Effects of Albuterol Isomers on the Contraction and Ca^{2+} Signaling of Small Airways in Mouse Lung Slices

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The β_2 -adrenergic agonist, albuterol, is used as a bronchodilator by patients with asthma and consists of a racemic mixture of (R)- and (S)-albuterol. However, the action of the individual enantiomers is poorly understood. Consequently, we investigated the effects of (R)-, (S)- and racemic-albuterol on airway smooth muscle cell (SMC) contraction and Ca^{2+} signaling in mouse lung slices with phase-contrast and confocal microscopy. (R)-albuterol relaxed airways contracted with methacholine (MCh) in a dose-dependent manner. By contrast, (S)-albuterol had no effect on airways. (R)-albuterol had a greater relaxant effect than a double concentration of racemic albuterol. Because MCh-induced contraction of airway SMCs is mediated by Ca^{2+} oscillations and an increase in Ca^{2+} sensitivity, the effects of albuterol on these responses were examined. Both (R)- and racemic albuterol decreased the frequency of the MCh-induced Ca^{2+} oscillations by a similar amount. However, (R)-albuterol was more effective than racemic albuterol in decreasing the Ca^{2+} sensitivity of the airway SMCs in "model" lung slices with a clamped $[\text{Ca}^{2+}]_i$. In contrast, (S)-albuterol had no effect on the Ca^{2+} oscillations or the Ca^{2+} sensitivity. In conclusion, (R)-albuterol consistently induced a greater airway relaxation than racemic albuterol, and (S)-albuterol appears to be responsible for this reduced efficacy.

Keywords: hyper-reactivity; β_2 -agonist; relaxation; Ca^{2+} oscillations; Ca^{2+} sensitivity

Inhalation of the β_2 -adrenergic agonist albuterol, a racemic mixture (~ 50%) of (R)- and (S)-albuterol (1), is commonly used to relieve bronchial constriction associated with asthma (1, 2). However, frequent use can reduce the efficacy of racemic albuterol and may, in fact, exacerbate bronchial constriction (3, 4). Several studies have compared (R)-albuterol and racemic albuterol, and have suggested that the use of the single (R)-enantiomer provided bronchodilator effects at a reduced doses and with fewer hospitalizations (5–7). However, other studies indicated that (R)-albuterol and racemic albuterol had a similar efficacy (1, 8–10). The (R)-enantiomer was found to strongly bind to β_2 receptors and mediate airway relaxation, while the (S)-enantiomer only weakly binds to β_2 receptors and was unable to relax airways (11–13).

It is likely that some of the ambiguity related to the action of albuterol has arisen because many of the earlier studies were performed with smooth muscle cells (SMCs) isolated from the trachea rather than small airways and because a simultaneous correlation of the contractile response with cellular changes in Ca^{2+} was not possible. Only a few studies have investigated the effects of albuterol on Ca^{2+} signaling. Mitra and coworkers (14)

CLINICAL RELEVANCE

Albuterol is commonly used to relieve bronchial constriction and acts by relaxing airway smooth muscle. This study focused on the influence of (R)- and (S)-enantiomers of albuterol on airway contraction in lung slice preparations from mice.

reported that (S)- and racemic albuterol slowly increased $[\text{Ca}^{2+}]_i$ in bovine tracheal SMCs and that (S)-albuterol bound to muscarinic receptors to activate inositol trisphosphate (IP_3) production and the release of internal Ca^{2+} . In the same study, Mitra and colleagues also found that in some SMCs (S)-albuterol induced a few Ca^{2+} oscillations. However, this effect resulted from the action of the drug on resting cells, while albuterol is usually used during an asthmatic attack when the SMCs are contracted and presumably exhibiting significant Ca^{2+} signaling. Albuterol-induced increases in $[\text{Ca}^{2+}]_i$ have also been reported for cultured human bronchial SMCs in the presence of MCh but only a single time point was recorded from a population of cells. In none of these studies was contraction and Ca^{2+} studied simultaneously in the same cell (11).

We (15, 16) and others (17, 18) have shown that Ca^{2+} oscillations are a key determinate of agonist-induced contraction in small airways and that SMC relaxation (often induced by elevating cAMP) can be achieved by slowing the frequency of the Ca^{2+} oscillations (16). A second important response of airway SMCs is their ability to alter their contractile sensitivity to Ca^{2+} (19–21). This response has been determined in "model" or "skinned" SMCs where the $[\text{Ca}^{2+}]_i$ was clamped at a constant level but different contractile forces were induced with different concentrations of agonist. We found in "model" lung slices (21) that high sustained Ca^{2+} induced a full relaxation of mouse airway SMCs, but in the presence of agonist (MCh), the contractile response to Ca^{2+} was fully restored. The important implication of these results is that rapid Ca^{2+} oscillations induce contraction via the activation of myosin light chain (MLC) kinase, whereas slow sustained Ca^{2+} increases can induce relaxation in mouse airways via the activation MLC phosphatase activity (21).

