Heterologous amplification of homologous beta-adrenoceptor desensitization in airway smooth muscle
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Chapter 5

Effects of R- and S-salbutamol on methacholine- and histamine-induced \( \text{Ca}^{2+} \)-responses in isolated bovine tracheal smooth muscle cells

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Summary

Regular treatment of asthmatic patients with racemic β₂-agonists may lead to an adverse effect of the S-enantiomer on intracellular calcium homeostasis in airway smooth muscle. In this study, the effects of R- and S-salbutamol were investigated on methacholine- and histamine-induced Ca²⁺-responses in bovine tracheal smooth muscle cell suspensions and adhered single cells. In cell suspension as well as in adhered single cells, no effect on basal intracellular calcium was found for both R- and S-salbutamol. In suspension, R- and S-salbutamol dose-dependently inhibited the 1 μM MCh-induced Ca²⁺-transient to approximately 50%, with a 250 to 400-fold potency difference. Up to 10 μM, both R- and S-salbutamol did not inhibit the sustained phase of the Ca²⁺-influx. In adhered single cells, 1 μM methacholine- and 10 μM histamine-induced Ca²⁺-transient and -plateau were significantly inhibited by 10 μM R-salbutamol, whereas no effect was observed with 0.1 μM R-salbutamol or with S-salbutamol. The inhibitory effect of R-salbutamol on methacholine-induced Ca²⁺-plateau was mainly due to inhibition of the amplitude of Ca²⁺-oscillations, which occurred in most of the adhered single cells. Histamine-induced Ca²⁺-responses of these cells were much more sensitive towards inhibition by 10 μM R-salbutamol than those induced by methacholine, as demonstrated by the marked inhibition of the Ca²⁺-plateau in non-oscillating cells and by the complete absence of histamine-induced Ca²⁺-oscillations in the presence of 10 μM R-salbutamol. Overall, both in airway smooth muscle cell suspension and in adhered single cells S-salbutamol did not increase basal intracellular Ca²⁺-levels and did not potentiate methacholine- and histamine-induced Ca²⁺-responses. Therefore, the results provide no explanation for the observed adverse effects of racemic β₂-adrenoceptor agonists in asthma. In single cell measurements R-salbutamol preferentially inhibits the contractile agonist-induced Ca²⁺-plateau by decreasing the amplitude of the Ca²⁺-oscillations. In addition, there is a differential susceptibility to β-agonist-induced inhibition of the Ca²⁺-transient and -plateau, depending both on the cellular condition (attached vs non-attached) and on the contractile agonist used.

Introduction

Regular treatment of asthmatic patients with β₂-adrenoceptor agonists may lead to a number of adverse effects, including rebound airway hyperreactivity to stimuli, including methacholine, histamine and propranolol [1-3]. The mechanisms of these adverse effects are presently unknown, but could involve a paradoxical up-regulation of a number of neural and non-neural processes that counteract β₂-adrenoceptor function, possibly by the S-enantiomer present in the racemic mixture of β₂-agonist drugs that are currently being used [4]. In this respect, changes in the cholinergic system may be of importance, as indicated by the observation that S-salbutamol-induced airway hyperreactivity to histamine in the guinea pig is inhibited by vagotomy [5]. In addition, enhanced contractile responses of isolated
human bronchial rings to histamine [6] and of guinea pig tracheal rings to carbachol [7] have been found after incubation with S-salbutamol, as well as enhanced Ca\(^{2+}\)-responses to carbachol in isolated bovine tracheal smooth muscle cells [8]. In addition, in bovine tracheal smooth muscle cells Mitra and co-workers observed an S-salbutamol-induced increase of intracellular Ca\(^{2+}\) in a concentration-dependent manner and an interaction of the S-enantiomer with the muscarinic M\(_3\)-receptor was suggested [9]. Recently, it has been reported that treatment of human bronchial smooth muscle cells for 24 hours with S-salbutamol significantly increased intracellular Ca\(^{2+}\)-responses of these cells upon stimulation with methacholine [10].

