}^{2+}$  sensitivity. This could also explain why the effect of the S-isomer is relatively slow acting in comparison with (R,R)- and racemic formoterol.

Importantly, (R,R)-formoterol was found to significantly decrease the  $\text{Ca}^{2+}$  sensitivity, although the relaxation induced by (R,R)-formoterol in  $\text{Ca}^{2+}$ -permeabilized airway was smaller than in normal airways. However, the difference in (R,R)-formoterol-induced relaxation between normal and  $\text{Ca}^{2+}$ -permeabilized airways is relatively small, and this suggests that most of the relaxant effect of (R,R)-formoterol can be mediated by a decrease of the  $\text{Ca}^{2+}$  sensitivity, even though the  $[\text{Ca}^{2+}]_i$  remains elevated. The combination of a low  $[\text{Ca}^{2+}]_i$  brought about by slowing or stopping the  $\text{Ca}^{2+}$  oscillations, together with a decrease in  $\text{Ca}^{2+}$  sensitivity, gave approximately 10–15% more relaxation. On the other hand, racemic formoterol was found to induce a similar relaxation in both normal and  $\text{Ca}^{2+}$ -permeabilized slices. The difference between racemic and (R,R)-formoterol on  $\text{Ca}^{2+}$ -permeabilized lung slices could be explained by the additional effect of the S-isomer on  $\text{Ca}^{2+}$  sensitivity, and supports the idea that a decrease in  $\text{Ca}^{2+}$  sensitivity is a major mechanism determining the relaxation induced by formoterol.

We also compared relaxation induced by (R,R)-formoterol and the SABA, (R)-albuterol. As expected, (R,R)-formoterol is much more potent than similar concentration of (R)-albuterol at relaxing mouse airways, and this is correlated with a stronger effect of (R,R)-formoterol on both  $\text{Ca}^{2+}$  oscillations and  $\text{Ca}^{2+}$  sensitivity.  $\beta_2$ -Agonists have different affinity and efficacy (25, 26), and this could explain why similar concentration of (R,R)-formoterol, (S,S)-formoterol, and (R)-albuterol induced a different amount of relaxation. Formoterol is believed to have a higher affinity for the  $\beta_2$ -adrenoceptor than albuterol (26). Therefore, we compared (R,R)-formoterol and (R)-albuterol at an equivalent relaxation. We found that, at 5 nM, (R,R)-formoterol induced a similar relaxation of mouse airways compared with 1,000 nM albuterol. Importantly, at these concentrations, formoterol exerted its relaxant effect by only decreasing the  $\text{Ca}^{2+}$  sensitivity; relaxation induced by (R)-albuterol was mediated by both a decrease in the  $\text{Ca}^{2+}$  sensitivity and by slowing the  $\text{Ca}^{2+}$  oscillations.



It is also interesting to note that 1  $\mu\text{M}$  (S,S)-formoterol induced a maximal relaxation of 21%, which is equivalent to the relaxation induced by approximately 3.5 nM (R,R)-formoterol (compare Figures 1D and 3D). In both cases, the relaxation of mouse airways was mediated by a decrease in  $\text{Ca}^{2+}$  sensitivity without a change in  $\text{Ca}^{2+}$  oscillations. However, unlike (R,R)-formoterol, (S,S)-formoterol had no ability to stop or slow the  $\text{Ca}^{2+}$  oscillations even at higher concentrations (up to 4  $\mu\text{M}$ ). (S,S)-formoterol did not appear to compete with its R-isomer, because racemic formoterol (at all the concentrations tested) induced a similar relaxation to that of (R,R)-formoterol (for equivalent concentrations of (R,R)-formoterol). In addition, the decrease in the  $\text{Ca}^{2+}$  sensitivity induced by (R,R)- and (S,S)-formoterol observed in  $\text{Ca}^{2+}$ -permeabilized lung slices was found to be additive. Whereas (S,S)-formoterol acts through  $\beta_2$ -adrenoceptors, but with a lower affinity than (R,R)-formoterol, our results suggest that (S,S)-formoterol exerts its action by a different process than that used by (R,R)-formoterol. The difference in molecular size and structure of the  $\beta_2$ -agonists, their different hydrophilic properties, and their residency time at the active site of the  $\beta_2$ -adrenoceptors are mainly responsible for their respective affinity and efficacy. However, our results also suggest that the  $\beta_2$ -adrenoceptors could activate different molecular pathways according to the  $\beta_2$ -agonists and isomers used. The regulation of the  $\text{Ca}^{2+}$  sensitivity would most likely reflect the activity of myosin light-chain phosphatase, and this appears to mediate most of the relaxant effect induced by albuterol and formoterol in mouse lung slices.

In summary, (R,R)- and racemic formoterol quickly induced sustained airway relaxation by a combination of two processes: a decrease in  $\text{Ca}^{2+}$  sensitivity, and a decrease in the frequency of  $\text{Ca}^{2+}$  oscillations. The strong efficacy of both (R,R)- and racemic formoterol appears to be associated primarily with its ability, especially at low concentrations, to substantially reduce  $\text{Ca}^{2+}$  sensitivity and bring about relaxation, even in the presence of ongoing  $\text{Ca}^{2+}$  oscillations. In this respect, (S,S)-formoterol had a similar, but weaker, behavior, and induced relaxation without the ability to influence  $\text{Ca}^{2+}$  oscillations. This capability of (S,S)-formoterol to decrease  $\text{Ca}^{2+}$  sensitivity also appears to enhance the effect of (R,R)-formoterol. The rapid onset of relaxation induced by higher concentrations of the (R,R)- and racemic formoterol appears to be mediated by the additional simultaneous reduction or stoppage of the frequency of the  $\text{Ca}^{2+}$  oscillations. These studies with short-term exposures to  $\beta_2$ -agonists in mouse lung slices also indicate that formoterol is a more effective bronchodilator than albuterol, and that its mechanism of action is not identical. A final important point is that variable degrees of relaxation were induced by the various isomers of formoterol; this implies that the isomer formulation of bronchodilators must be taken into consideration when evaluating their therapeutic potential.

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