To gain a better understanding of the effects of single isomers of β_2 -adrenergic compounds at the cellular level, we compared the effects of (R)-, (S)- and racemic albuterol on airway SMC contraction and Ca^{2+} signaling. We found that (R)-albuterol relaxed MCh-induced airway contraction in a dose-dependent manner and that (R)-albuterol had a greater relaxant effect than racemic albuterol, even with a doubled dose of racemic albuterol (to match the concentration of (R)-albuterol). By contrast, (S)-albuterol alone had no effect on airway contraction. We show that (R)- and racemic albuterol can relax airways contracted with MCh by slowing down the frequency of the Ca^{2+} oscillations and by decreasing the sensitivity of the contractile apparatus to Ca^{2+} . (S)-albuterol alone had no effect on the frequency of Ca^{2+} oscillations or the sensitivity to Ca^{2+} but when associated with (R)-albuterol, reduced the efficacy of the racemic albuterol to

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relax the airways. The initial efficacy of (R)-albuterol correlates with its ability to quickly reduce the frequency of Ca^{2+} oscillations while the greater reduction in the Ca^{2+} sensitivity induced by (R)-albuterol is responsible for the difference in the sustained relaxation state induced by (R)- or racemic albuterol.

MATERIALS AND METHODS

Solutions and Chemicals

Hanks' balanced salt solution without phenol red (HBSS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO/Invitrogen Corp. (Carlsbad, CA). HBSS was supplemented with 25 mM HEPES (sHBSS, pH 7.4). Oregon Green 488 BAPTA-1-AM was obtained from Molecular Probes/Invitrogen Corp. (Eugene, OR), and Pluronic F-127 was obtained from Calbiochem (La Jolla, CA). Sulfobromophthalein and DMSO were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Agarose was either obtained from Sigma (type VII-A, low gelling temperature) or Invitrogen (low melting point, cat 15517-014). It may be necessary to perform a batch analysis with the agarose to verify the lung slice response. (R)-, (S)- and racemic albuterol were donated by Sepracor, Inc. (Marlborough, MA). Oregon Green 488 BAPTA-1-AM (50 $\mu\text{g}/20\ \mu\text{l}$) and Pluronic F-127 (20%) were dissolved in DMSO and diluted in sHBSS to the final working concentrations.

Preparation of Mouse Lung Slices

Lung slices were prepared by the methods previously described (15). Lung slices were prepared from BALB/C mice (7 to 9 wk old; Charles River Breeding Labs, Needham, MA), which were killed by intraperitoneal injection of pentobarbital sodium (Nembutal) as approved by the IACUC of the University of Massachusetts Medical School. The trachea was cannulated and the lungs were inflated (to approximately total lung capacity) 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. While maintaining the airway pressure, the lungs were cooled with cold sHBSS to gel the agarose. A lung lobe was mounted in a vibratome (model EMS-4000; Electron Microscopy Sciences, Hatfield, PA) with the peripheral region uppermost and lung slices approximately 130 μm thick were cut in cold sHBSS. Lung slices were maintained in DMEM supplemented with antibiotics and antimycotics at 37°C in 10% CO_2 . Lung slices were used within 3 days.

Measurement of the Contractile Response of Airways

Lung slices were mounted in a simple custom-made perfusion chamber consisting of two coverslips. The lung slice was placed on a large coverslip and held in place with a small sheet of nylon mesh with a central opening. A second coverslip, edged with silicone grease, was placed over the slice. Perfusion of the slice in the chamber was performed by a gravity-fed perfusion system (15). Lung slices were observed with phase-contrast microscopy on an inverted microscope with a $\times 10$ objective and images were recorded using a CCD camera and image acquisition software ("Video Savant"; IO Industries, London, Ontario, Canada). Digital images were recorded in time-lapse (0.5 Hz), stored directly 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 pre-stimulation lumen area (initial area). All measurements were performed at room temperature.