Over the years, clinical data have suggested that the S-enantiomer present in the racemic \(\beta_2\)-adrenoceptor agonist mixture may cause airway hyperreactivity [11] and even contribute to increased asthma death [12]. Results obtained in animal models and in isolated cells have substantiated the possible effect of S-salbutamol on airway responsiveness [13-15]. Furthermore, studies in children [16-18] and adults [19,20] with asthma or chronic obstructive pulmonary disease (COPD) have suggested that R-salbutamol offers efficacy and safety benefits compared with racemic salbutamol. However, notwithstanding the above-mentioned evidence, adverse effects of the S-enantiomer in racemic \(\beta\)-agonist drugs are still subject of controversy. Thus, in guinea pigs it has been found that both basal airway reactivity and allergen-induced airway hyperreactivity towards histamine were not affected by S-salbutamol [21]. In patients with mild to moderate asthma, other groups have failed to find any adverse effects of S-salbutamol on bronchoprotection and bronchodilatation [22,23]. Moreover, several clinical studies in patients with asthma or COPD were unable to demonstrate advantage of R-salbutamol over the racemate regarding bronchodilation and airway reactivity [24-28].

Among the observations made, the finding that S-salbutamol increases the intracellular free Ca\(^{2+}\)-concentration and enhances agonist-induced Ca\(^{2+}\)-mobilization in dissociated cells from airway smooth muscle, particularly through an effect on muscarinic receptors, is fascinating and deserves further investigation. To this purpose, we have thoroughly compared the effects of R- and S-salbutamol on methacholine- and histamine-induced Ca\(^{2+}\)-responses in Fura-2AM-loaded bovine tracheal smooth muscle cells, using both cell suspension spectrofluorometry and single cell fluorescence microscopy.

**Materials and Methods**

**Tissue preparation**

Fresh bovine tracheas were obtained from the slaughterhouse and were transported to the laboratory within 30 min at room temperature in Krebs-Henseleit (KH) buffer of the following composition (nM): NaCl 177.5, KCl 5.6, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5, NaH\(_2\)PO\(_4\) 1.3, NaHCO\(_3\) 25.0, glucose 5.5, pregassed with 95% O\(_2\) and 5% CO\(_2\); pH 7.4. The tracheal smooth muscle was dissected carefully and prepared free of mucosa and serosal connective tissue in KH buffer gassed with 95% O\(_2\)/5% CO\(_2\) at room temperature. Subsequently, the
tracheal smooth muscle was chopped, using a McIlwain tissue chopper, two times at the size of 300 μm and three times at 100 μm. Tissue fragments were washed under sterile conditions (three times) and maintained overnight in Dulbecco’s modified Eagle’s medium (DMEM; 2.5 mg/ml) supplemented with 10 mM NaHCO₃, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS at 37°C (55 rpm).

Isolation of tracheal smooth muscle cells

Tissue fragments were washed three times in Krebs-Ringer-Henseleit (KRH) buffer containing (mM); NaCl 125.0, KCl 6.0, MgCl₂ 2.5, CaCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.0, HEPES 25.0, pH 7.4, supplemented with 2.0 mM (±)-dithiothreitol (DTT) and were then resuspended in a digestion mixture of collagenase P (0.75 mg/ml), papain (1 mg/ml) and trypsin inhibitor (1 mg/ml) in KRH buffer. The suspension was incubated for 20 min at 37°C (55 rpm) and then gently dispersed with a wide-bored pipette. After another 10 min of incubation at 37°C (70 rpm), the suspension was gently dispersed again and filtered over a 50 μm gauze and the cells were collected by centrifugation (1000g, 10 min). The isolated cells were washed three times with KRH, pH 7.4, supplemented with 2 mg/ml fetal bovine serum (KRH/FBS) and were allowed to regenerate for 1h at 37°C (55 rpm).

Intracellular Ca²⁺ measurements in cell suspensions

The freshly isolated dispersed bovine tracheal smooth muscle cells were incubated with the fluorescent dye, Fura-2/AM (3 μM) for 30 min at 37°C (55 rpm). The loaded cells were washed three times in KRH/FBS and resuspended to a density of 1 x 10⁶ cells/ml. The cells were kept at room temperature on a Rock-N-Roller (Breda Scientific) and used for measurement of the intracellular calcium concentration ([Ca²⁺]) within 2-4 h, during which they remained viable and responsive. Fura-2 fluorescence of the cells (excitation wavelengths: 340 and 380 nm; emission wavelength: 510 nm) was measured at 37°C with a Perkin Elmer Spectrometer (LS-50B). Each cuvet contained 2 ml of magnetically stirred cell suspension. In all experiments, Ca²⁺-transient (initial rise in [Ca²⁺]) and -plateau (sustained rise in [Ca²⁺]) were induced by 1 μM methacholine, added at t = 360 seconds after starting the Ca²⁺-measurement. To establish the effects of R- and S-salbutamol on methacholine-induced Ca²⁺-responses, single doses of R- or S-salbutamol (1 nM – 100 μM) were administered 5 minutes before methacholine (t = 60 seconds). The first 60 seconds were used to measure basal Ca²⁺-level. [Ca²⁺] was calculated every 0.2 s according to Grynkiewicz [29]. At the end of the experiment, the maximal fluorescence ratio (Rmax) was determined after adding 0.01% of Triton-X-100 as a permeabilizing agent. The minimal fluorescence ratio (Rmin) was determined by addition of 5 mM EGTA to the permeabilized cells.
Intracellular Ca\(^{2+}\) measurements in single cells

Freshly isolated bovine tracheal smooth muscle cells were adhered to poly-L-lysine-coated round coverslips (1 h, 37°C), after which they were loaded with Fura-2AM (0.5 μM; 1 h, 37°C). The coverslips were transferred to a thermostatted (37°C) microscope recording chamber equipped with a special perfusion system, and Ca\(^{2+}\)-responses to 1 μM methacholine or 10 μM histamine were measured after preincubation (5 min, 37°C) with buffer solution or 0.1 and 10 μM R-, and S-salbutamol, using a Zeiss Axiocvert 35M fluorescent imaging microscope with an on-line SensiCam cooled imaging system. Fluorescence ratio’s (excitation wavelengths: 340 and 380 nm; emission wavelength 510 nm) were plotted for each individual cell.

Data analysis

In cell suspension measurements, the methacholine-induced transient rise of [Ca\(^{2+}\)]\(_i\), representing Ca\(^{2+}\)-mobilization from internal stores, was expressed as the maximal increase of [Ca\(^{2+}\)]\(_i\), above prestimulatory level after the addition of 1 μM methacholine. The plateau level of [Ca\(^{2+}\)]\(_i\), representing Ca\(^{2+}\)-influx, was expressed as the [Ca\(^{2+}\)]\(_i\), above prestimulatory level at 2 min after contractile agonist addition.

In single cell measurements, methacholine- and histamine-induced Ca\(^{2+}\)-transients were expressed as the maximal increase of [Ca\(^{2+}\)]\(_i\) (fluorescence ratio) above prestimulatory level following the addition of agonist. Due to methacholine- and histamine-induced slow Ca\(^{2+}\)-oscillations in many cells, Ca\(^{2+}\)-plateau (influx including oscillations) was expressed as the area under the curve above prestimulatory level using a fixed period of 20 seconds (AUC\(_{20}\)) for each cell, starting from the inflection point after the first Ca\(^{2+}\)-transient. This fixed period of 20 seconds was used because not all cells responded at the same time after stimulation with methacholine or histamine. The oscillation frequency was measured using the same period of 20 seconds.