Measurement of Intracellular Ca^{2+}

Lung slices (about 12–15) were incubated with Oregon Green 488 BAPTA-1-AM (20 μM , 2 ml), 100 μM sulfobromophthalein (an inhibitor that prevents dye extrusion via anion exchangers), and 0.05% Pluronic F-127 for 40 minutes at 30°C. Final DMSO concentration was 1.25%. For de-esterification, the slices were washed for 40 minutes in sHBSS containing 100 μM sulfobromophthalein at 30°C. Dye-loaded lung slices were mounted in a custom perfusion chamber and imaging was performed using a video-rate confocal microscope (15, 22, 23). In brief, a 488-nm laser provided the excitation light and the emitted fluorescence ($> 510\ \text{nm}$) was detected by a photomultiplier tube and a frame capture board to form an image.

Fluorescence images were recorded to a hard disk using Video Savant software at 15 or 30 fps. Changes in fluorescence were analyzed by selecting a region of interest (ROI, 5×5 pixels) in a single SMC. Changes in fluorescence (F) were normalized to the initial level of fluorescence (F_0) and final fluorescence values were expressed as a ratio (F/F_0).

Preparation of "Model" Lung Slices

"Model" lung slices were prepared by perfusing "normal" lung slices with sHBSS containing 20 mM caffeine and 50 μM ryanodine for at least 4 minutes, followed by washout with sHBSS. In this procedure, caffeine activates or opens the ryanodine receptors (RyR) of the sarcoplasmic reticulum (SR), and this allows ryanodine to lock the RyR in an open state. This results in the depletion of the intracellular Ca^{2+} store of the SR, which in turn, increases the Ca^{2+} permeability of the plasma membrane by the activation of store-operated Ca^{2+} channels (21). Under these conditions, the $[\text{Ca}^{2+}]_i$ is determined by the extracellular Ca^{2+} concentration, which was set at 1.3 mM (sHBSS). Importantly, the effect of the caffeine-ryanodine treatment was not reversible.

Statistics

Data are expressed as means $\pm$ SD. Statistical analysis was assessed by ANOVA. Comparison of paired-data was done using a paired-samples *t* test. A value of $P < 0.05$ was considered statistically different.

RESULTS

To determine if albuterol isomers influence airway contraction (increase or decrease), it is necessary to set the airway contraction near the mid-point of their contractile response. Consequently, we initially confirmed the contractile response of the small airways of lung slices to the agonist MCh. As the MCh concentration was sequentially increased, the extent of airway contraction increased and a maximal contraction was induced by 1 μM MCh. As a result, an MCh concentration of 400 nM was used for experiments on airway contraction because this induced a substantial but not maximal contraction.

Effect of (R)-Albuterol on Airway Contraction

The acute effect of (R)-albuterol on contracted airways was investigated by repeatedly exposing an airway to 400 nM MCh followed by (R)-albuterol over a range of concentrations from 10 nM to 2 μM (Figure 1A). With this approach, we found that (R)-albuterol was able to relax MCh-induced airway contraction in a dose-dependent manner (Figure 1B). A maximal relaxation of $26.6 \pm 4.2\%$ ($n = 10$ slices from three mice) was induced by 1 μM (R)-albuterol (expressed as a percentage of the contraction induced by 400 nM MCh). Frequently, the relaxation was followed by a small re-contraction of the airway. The relaxant effect of (R)-albuterol was completely reversible; the airway re-contracted to its previous level after albuterol removal (Figure 1A).

In a second series of experiments, the efficacy of (R)-albuterol (1 μM) was tested against weak or strong airway contraction (induced by low or high doses of MCh) (Figure 2A). (R)-albuterol was able to almost fully relax airways contracted with low doses of MCh (up to 200 nM), while at higher doses of contractile agonist, (R)-albuterol was only able to partially relax the airways ($5.54 \pm 2.63\%$ of the contraction induced by 1000 nM MCh, $n = 9$ from three mice, $P < 0.05$) (Figure 2B).

Effect of Racemic and (S)-Albuterol on Airway Contraction

A similar series of experiments were performed in which airways were contracted with MCh and exposed to a range of concentrations of racemic albuterol or (S)-albuterol (Figure 1B). At most concentrations racemic albuterol (compared at equal concentrations of (R)-albuterol) induced significantly less air-

Figure 1. The relaxant effect of a range of (R)-albuterol concentrations on airways contracted with methacholine (MCh). (A) The airways were contracted with 400 nM MCh and exposed to a series of (R)-albuterol concentrations (10 nM to 2 μ M). In response to albuterol, the airways quickly relaxed, in dose-dependent manner. After the initial relaxation, the airways displayed a small recontraction. Between each application, the airways were washed with sHBSS to return the airway to the relaxed state. (B) The mean concentration-dependent relaxation response of airways contracted with MCh (400 nM) to (R)-albuterol (10–2,000 nM), racemic albuterol (20–4,000 nM) and (S)-albuterol (10–2,000 nM), plotted with respect to the (R)-albuterol concentration in each formulation. With increasing concentration, (R)-albuterol induced a greater relaxation than racemic albuterol. Each point represents 10 experiments (mean $\pm$ SD) in different airways from three mice. *The difference between (R)-albuterol and racemic albuterol is statistically significant ($P < 0.05$).

way relaxation than (R)-albuterol (Figure 1B). A maximal difference of $6.5 \pm 1.6\%$ was found between racemic (2 μ M) and (R)-albuterol (1 μ M) ($n = 10$ from three mice, $P < 0.05$). On the other hand, (S)-albuterol at concentrations up to 2 μ M had no effect on airway contraction (10 experiments in different airways from three mice).