All data are presented as means ± s.e.m. Statistical analysis was performed by means of the two-tailed Student’s *t* test for paired or unpaired observations as appropriate. *P* values < 0.05 were considered statistically significant.

Materials

Dulbecco’s modification of Eagle’s Medium (DMEM), NaHCO\(_3\) solution (7.5%), penicillin/streptomycin solution (5000 U/ml ; 5000 μg/ml) and HEPES solution (1 M) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). (S)-, (R)- and (R/S)-salbutamol were obtained from Sepracor Inc. (Marlborough, MA, U.S.A.). Fetal bovine serum (FBS), dithiothreitol (DTT), trypsin inhibitor (type II-S), Fura-2/AM, methacholine chloride and histamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and papain and collagenase P were purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.
Results

Cell suspension measurements

Figure 1a shows a typical intracellular Ca\textsuperscript{2+}-response to 1 µM methacholine in bovine tracheal smooth muscle cells in suspension. The response is characterised by a transient-rise in [Ca\textsuperscript{2+}], representing Ca\textsuperscript{2+}-mobilization, followed by a sustained plateau level of [Ca\textsuperscript{2+}], representing Ca\textsuperscript{2+}-influx. Obviously, 10 µM S-salbutamol had no effect on basal Ca\textsuperscript{2+}-level, while a clear inhibition of the methacholine-induced Ca\textsuperscript{2+}-transient was observed at this concentration (Fig. 1b).

![Figure 1](image_url)  
**Figure 1** Typical intracellular Ca\textsuperscript{2+}-responses in bovine tracheal smooth muscle cell suspensions, showing the effect of 1 µM methacholine (MCh) in the absence (a) and presence (b) of 10 µM S-salbutamol.

Whereas no significant effects were found on basal [Ca\textsuperscript{2+}], after preincubation with various concentrations of both R- and S-salbutamol, both enantiomers dose-dependently inhibited the 1 µM methacholine-induced Ca\textsuperscript{2+}-transient by approximately 50% (Fig. 2b). However, S-salbutamol inhibited the Ca\textsuperscript{2+}-response at approximately 250 to 400-fold higher concentrations than R-salbutamol (pEC\textsubscript{50} R-salbutamol = 7.8, estimated pEC\textsubscript{50} S-salbutamol = 5.4). No significant inhibition of the Ca\textsuperscript{2+}-plateau level was observed for both R- and S-salbutamol up to 10 µM, although the plateau level tended to be reduced in the presence of increasing concentrations of R-salbutamol (Fig. 2c). At the highest concentration used (100 µM), however, both enantiomers significantly decreased the Ca\textsuperscript{2+}-plateau by about 25%.
Effects of R- and S-salbutamol on intracellular Ca\(^{2+}\)-responses

Figure 2 Effects of five minutes preincubation with various concentrations R- and S-salbutamol on basal intracellular Ca\(^{2+}\)-levels (a) and on methacholine (1 \(\mu\)M) induced Ca\(^{2+}\)-transient (b) and -plateau (c) in bovine tracheal smooth muscle cell suspensions. Ca\(^{2+}\)-transient and -plateau were measured above prestimulatory level and expressed as percentage of control without salbutamol preincubation. Results are means ± S.E.M. of 3 experiments.

Single cell measurements

Figure 3a illustrates that, as in cell suspension, no effects were found of 0.1 and 10 \(\mu\)M R- and S-salbutamol on basal Ca\(^{2+}\)-levels in adhered single cells. In addition, the 1 \(\mu\)M methacholine-induced Ca\(^{2+}\)-transient was unaffected by 0.1 and 10 \(\mu\)M S-salbutamol as well as by 0.1 \(\mu\)M R-salbutamol, whereas a significant decrease was observed in the presence of 10 \(\mu\)M R-salbutamol (Fig. 3b). The Ca\(^{2+}\)-transient in response to 10 \(\mu\)M histamine was also inhibited by 10 \(\mu\)M R-salbutamol, whereas 10 \(\mu\)M S-salbutamol tended to reduce this response.