To confirm these differential effects of albuterol isomers, we examined the effects of the three formulations of albuterol on the same airway contracted with MCh 400 nM (Figure 3A). The three formulations were also tested in multiple different orders to confirm that a previous exposure to one enantiomer did not affect the responses of a subsequent enantiomer. In all cases, we found that (S)-albuterol had no effect on airway contraction, but that (R)-albuterol (1 μ M) had a greater relaxant effect than racemic albuterol (2 μ M) (Figure 3A).

Figure 2. The relaxant effect of 1 μ M (R)-albuterol on airways contracted with increasing concentration of MCh. (A) Effect of 1 μ M (R)-albuterol on the same airway contracted with increasing concentrations of MCh (100, 200, and 1,000 nM). The increasing MCh concentration induced greater contraction and this reduced the amount of relaxation that could be induced by albuterol. (B) A summary of the experiments shown in A indicating the relative relaxation induced by 1 μ M (R)-albuterol in airways contracted with increasing concentrations of MCh. Each bar represents nine experiments (mean $\pm$ SD) in different airways from three mice. In all graphs, the extent of contraction is expressed as a percentage relative to the lumen area (initial area, 100%) before stimulation. *The difference between the MCh and MCh + (R)-albuterol experiments was statistically significant ($P < 0.05$).

An implication of this difference between (R)- and racemic albuterol is that (S)-albuterol has an “antagonist” effect on relaxation. However, because no change in the contractility of the airways was observed in response to (S)-albuterol alone, we investigated the effect of an increasing concentration of (S)-albuterol (from zero to 20 μ M) on the airway relaxation induced by a constant concentration of (R)-albuterol (1 μ M) (Figure 3B). The relaxation induced by (R)-albuterol was proportionally decreased by increasing concentrations of (S)-albuterol up to 1 μ M. A maximal significant effect of $6.13 \pm 1.24\%$ (compared with (R)-albuterol 1 μ M alone) was achieved by 1 μ M (S)-albuterol ($n = 6$, $P < 0.05$). This decrease in the relaxant effect of (R)-albuterol by (S)-albuterol is similar to the difference in relaxation induced by racemic and (R)-albuterol. Experiments with higher concentrations of (S)-albuterol (10 and 20 μ M) did not show any further decrease in the relaxation induced by (R)-

Figure 3. A comparison of the effects of (R)-, (S)- and racemic albuterol on airway contraction. (A) Effect of racemic albuterol (2 μ M), (S)-albuterol (1 μ M), and (R)-albuterol (1 μ M) on the same airway contracted with 400 nM MCh. (S)-albuterol had no effect; racemic albuterol induced relaxation, while (R)-albuterol induced a greater relaxation. (B) The mean concentration-dependent relaxation of airways contracted with 400 nM MCh in response to 1 μ M (R)-albuterol in the presence of a range of (S)-albuterol concentrations (10–4,000 nM). The relaxation of the airways induced by (R)-albuterol was reduced with increasing concentrations of (S)-albuterol; the maximal effect of (S)-albuterol occurred at 1 μ M ($n = 6$, mean $\pm$ SD). *The difference between (R)-albuterol and (R)-albuterol + (S)-albuterol concentrations was significant ($P < 0.05$).

albuterol 1 μ M. These results suggest that (S)-albuterol interferes with the relaxant effect of (R)-albuterol (Figure 3B).

Effect of Albuterol Isomers on Ca^{2+} Oscillations Induced by MCh

Because the frequency of the Ca^{2+} oscillations within airway SMCs is an important parameter determining their contractility (15, 16), we investigated if the frequency of these Ca^{2+} oscillations could be modified by (R)- and racemic albuterol. In response to 200 nM MCh, the airway SMCs demonstrated an initial increase of intracellular Ca^{2+} , which was followed by rhythmic Ca^{2+} oscillations within 1 minute (12.26 ± 1.89 oscillations per minute, $n = 15$, Figures 4A and 4B). These Ca^{2+} oscillations were maintained on a slightly elevated baseline of $[\text{Ca}^{2+}]_i$. Immediately after addition of (R)-albuterol (1 μ M), the Ca^{2+} oscillations quickly slowed down and often stopped during

Figure 4. The effect of albuterol isomers on Ca^{2+} signaling of airway smooth muscle cells (SMCs). (A and B) Representative experiments showing the Ca^{2+} oscillations induced in individual airway SMCs of a lung slice by 200 nM MCh (recording rate: 30 frames per second). (A) Upon exposure to (R)-albuterol (1 μ M) the Ca^{2+} oscillations were abolished, but with time, the Ca^{2+} oscillations were reestablished although with a slower frequency. (B) Upon exposure to racemic albuterol (2 μ M), the Ca^{2+} oscillation frequency was initially reduced before recovering to a slower steady rate. (C) A summary graph showing the change in the frequency of Ca^{2+} oscillations in SMCs in response to 200 nM MCh and albuterol. (R)-albuterol (1 μ M) transiently abolished the Ca^{2+} oscillations, while racemic albuterol (2 μ M) decreased the frequency of Ca^{2+} oscillations. After 180 seconds the Ca^{2+} oscillation frequency had stabilized at a similar but slower rate. (S)-albuterol (1 μ M) had no effect on the Ca^{2+} oscillation frequency. Values expressed are means $\pm$ SD ($n = 11$ for (R)-albuterol, $n = 7$ for racemic albuterol, and $n = 7$ for (S)-albuterol).

the first 1 to 2 minutes (Figures 4A and 4C). A similar response was induced by racemic albuterol (2 μ M), except that the Ca^{2+} oscillations only slowed and did not stop (Figure 4B). An average of 2.8 ± 0.51 and 6.47 ± 0.85 oscillations per minute

was found for the first 2 minutes of exposure to (R)- ($n = 11$) and racemic albuterol ($n = 7$), respectively ($P < 0.05$, Figure 4C). With continued exposure to (R)- or racemic albuterol, the Ca^{2+} oscillations resumed and increased their frequency and approached a steady, but significantly slower rate than with MCh alone (7.1 ± 0.92 for (R)-albuterol, $n = 11$ and 7.7 ± 0.54 oscillations per min for racemic albuterol, $n = 7$, $P < 0.05$ compared to MCh alone) (Figure 4C). However, the frequency of oscillations was not significantly different between racemic and (R)-albuterol after 2 minutes of exposure. This slowing down of the Ca^{2+} oscillations was correlated with the relaxation of the airways. After removal of the albuterol, the frequency of Ca^{2+} oscillations returned to its original rate (data not shown).

Similar experiments were performed to investigate the effect of (S)-albuterol on the Ca^{2+} signaling of SMCs. However, (S)-albuterol neither increased baseline $[\text{Ca}^{2+}]_i$ (data not shown) nor influenced the frequency of Ca^{2+} oscillations induced by MCh (Figure 4C).

Effect of Albuterol Isomers on the Ca^{2+} Sensitivity of the Airway SMCs

A second mechanism that regulates the contractility of airway SMCs is the sensitivity of the contractile apparatus to Ca^{2+} or " Ca^{2+} sensitivity" (20, 21). To investigate the effects of albuterol isomers on this Ca^{2+} sensitivity, we compared the responses of the lung slice to albuterol isomers before ("normal") and after conversion into "model" lung slices by treatment with caffeine and ryanodine (Figure 5). In "model" lung slices, in the presence of extracellular Ca^{2+} , the $[\text{Ca}^{2+}]_i$ of the SMCs remains constantly elevated (21). The consequence of this elevated $[\text{Ca}^{2+}]_i$ is that the SMCs of the mouse airway are de-sensitized to Ca^{2+} , and this results in a fully relaxed airway. We have extensively characterized this response in a previous study (21). However, the addition of MCh to the "model" lung slice induced airway contraction, which indicates that the Ca^{2+} sensitivity of the SMCs was substantially increased. Interestingly, the contraction of the "model" lung slice in which the SMCs have a high clamped $[\text{Ca}^{2+}]_i$ was almost identical to that of "normal" lung slices in which the SMCs display Ca^{2+} oscillations (Figure 5A). When (R)-albuterol was subsequently added, we found that the "model" airway partially relaxed indicating a decrease in Ca^{2+} sensitivity (Figure 5A). Consistent with our earlier results, (S)-albuterol alone had no effect on the MCh-induced contractile state of "model" airways (Figure 5B).