In a fraction of the cells, the methacholine- and histamine-induced Ca\(^{2+}\)-transient was followed by slow Ca\(^{2+}\)-oscillations with varying characteristics. Figure 4 illustrates typical registrations of the most prevalent oscillation patterns induced by methacholine; similar patterns were observed for histamine. To determine the effects of R- and S-salbutamol on methacholine- and histamine-induced Ca\(^{2+}\)-plateau (influx including oscillations), we calculated the area under the curve above prestimulatory level for a period of 20 seconds immediately after the first Ca\(^{2+}\)-transient. As is demonstrated in Figure 3c, only 10 \(\mu\)M R-salbutamol inhibited methacholine- and histamine-induced Ca\(^{2+}\)-plateau. No effects were observed for 0.1 and 10 \(\mu\)M S-salbutamol and for 0.1 R-salbutamol. Further analysis of the Ca\(^{2+}\)-oscillations indicated that both R- and S-salbutamol had no significant effects on methacholine-induced oscillation frequency, though 10 \(\mu\)M R-salbutamol tended to reduce it (Fig. 3d). Interestingly, in the presence of 10 \(\mu\)M R-salbutamol histamine-induced Ca\(^{2+}\)-oscillations were completely abolished.
Figure 3 Effects of five minutes preincubation with 0.1 and 10 μM R- and S-salbutamol on basal intracellular Ca\textsuperscript{2+}-levels (a) and on methacholine (1 μM)- and histamine (10 μM)-induced Ca\textsuperscript{2+}-transient (b), -plateau (c) and -oscillation frequency (d) in single bovine tracheal smooth muscle cells. The Ca\textsuperscript{2+}-transient was measured as the maximal increase in [Ca\textsuperscript{2+}]\textsubscript{i} above prestimulatory level. The Ca\textsuperscript{2+}-plateau was measured as the area under the curve above prestimulatory level, using a fixed period of 20 seconds (AUC\textsubscript{20}) for each cell after the first Ca\textsuperscript{2+}-transient. The oscillation frequency was measured using the same fixed period of 20 seconds. Results are means ± S.E.M. of 6-7 experiments for methacholine (5-15 cells per experiment) and 3 experiments for histamine (8-17 cells per experiment). Significantly different from control: * P<0.05; *** P<0.001.

Given the observation that 10 μM R-salbutamol inhibited the methacholine-induced Ca\textsuperscript{2+}-plateau, whereas the oscillation frequency remained unaffected, we sought to determine which effect of 10 μM R-salbutamol was responsible for the observed inhibition of the Ca\textsuperscript{2+}-plateau. First, for each preincubation condition we pooled the results from all individual cells (from 6-7 animals), and calculated the average Ca\textsuperscript{2+}-plateau (AUC\textsubscript{20}) per condition. Not surprisingly, qualitatively similar results were obtained as described above. Thus, 10 μM R-salbutamol significantly inhibited both methacholine- and histamine-induced Ca\textsuperscript{2+}-plateau, but no effects were observed for 10 μM S-salbutamol (Fig. 5).
Effects of R- and S-salbutamol on intracellular Ca\(^{2+}\)-responses

**Figure 4** Typical registrations of methacholine (1 µM)-induced Ca\(^{2+}\)-responses in single bovine tracheal smooth muscle cells, demonstrating non-oscillating (a) and oscillating (b-d) cells.

**Figure 5** Effects of five minutes preincubation with 10 µM R- or S-salbutamol on methacholine (1 µM)- and histamine (10 µM)-induced Ca\(^{2+}\)-plateau in single bovine tracheal smooth muscle cells. The Ca\(^{2+}\)-plateau was measured as the area under the curve above prestimulatory level, using a fixed period of 20 seconds (AUC\(_{20}\)) for each cell after the first Ca\(^{2+}\)-transient. Individual cells from all experiments were pooled and results are means ± S.E.M. of 53-72 cells for methacholine and 33-37 cells for histamine. Significantly different from control: * P<0.05; *** P<0.001.
Next, we divided the cells into two groups. In the first group all non-oscillating cells (see Fig. 4a) were pooled. The second group was represented by the remaining cells, which showed different oscillating patterns (see Figures 4b-d). Both under control condition and after pretreatment with 10 μM R-salbutamol, the fraction of oscillating cells was much higher for methacholine, as compared to histamine. Thus, for methacholine and histamine we found 46 and 13 oscillating cells, respectively, in the absence and 40 and 0 oscillating cells in the presence of 10 μM R-salbutamol, whereas 26 and 22 non-oscillating cells were found in the absence and 27 and 37 non-oscillating cells in the presence of 10 μM R-salbutamol.

![Graph](image)

**Figure 6** Effects of five minutes preincubation with 10 μM R-salbutamol on methacholine (1 μM)- and histamine (10 μM)-induced Ca^{2+}-plateau in non-oscillating (a) and oscillating (b) single bovine tracheal smooth muscle cells. The Ca^{2+}-plateau was measured as the area under the curve above prestimulatory level, using a fixed period of 20 seconds (AUC_{20}) for each cell after the first Ca^{2+}-transient. Individual oscillating cells (46 and 13 cells in the absence and 40 and 0 cells in the presence of 10 μM R-salbutamol for methacholine and histamine, respectively) and non-oscillating cells (26 and 22 cells in the absence and 27 and 37 cells in the presence of 10 μM R-salbutamol for methacholine and histamine, respectively) from all experiments were pooled and results are means ± S.E.M. Significantly different from control: **P<0.01; *** P<0.001.

Remarkably, in non-oscillating cells 10 μM R-salbutamol did not inhibit the methacholine-induced Ca^{2+}-plateau (Fig. 6a), whereas a significant effect was found in the oscillating cells (Fig. 6b), without an effect on oscillating frequency (not shown). This indicates that the overall inhibition of the methacholine-induced Ca^{2+}-plateau by 10 μM R-salbutamol shown in Figure 5 refers to oscillating cells only. Although the methacholine-induced Ca^{2+}-plateau in non-oscillating cells was not affected by 10 μM R-salbutamol, the histamine-
induced Ca\textsuperscript{2+}-plateau was significantly inhibited in these cells. Moreover, in the presence of 10 μM R-salbutamol all histamine-induced oscillation patterns were abolished, indicating that histamine-induced Ca\textsuperscript{2+}-responses are more sensitive towards 10 μM R-salbutamol as compared to methacholine.