To further determine the relative contribution of the decrease in Ca^{2+} sensitivity to the overall relaxant effect of albuterol isomers, we averaged ($n = 3$) and compared the relaxation of airways in "normal" lung slices with those of "model" lung slices in response to (R)- or racemic albuterol (Figure 5C). We found that (R)-albuterol induced a slower and initially smaller relaxation of airways in "model" lung slices compared with "normal" slices. Although we also found a similar difference for racemic albuterol between "normal" and "model" airways, the overall relaxation induced by racemic albuterol was much smaller than that induced by (R)-albuterol (Figure 5C).

To confirm these results, we examined the relaxation induced by racemic and (R)-albuterol in the same "model" airway (Figure 6). As expected, the relaxant effect of racemic albuterol was smaller than that of (R)-albuterol in the same airway (Figure 6A), but the temporal aspects of the relaxation were similar ($n = 3$, Figure 6B). The order in which the drugs were added was found to have no effect on the results. A maximal absolute difference of $6.5 \pm 0.36\%$ was observed between racemic and (R)-albuterol ($n = 3$, $P < 0.05$). This difference in the relaxant efficacy of racemic and (R)-albuterol appears to

be related to changes in the Ca^{2+} sensitivity and not dependent on the $[\text{Ca}^{2+}]_i$ because in "model" SMCs the $[\text{Ca}^{2+}]_i$ remained unchanged during these treatments with albuterol (data not shown).

DISCUSSION

Although β_2 -agonists are a standard acute treatment to relieve bronchial constriction associated with asthma (2, 24), it is surprising that their mechanism of action at the cellular level has remained poorly defined. This lack of understanding has, perhaps, been acceptable in view of the efficacy of these agonists. However, albuterol, a β_2 -agonist that is frequently used, consists of a racemic mixture of (R)- and (S)-albuterol ($\sim 50\%$) and several studies have raised concerns regarding the use of racemic albuterol. These reports suggest that (S)-albuterol activates pro-inflammatory pathways (11, 25) or increases allergen-induced edema and hyper-responsiveness (26) and may, in fact, have a detrimental effect in asthma management. In addition, the use of the single (R)-enantiomer may provide similar bronchodilator effects as racemic albuterol but at a reduced dose (5–7). By contrast, others studies have indicated that (R)- and racemic albuterol have similar efficacy (1, 8–10).

To address some of these concerns, we compared the acute effects of (R)-, (S)-, and racemic albuterol on airway contractility and the Ca^{2+} signaling of airway SMCs in mouse lung slices. Lung slices have considerable advantages in determining how β_2 -adrenergic agonists affect airway contractility. The lung slice retains most of the *in situ* features of lung tissue and importantly provides access to the small airways. The airways are lined with epithelial cells and tethered to the surrounding alveoli tissue, and can undergo multiple cycles of contraction and relaxation. The lung slice is highly compatible with microscopy techniques, which allow a quantitative analysis of changes in airway contractility and the simultaneous changes in $[\text{Ca}^{2+}]_i$ within the individual cells.

In this study, we found that (R)- and racemic albuterol induced a rapid relaxation of small airways that were contracted with MCh. Relaxation was evident at low doses (about 100 nM) of (R)- and racemic albuterol while a maximal relaxation was induced by 1 μM . We found that (R)-albuterol was more effective at relaxing the airways than racemic albuterol at most equivalent concentrations. These responses suggested that (S)-albuterol may antagonize the effects of (R)-albuterol, yet (S)-albuterol alone was found to have no direct effect on the contractility of airways (either partially contracted with MCh or relaxed), even at high doses. The implication of these results is that action of (S)-albuterol requires the presence of (R)-albuterol. Support for this idea is provided by the observation that the efficacy of (S)-albuterol to antagonize airway relaxation induced by a fixed concentration of (R)-albuterol (1 μM) was concentration dependent from 50 to 1,000 nM. A maximal antagonism by (S)-albuterol was achieved when the concentrations of (S)- and (R)-albuterol were equal. Increasing the (S)-albuterol concentration to be greater than the (R)-albuterol concentration had no further effect. Consequently, reduced airway relaxation in the presence of (S)-albuterol cannot be simply explained by a contractile effect of (S)-albuterol as suggested by others (11). However, we have not ruled out the possibility that (S)-albuterol may require prolonged exposure, presumably to sensitize the airways to the (S)-enantiomer, before it can exert a contractile effect.