**Discussion**

The present study indicated that S-salbutamol does not have adverse effects on basal [Ca\textsuperscript{2+}]	extsubscript{i} and agonist-induced Ca\textsuperscript{2+}-responses in bovine tracheal smooth muscle cells, using measurements both in cell suspensions and in single cells. In cell suspension it was demonstrated that 1 μM methacholine-induced Ca\textsuperscript{2+}-transient was effectively and dose-dependently inhibited by R-salbutamol, as may be expected from previous observations using R-salbutamol in single bovine tracheal smooth muscle cell measurements [8] and (racemic) isoprenaline in bovine tracheal smooth muscle cell suspensions [30]. S-salbutamol also inhibited the methacholine-induced response, but at 250-400-fold higher concentrations, illustrating the stereoselectivity of action of salbutamol at the β-adrenoceptor. The latter observation does not agree with the previous observation of Yamaguchi and McCullough (1996), in single cells, showing that S-salbutamol caused potentiation of carbachol-induced Ca\textsuperscript{2+}-responses. In addition, in our study S-salbutamol did not enhance basal Ca\textsuperscript{2+}-level in airway smooth muscle cell suspension, whereas such effects were observed by others using single bovine tracheal smooth muscle cells [8,9]. Therefore, we decided to assess the effects of the enantiomers under single cell conditions as well. In line with our findings in cell suspension, however, no effects of 0.1 and 10 μM S- and R-salbutamol were observed on basal Ca\textsuperscript{2+}-levels, indicating that in both experimental setups S-salbutamol is not able to elevate intracellular calcium levels by itself. In our single cell measurements, the 1 μM methacholine-induced Ca\textsuperscript{2+}-transient was significantly inhibited by 10 μM R-salbutamol, whereas 0.1 μM R-salbutamol and 0.1 and 10 μM S-albuterol had no effect. This is in contrast with the cell suspension measurements, where both 0.1 μM R- and 10 μM S-salbutamol significantly inhibited the 1 μM methacholine-induced Ca\textsuperscript{2+}-transient, indicating the methacholine-induced Ca\textsuperscript{2+}-transient to be more resistant to inhibition by R- and S-salbutamol in single cells as compared to cell suspension. However, similar to cell suspension, no enhanced methacholine-induced Ca\textsuperscript{2+}-transient was found in the presence of S-salbutamol. Similarly, only R-salbutamol significantly inhibited the Ca\textsuperscript{2+}-transient induced by 10 μM histamine, whereas preincubation with S-salbutamol had no effect. Collectively, our data clearly indicate that both in cell suspension and in single cell measurements S-salbutamol does not enhance contractile agonist-induced Ca\textsuperscript{2+}-transients. Interestingly, after stimulation with methacholine or histamine a considerable number of the attached cells showed slow Ca\textsuperscript{2+}-oscillations with different patterns (Fig. 4), irrespective of the preincubation conditions used. Several studies have investigated the nature and origin of contractile-agonist induced intracellular Ca\textsuperscript{2+}-oscillations. Initially, in airway smooth muscle cell suspension [30-32] and intact tissue [33,34], changes in intracellular Ca\textsuperscript{2+}-
concentration upon contractile agonist stimulation were shown to be biphasic. The rapid transient rise of intracellular Ca\(^{2+}\), representing Ca\(^{2+}\)-release from the sarcoplasmic reticulum store [35], was found to be mediated by IP\(_3\)-receptors [36], while the subsequent sustained elevation (plateau) of intracellular Ca\(^{2+}\), representing extracellular Ca\(^{2+}\)-influx [31,37], is considered to be mediated by store-operated Ca\(^{2+}\)-channels [38,39]. However, in additional studies using real-time confocal microscopy, both in intact airway smooth muscle [40] and in adhered airway smooth muscle cells [41-43] many contractile agonists have been shown to induce intracellular Ca\(^{2+}\)-oscillations, superimposed on the plateau phase. It has been demonstrated that activation of IP\(_3\)-receptors is critical for the initiation of these oscillations and that ongoing oscillations are mediated by ryanodine receptors (RyRs), localized in the sarcoplasmic reticulum as well [44,45]. The release of Ca\(^{2+}\) through RyRs appears to be sensitive to Ca\(^{2+}\) (calcium-induced calcium release; CICR) [46] and there is recent evidence that agonist-induced cADP-ribose (cADPR), an endogenous calcium-releasing messenger, may be involved in activating RyRs and modulating CICR [47].