Albuterol is believed to act on β_2 adrenoceptors and to rule out the possibility that (R)-albuterol or/and (S)-albuterol could act as a β_1 agonist, we have examined the effect of the albuterol on our mouse slices using β adrenoceptor antagonists. Atenolol,

Figure 5. Effect of albuterol isomers on Ca^{2+} sensitivity in "model" lung slices. (A) An airway under "normal" conditions was contracted with MCh. The addition of (R)-albuterol induced relaxation of the airway and washing of the slice with sHBSS returned the airway to the relaxed state. To convert this lung slice to the "model" state, the slice was perfused with 20 mM caffeine and 50 μM ryanodine, which induced a transient contraction. A second caffeine application did not evoke a contraction confirming the "model" status of the lung slice. Exposure to 200 nM MCh induced contraction of the same airway in a similar manner to that induced in the "normal" airway, indicating an increase in Ca^{2+} sensitivity. Exposure to (R)-albuterol relaxed the contracted airway indicating a decrease in Ca^{2+} sensitivity. The noncontractile response to the final exposure to caffeine reaffirms the continued "model" state of the slice. (B) A similar experiment showing that (S)-albuterol has no effect on the contraction induced by MCh in a "normal" or "model" slice. (C) A summary graph of three experiments comparing the contractility of airways in "model" slices contracted with 200 nM MCh and relaxed with either racemic (2 μM , right) or (R)-albuterol (1 μM , left). Data were extracted from experiments performed as shown in A and correspond to the time of exposure (6 min) to racemic and (R)-albuterol. Area ratios are expressed as means ($n = 3$) and were normalized to the initial level of contraction with MCh 200 nM (Area contracted, A_c). SD bars are indicated each 30 seconds.

a specific β_1 antagonist, failed to modify the response of airways to racemic or (R)-albuterol, while propranolol, a β_1/β_2 antagonist, inhibited the effects of the albuterol (data not shown). This is consistent with previous data indicating that the β_2 receptor primarily mediates the responses to albuterol.

Because the primary mechanism of action of β -agonists is believed to be the reversal of increases in $[\text{Ca}^{2+}]_i$ in airway SMCs, it is essential to understand the effects of (R)-, (S)-, and racemic albuterol on the Ca^{2+} signaling of airway SMCs. We previously found with mouse and rat lung slices that agonists such as MCh and 5-hydroxytryptamine (5-HT) induce contraction by initiating Ca^{2+} oscillations in airway SMCs (15, 16). Importantly, an increasing frequency of these Ca^{2+} oscillations

correlated with an increasing extent of contraction. Conversely, we have also found that airway relaxation induced by the β_2 -agonist isoproterenol (or agents that elevate cAMP to mimic the action of β_2 -agonists) correlated with a reduction in the frequency of agonist-induced Ca^{2+} oscillations (16). This slowing of the Ca^{2+} oscillations appeared to be mediated by a reduction in the sensitivity of the IP_3 receptor (IP_3R) to IP_3 rather than by a reduction of Ca^{2+} influx (16).

(R)-albuterol appears to have a similar but stronger action than isoproterenol. During the initial response to (R)-albuterol, the frequency of the MCh-induced Ca^{2+} oscillations was dramatically reduced but after a short time, the Ca^{2+} oscillation frequency began to slowly increase to a new steady rate that was

Figure 6. Effects of racemic and (R)-albuterol on "model" lung slices. (A) An airway was exposed to caffeine and ryanodine in sHBSS to induce the formation of a "model" slice. The second caffeine application did not evoke a contraction confirming the "model" status of the lung slice. The sequential addition of racemic and (R)-albuterol indicated that (R)-albuterol reduced the Ca^{2+} sensitivity of the airway (greater relaxation) more than racemic albuterol. The noncontractile response to the final exposure to caffeine reaffirms the continued "model" state of the slice. (B) A summary graph of three experiments comparing the contractility of airways in "model" slices contracted with 200 nM MCh and relaxed with either racemic (2 μM) or (R)-albuterol (1 μM) (in presence of MCh 200 nM). Data were extracted from experiments shown in A and correspond to the time of exposure (6 min) to racemic and (R)-albuterol. Area values are expressed as means ($n = 3$) and were normalized to the initial level of contraction (MCh 200 nM, A_c). SD bars are indicated each 30 seconds.

slower than with MCh alone. The fast initial relaxation of the airway can be correlated with the rapid decrease in the frequency of Ca^{2+} oscillations. Similar results were found with racemic albuterol. However, (R)-albuterol was more effective at slowing the Ca^{2+} oscillation frequency during the initial stages of the response. By contrast, (S)-albuterol alone had no effect on the $[\text{Ca}^{2+}]_i$ of relaxed airway SMCs (data not shown) or on the frequency of the Ca^{2+} oscillations induced by MCh. While these results are consistent with the observed contractile state of the airway in our lung slices, they significantly differ to from those using isolated bovine tracheal SMCs where

(S)-albuterol was reported to increase $[\text{Ca}^{2+}]_i$ (14) or initiate Ca^{2+} oscillations.

An additional mechanism by which (R)- and racemic albuterol may induce SMC relaxation is by altering the sensitivity of the SMCs to the Ca^{2+} . This possibility was investigated with "model" lung slices in which the $[\text{Ca}^{2+}]_i$ of the SMCs remains elevated and unchanged during exposure to contractile agonists. With this approach, we (21) and others (20) have shown that a sustained elevation of $[\text{Ca}^{2+}]_i$ resulted in airway relaxation. We hypothesized that this behavior results from a reduction in the Ca^{2+} sensitivity of the SMCs by a Ca^{2+} -dependent activation of myosin light chain phosphatase (MLCP). Increased activity of this enzyme would de-phosphorylate the regulatory myosin light chain (rMLC) and reduce force development. However, the addition of agonist to relaxed airways of "model" slices induced a strong airway contraction even though the $[\text{Ca}^{2+}]_i$ remained unchanged. Our interpretation of this response is that the contractile agonist (MCh) subsequently increases the SMC Ca^{2+} sensitivity by activating RhoA/ROK and/or PKC/CPI-17 pathways to decrease MLCP activity.

Using these "model" lung slices, we found that (R)- and racemic albuterol relaxed airways contracted with MCh, even though the intracellular Ca^{2+} remained elevated and unchanged (no Ca^{2+} oscillations). The relaxation induced by racemic albuterol was smaller than that induced by (R)-albuterol, but (S)-albuterol alone had no effect on the Ca^{2+} sensitivity. These results suggest that (R)-albuterol alone is more effective at reducing the sensitivity to the Ca^{2+} and that this effect can be antagonized by (S)-albuterol.

In our studies, we also found that the relaxing efficacy of albuterol was decreased by increasing concentrations of the contractile agonist MCh. Furthermore, a full relaxation of the MCh-induced contraction was never observed. Because SMC contraction is determined by the phosphorylation of rMLC which, in turn, results from the balance between MLCP and MLC Kinase activity, relaxation requires the full dephosphorylation of the rMLC. However, albuterol did not fully inhibit the Ca^{2+} oscillations or fully inactivate the Ca^{2+} sensitivity mechanisms. Our previous studies with the β -agonist isoproterenol indicated that increases in cAMP reduced the sensitivity of the IP_3R to slow the Ca^{2+} oscillations. However, this reduced sensitivity was overcome by increases in IP_3 . Therefore it would seem likely that higher concentrations of MCh lead to higher elevations in IP_3 that compensate for the reduced sensitivity of the IP_3R . The observation that the frequency of the Ca^{2+} oscillations begins to increase after the initial slowing also fits with the idea that the albuterol response is subject to tachyphylaxis. The conclusion is that albuterol modulates the MCh-mediated contractile process rather than directly antagonizing the activation of the M_3 receptor.

Overall, it appears that the decrease in the Ca^{2+} sensitivity rather than the change in the Ca^{2+} oscillations accounts for the sustained difference in the relaxation induced by (R)- and racemic albuterol. This dependence of contraction on the Ca^{2+} sensitivity is emphasized by the fact that after prolonged exposure to (R)-albuterol (or racemic albuterol), we observed a similar relaxation in both "normal" and "model" slices. Under these conditions, in the "normal" slice, the $[\text{Ca}^{2+}]_i$ is oscillating at a reduced frequency, whereas in the "model" slice the $[\text{Ca}^{2+}]_i$ remains elevated. Thus, the reduction in Ca^{2+} sensitivity by albuterol has greatly attenuated the influence of different levels of $[\text{Ca}^{2+}]_i$. However, the dynamics of the Ca^{2+} oscillations significantly influence the temporal aspects of the relaxation process. The rapid cessation of the Ca^{2+} oscillations effectively reduces the $[\text{Ca}^{2+}]_i$ before changes in Ca^{2+} sensitivity occur, and this accounts for the early relaxation responses.

In summary, (R)- and racemic albuterol can relax airways contracted with MCh by initially slowing down the frequency of the Ca^{2+} oscillations and by subsequently decreasing the sensitivity of the contractile apparatus to Ca^{2+} . Overall (R)-albuterol is more effective than racemic albuterol at relaxing the airways. By contrast, (S)-albuterol alone is without effect and requires the presence of (R)-albuterol to exert any influence. The exact mechanism of antagonism by (S)-albuterol is unknown. However, these data indicate that (S)-albuterol is interfering with the activation of the β_2 -adrenergic response by (R)-albuterol.

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