The agonist-induced intracellular Ca\(^{2+}\)-oscillations observed in our single bovine tracheal smooth muscle cells are in line with these findings, while the observed Ca\(^{2+}\)-plateau in response methacholine in suspension most likely reflects the integrated oscillations of all individual cells. Indeed, integration of all Ca\(^{2+}\)-signals collectively obtained in our single cell experiments showed a biphasic overall response with no oscillation patterns (data not shown). It was also found that only 10 μM R-salbutamol inhibited methacholine- and histamine-induced Ca\(^{2+}\)-plateau (influx plus oscillations), whereas other preincubations had no effect (Fig. 3c). Remarkably, the inhibitory effect of 10 μM R-salbutamol on the Ca\(^{2+}\)-plateau from adhered cells was not observed in suspension measurements. The differences in susceptibility of the Ca\(^{2+}\)-plateau as well as the Ca\(^{2+}\)-transient to modulation by β-adrenoceptor stimulation might be explained by different arrangements of the cytoskeleton and intracellular Ca\(^{2+}\) pools in attached versus non-attached cells [48]. In line with the suspension measurements, however, S-salbutamol had no adverse effects on methacholine- or histamine-induced Ca\(^{2+}\)-plateau.

Analysing the methacholine-induced Ca\(^{2+}\)-oscillations, we found no significant effect of R- and S-salbutamol on the fraction of oscillating cells (data not shown) and on oscillation frequency (Fig. 3d). In addition, when we pooled all non-oscillating cells and all oscillating cells, we found that 10 μM R-salbutamol did not inhibit the methacholine-induced Ca\(^{2+}\)-plateau in non-oscillating cells (Fig. 6a), whereas this plateau was significantly inhibited in oscillating cells (Fig. 6b), without effect on the oscillation frequency (data not shown). Therefore, it can be concluded that the observed inhibition of the methacholine-induced Ca\(^{2+}\)-plateau by 10 μM R-salbutamol only affects the amplitude of the Ca\(^{2+}\)-oscillations. Interestingly, the histamine-induced Ca\(^{2+}\)-responses were more sensitive towards 10 μM R-salbutamol than those induced by methacholine. Thus, no histamine-induced Ca\(^{2+}\)-oscillations were found at all in the presence of 10 μM R-salbutamol. Furthermore, in the non-oscillating cells the histamine-induced Ca\(^{2+}\)-plateau was markedly inhibited by 10 μM
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R-salbutamol. This is in line with previous observations in bovine tracheal smooth muscle showing that histamine-induced Ca\(^{2+}\)-responses and contraction are more susceptible to β-agonist-induced inhibition than those induced by methacholine [30,49] which may be explained by the higher potency of methacholine in inducing inositol phosphates formation and subsequent intracellular Ca\(^{2+}\)-changes [30].

Interestingly, both under control condition and after pretreatment with 10 μM R-salbutamol, the fraction of oscillating cells was much higher for methacholine as compared to histamine. Recently, it has been demonstrated that signalling pathways involved in intracellular Ca\(^{2+}\)-oscillations may operate in an agonist-specific manner in airway smooth muscle. Thus, in porcine airway smooth muscle cells preincubated with 100 μM 8Br-cADPR (an antagonist of cADPR) the intracellular Ca\(^{2+}\)-responses to acetylcholine were significantly attenuated, whereas responses to histamine were not [50]. The presence of this additional signalling pathway might explain why methacholine induces a higher fraction of oscillating cells and might also be an additional explanation why histamine-induced Ca\(^{2+}\)-oscillations are more sensitive towards R-salbutamol.

In conclusion, both in airway smooth muscle cell suspension and in adhered single cells S-salbutamol does not increase basal intracellular Ca\(^{2+}\)-levels and has no potentiating effect on methacholine- and histamine-induced intracellular Ca\(^{2+}\)-responses. In this respect, our results offer no explanation for the observed adverse effects of β\(_2\)-adrenoceptor agonists in asthma. In addition, in single cell measurements we have demonstrated that R-salbutamol preferentially inhibits contractile agonist-induced Ca\(^{2+}\)-plateau by decreasing the amplitude of the Ca\(^{2+}\)-oscillations. Finally, the susceptibility of the Ca\(^{2+}\)-transient and -plateau for modulation by β-adrenoceptor agonists appears to depend both on the cellular condition (attached vs non-attached) and the contractile agonist used.

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